Icksoo Lee

Regulation of Electron Transport and Proton Translocation in Mammalian Cytochrome c Oxidase by ATP, Palmitate, and Protein Phosphorylation

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Für meine Eltern

To my parents

부모님께

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1. Introduction

1.1. Mitochondria and energy metabolism

Life on earth requires energy for its growth, proliferation, and for any expression of liveliness. To earn more of the cellular energy ATP, aerobic cells have developed a specific mechanism, oxidative phosphorylation. Most aerobic bacteria and all eukaryotic cells generate about 15 times more ATP by oxidative phosphorylation compared to glycolysis, which produces lactic acid or alcohol as the end product. In eukaryotes oxidative phosphorylation takes place in mitochondria, cellular organelles which contain their own genome (e.g. human mtDNA = 16569 bp) which is inherited maternally. Only 13 polypeptides are encoded by mitochondrial DNA which all represent subunits of the enzyme complexes of oxidative phosphorylation. Three enzyme complexes of the mitochondrial respiratory chain (NADH:ubiquinone oxidoreductase = complex I, cytochrome c reductase = complex III, and cytochrome c oxidase = complex IV) are electron transport-driven proton pumps which transfer reducing equivalents from nutrition to dioxygen accompanied by transfer of protons across the inner mitochondrial membrane, and generate a proton motive force. The proton motive force consists of an electrochemical gradient and a pH gradient.

$$\Delta p = \Delta\psi - Z \cdot \Delta pH, \quad Z = 2.303 \cdot RT / F, \Delta p \cdot F = \Delta\mu H^+$$

The ATP synthase in mitochondria uses the proton motive force to produce ATP from ADP and inorganic phosphate [Abrahams et al., 1994; Junge et al., 1997]. Cytochrome c oxidase is the terminal enzyme of the mitochondrial respiratory chain which transfers electrons from cytochrome c to molecular oxygen, coupled with the uptake of protons from the matrix forming water, and with the translocation of protons across the mitochondrial inner membrane.

1.2. Cytochrome c oxidase

1.2.1. Structure of cytochrome c oxidase

The mammalian enzyme is composed of 13 subunits which are encoded on the mitochondrial (subunits I, II, and III) and nuclear genome (subunits IV, Va, Vb, VIa, VIb, VIc, VIIa, VIIb, VIIc, VIII). The bovine heart enzyme crystallizes as a dimer [Tsukihara et al., 1996],

as shown in Fig. 1. In contrast, the bacterial enzyme is monomeric, consisting of only 2-4 subunits [Iwata et al., 1995].

Subunit I contains the redox center heme a and the oxygen binding binuclear center heme a_3-Cu_B, and subunit II contains Cu_A and the cytochrome c binding site. Heme a and heme a_3 are chemically identical, but differ in their spectral properties, due to the different environment in subunit I. The properties of subunits I and II are nearly identical from bacterial to mammalian cytochrome c oxidase and have been shown to resemble each other in the crystal structures of the bovine heart enzyme [Tsukihara et al., 1995] and *Paracoccus denitrificans* enzyme [Iwata et al., 1995]. Additionally 1 Zn, 1 Mg, and 1 Na (or Ca) are observed in the mammalian enzyme [Yoshikawa et al., 1998], but their functions are unknown.

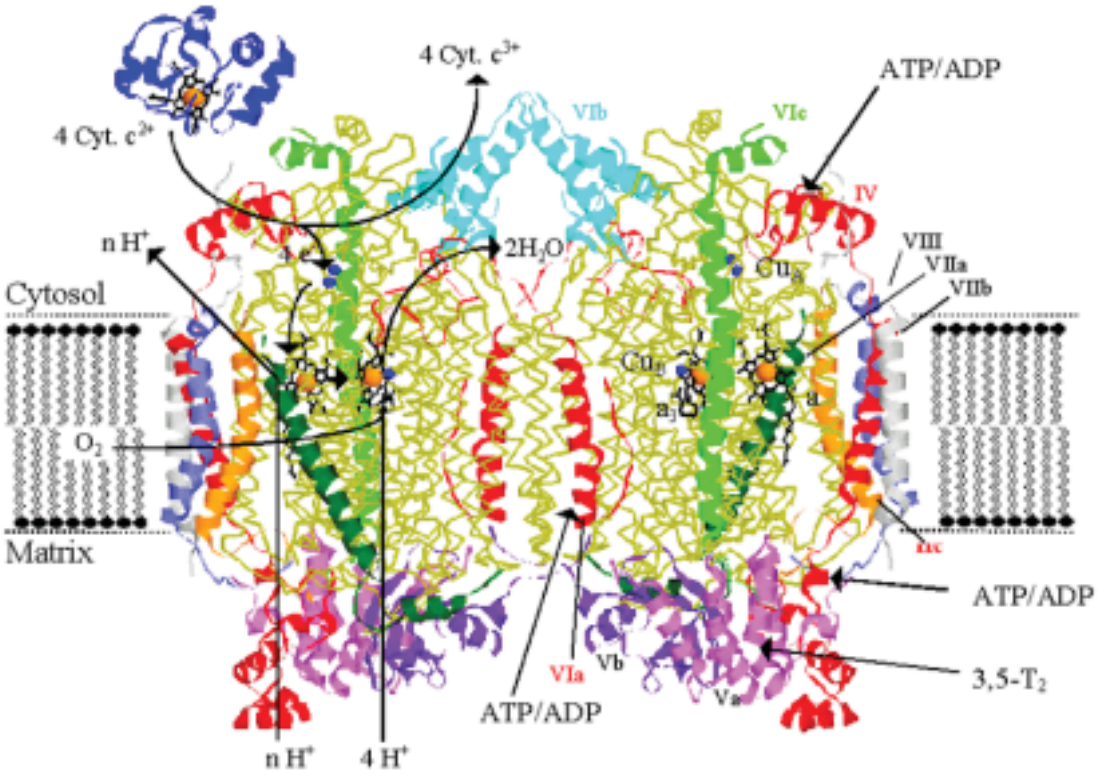


Fig. 1. Crystal structure of cytochrome c oxidase and cytochrome c from bovine heart. Each monomer of the dimeric enzyme contains 3 mitochondrial coded subunits (I, II, and III, yellow backbone), containing the cytochrome c binding site and two copper atoms (Cu_A) at subunit II, and heme a and the dioxygen binding site at the binuclear center heme a_3/Cu_B in subunit I. The copper atoms are presented in blue, heme groups in black and the iron atoms in the heme groups in orange. The 10 nuclear coded regulatory subunits (ribbons) are indicated in different colors (subunits IV, Va, Vb, VIa, VIb, VIc, VIIa, VIIb, VIIc, and VIII). Left monomer: indicates the catalytic reaction. Right monomer: shows the binding sites for ATP or ADP and 3,5-diiodothyronine. Data source for cytochrome c oxidase: protein data bank Brookhaven, Tsukihara et al. [1996]. This modeling was prepared with the program "RasMol 2.6".

In Fig. 1 the nuclear coded subunits are presented in different colors. Seven of them are located as transmembraneous polypeptides and the other three are located outside of the inner membrane: subunit VIb at the cytosolic side and subunits Va and Vb at the matrix side.

The subunits of cytochrome c oxidase from turkey [Hüttemann et al., 2000] and tuna [Arnold et al., 1997] contain also thirteen subunits, similar to the enzyme from mammals. On the other hand, 11 subunits were found in the enzyme from yeast [Geier et al., 1995], seven from *Dictiostelium discoideum* [Bisson et al., 1986], but only four subunits occur in cytochrome c oxidase from the bacteria, *P. denitrificans* [Iwata et al., 1995].

For a long time, the regulatory function of the 10 nuclear coded subunits in mammalian cytochrome c oxidase [Kadenbach and Merle, 1981; Kadenbach et al., 1983; Tsukihara et al., 1996], which do not occur in the bacterial enzyme [Iwata et al., 1995], was questioned [Saraste, 1983]. The identification of tissue-, species-, and developmental-specific isoforms of nuclear coded subunits, however, suggested specific regulatory functions. The heart type subunits of VIa, VIIa, and VIII (VIaH, VIIaH, and VIIIH) are expressed in heart and skeletal muscle, whereas the liver type subunits (VIaL, VIIaL, and VIIIL) are ubiquitously expressed [Schlerf et al., 1988; Lightowlers et al., 1990; Kennaway et al., 1990; Seelan and Grossman, 1991; Linder et al., 1995]. Recently the cDNA of an isoform of subunit IV (IV-2) was found and its transcript was observed in the lungs of adult and fetal human and adult rat, as well as in the muscle of fetal human by Northern Blot analysis. In contrast, subunit IV-1 was ubiquitously transcribed in all tissues, including lung [Hüttemann et al., 2001]. Only one isoform was found for subunit VIII in human, for subunit VIIa in rat and for subunit VIa in fish [Grossman and Lomax, 1997; Linder et al., 1995; Hüttemann et al., 1997]. In the adult rat heart, the cytochrome c oxidase subunit VIa consists of two thirds of the heart type isoform (VIaH) and one third of the liver type isoform (VIaL) [Kadenbach et al., 1990], whereas in the skeletal muscle almost 100 % of subunit VIaH is expressed [Anthony et al., 1990]. In fetal heart and skeletal muscle mostly the liver type of subunits VIa and VIIa (VIaL and VIIaL) are expressed, but switch to the heart type isoforms after birth [Taanman et al., 1992; Bonne et al., 1993; Grossman et al., 1995; Parsons et al., 1996]. Interestingly, a different subunit composition of cytochrome c oxidase from wheat germ and wheat seedling suggests the existence of developmental- or tissue-specific isoforms also in the plant enzyme [Peiffer et al., 1990]. In yeast, the expression of subunit V isoforms (corresponding to subunit IV in mammals), Va and Vb, are dependent on the oxygen concentration in the growth medium [Burke and Poyton, 1998]. Different compositions of subunits and isoforms of cytochrome oxidases from various species are listed in Table 1.

Table 1. The comparison of subunits and isoforms of cytochrome oxidases from various species.
The number of subunits including isoforms are listed. The different nomenclatures of subunits from mammals are described next to the subunit number. [1] [Witt and Ludwig, 1997], [2] [Taanmann and Capaldi, 1992], [3] [Bisson and Schiavo, 1986], [4] [Peiffer et al., 1990], [5] [Arnold et al., 1997], [6] [Kadenbach et al., 1986 and 1987; Kadenbach and Reimann, 1992; Hüttemann et al., 2001; Capaldi, 1990 and 1996; Schlerf et al., 1988; Fabrici et al., 1992; van Kuilenburg et al., 1990].

Sub-units	*Paracoccus denitrificans*	*Saccharomyces cerevisiae* (yeast)	*Dictyostelium discoideum* (Slime mold)	*Triticum aestivum* (wheat)	*Thunnus obesus* (tuna)	*Bos taurus* (bovine)	*Rattus norvegicus* (rat)	*Homo sapiens* (human)
	[1]	[2]	[3]	[4]	[5]	[6]		
I	1	1	1	1	1	1	1	1
II	1	1	1	1	1	1	1	1
III	1	1	1	1	1	1	1	1
IV	-	2 Va, b	1 VI	1	2	2	2	2
Va	-	1 VI	1 IV	2	1	1	1	1
Vb	-	1 IV	1 V	1	1	1	1	1
VIa	-	1	-	-	2	2	2	2
VIb	-	1	-	-	1	1	1	1
VIc	-	1 VIIa	2 VII	1 Vc	1	1	1	1
VIIa	-	1 VII	-	-	2	2	1	2
VIIb	-	-	-	-	1	1	1	1
VIIc	-	1 VIII	-	-	1	1	1	1
VIII	-	-	-	-	1	2	2	1
?	1							

1.2.2. Catalytic activity of cytochrome c oxidase

The electron transfer by cytochrome c oxidase from ferrocytochrome c to dioxygen occurs without the formation of intermediate reactive oxygen species (ROS). The reaction is coupled with the translocation of protons from the matrix to the intermembrane space of the mitochondria, and has the following stoichiometry:

$$4 \text{ cytochrome } c^{2+} + 4\ H^{+} + n\ H^{+}_{matrix} + O_2 \rightarrow 4 \text{ cytochrome } c^{3+} + n\ H^{+}_{cytosol} + 2\ H_2O$$

The pathway of electrons from cytochrome c via Cu_A (two electronically coupled copper atoms), heme a, and the binuclear center, composed of heme a_3 and Cu_B, is mostly established [Ludwig et al., 2001]. The fact that cytochrome c oxidase represents the only enzyme which reacts with oxygen without the formation of ROS, is based on the unique structure of the oxygen binding binuclear center, which simultaneously transfers four electrons to the bound dioxygen molecule (see Fig. 2). Oxygen, bound to the reduced binuclear center (Fe^{2+}/Cu_B^{1+}), is immediately reduced to the redox state of water by the uptake of 4 electrons: two from iron (forming the ferri state,

Fe^{4+}), one from copper (forming Cu^{2+}), and the fourth from a tyrosine side chain, forming a radical. The formation of a tyrosine radical is possible by a covalent bridge between Tyr^{280} and His^{276} (nomenclature of the *P. denitrificans* enzyme) which stabilizes the tyrosine radical.

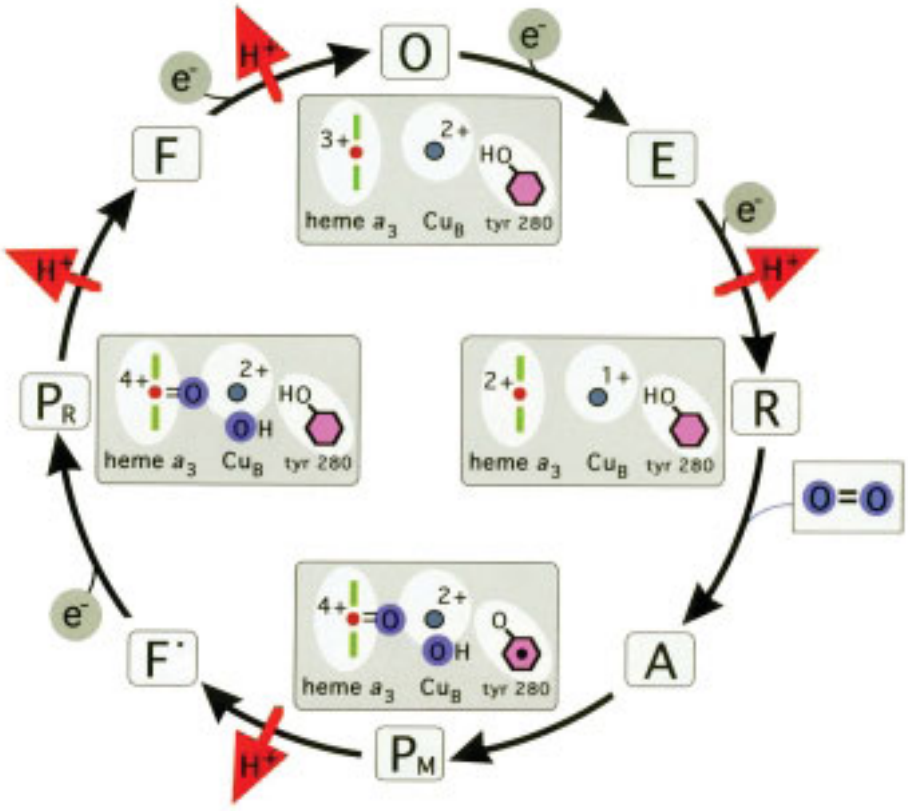


Fig. 2. Simplified scheme of the O_2 reduction cycle catalyzed by cytochrome c oxidase. Key intermediates (white boxes labeled "O", "E", "R" etc.) are listed clockwise along with the input steps for the four electrons (gray circles), the binding of dioxygen (blue), and the presumed transmembrane proton translocation steps (red arrows; according to Michel [1999]). The four gray shaded boxes within the reaction circle detail the states of cytochrome c oxidase, "O", "R", "R_M", "P_R". Proton uptake steps, water release, and the assignment of formal charges to the oxygen atoms have been omitted for clarity.

In contrast to the known mechanism of electron transfer in cytochrome c oxidase, the mechanism of its coupling to the translocation of protons across the membrane is mostly unknown [Michel, 1999]. Two proton pathways, consisting of hydrophilic amino acids and water molecules, have been identified in the bacterial enzyme [Iwata et al., 1995] and were corroborated in the bovine heart enzyme [Tsukihara et al., 1996]. But a third proton pathway, including Asp^{51} as the possible outlet amino acid at the cytosolic side, based on its structural movement between the oxidized and reduced crystal structures of the bovine heart enzyme

[Yoshikawa et al., 1998], could not be found in the bacterial enzyme [Pfitzner et al., 1998; Lee et al., 2000]. It appears that in animals the mechanism of proton translocation in cytochrome c oxidase has been modified during evolution, in order to regulate the stoichiometry of coupling between electron transport and proton translocation (see the results section).

1.2.3. Nucleotide binding sites of cytochrome c oxidase

In bovine heart cytochrome c oxidase seven high affinity binding sites for ATP or ADP and three additional sites only for ADP were identified by equilibrium dialysis with radioactive ATP and ADP [Napiwotzki et al., 1997; Napiwotzki and Kadenbach, 1998]. In the enzyme of *P. denitrificans* only one ATP binding site was identified [Rieger et al., 1995]. One binding site for ADP at the matrix domain of subunit VIa in the bovine heart cytochrome c oxidase had already been postulated by Anthony et al. [1993], based on kinetic, spectroscopic, and immunological data. In the crystal structure of the bovine heart enzyme a bound cholate molecule was observed at this site [Tsukihara et al., 1996]. Cholate has a very similar structure to ADP. Later, ten tightly bound cholate molecules were verified in the crystallized bovine heart enzyme using radioactive cholate [Napiwotzki et al., 1997]. These bound cholates seemed to prevent regulation of enzyme activity by nucleotides (ATP/ADP-ratios) and were very slowly exchanged by ATP, in contrast to ADP [Napiwotzki and Kadenbach, 1998].

1.2.4. Allosteric ATP-inhibition of cytochrome c oxidase

The activity of cytochrome c oxidase is inhibited at high intramitochondrial ATP/ADP-ratios. At high ATP/ADP-ratios, the hyperbolic type kinetics of the enzymes from tuna and bovine were converted into sigmoidal ones (activity/[cytochrome c] or v/[s] relationship) [Arnold and Kadenbach, 1997; Kadenbach and Arnold, 1999]. Based on the crystal structure of the bovine heart enzyme [Tsukihara et al., 1996], a pocket close to the membrane formed by amino acids of subunits I, II, and IV was proposed for the ATP or ADP binding site [Hüttemann et al, 2001]. Allosteric ATP-inhibition was correlated to binding of ATP to the matrix domain of subunit IV, since preincubation of the bovine enzyme with a monoclonal antibody against subunit IV prevented the allosteric ATP inhibition. Half-maximal inhibition of activity was obtained at an intramitochondrial ATP/ADP-ratio of 28, and the inhibition was independent of the proton motive force [Arnold and Kadenbach, 1999]. The maximal Hill-coefficient of 2

[Arnold and Kadenbach, 1997] indicates cooperativity of two cytochrome c binding sites which are assumed to be located at each monomer of the dimeric enzyme.

An allosteric ATP-inhibition was also found with the yeast enzyme, but not with the enzyme from *Rhodobacter sphaeroides* [Follmann et al., 1998] which lacks a subunit similar to subunit IV of the eukaryotic enzyme. Regulation of activity by addition of ATP was also measured with cytochrome c oxidase from cyanobacteria. This oxidase contains a fourth subunit which is homologous to the eukaryotic subunit IV [Alge et al., 1999].

1.2.5. Reversible cAMP-dependent phosphorylation of cytochrome c oxidase

The extent of full ATP-inhibition of cytochrome c oxidase activity at low cytochrome c concentrations varied among different preparations of mitochondria or of the enzyme. It was demonstrated, however, that the concentration range of cytochrome c, where full inhibition of enzyme activity is obtained, can be extended by cAMP-dependent phosphorylation and is abolished by Ca^{2+}-activated dephosphorylation of the enzyme [Bender and Kadenbach, 2000]. Preincubation of isolated bovine liver mitochondria with cAMP, ATP, and an ATP-regenerating system, increased the allosteric ATP-inhibition of the enzyme after solubilization in Tween 20. Remarkably, the enzyme activity was completely inhibited up to 10 μM cytochrome c when EGTA and potassium fluoride, an unspecific inhibitor of protein phosphatases were present during preincubation. Subsequent incubation of mitochondria with Ca^{2+}, after preincubation with cAMP, ATP, and an ATP-regenerating system, abolished the ATP-inhibition of the enzyme. It has been postulated that *in vivo* an equilibrium between cAMP-dependent phosphorylation and dephosphorylation via Ca^{2+}-activated protein phosphatases exists. In isolated cytochrome c oxidase from bovine heart, subunits I, II (and/or III), and Vb were specifically labelled by [γ-^{32}P]ATP after incubation with protein kinase A, cAMP, and ATP [Bender and Kadenbach, 2000].

In other studies, the specific binding of the recombinant regulatory subunit of protein kinase A to cytochrome c oxidase subunit Vb of CHO (Chinese Hamster Ovary) cells was described [Yang et al., 1998]. In addition, phosphorylation of cytochrome c oxidase subunit IV from rat liver by an endogenous cAMP-independent kinase was observed [Steenaart and Shore, 1997]. cAMP-dependent protein kinases in mitochondria have been described repeatedly [Müller and Bandlow, 1987a and 1987b; Burgess and Yamada, 1987; Schwoch et al., 1990; Vallejo and Seguido, 1997], but phosphorylation of a specific subunit of respiratory chain complexes has only been shown for the 18 kDa subunit of the NADH dehydrogenase [Papa et al., 1996].

1.2.6. Variable H^+/e^- stoichiometries in cytochrome c oxidase

The coupling mechanism of electron transport with proton translocation in cytochrome c oxidase is still a matter of discussion [Michel, 1999]. The proton pumping activity of cytochrome c oxidase was first demonstrated by Wikström and coworker [Wikström and Saari, 1977; Krab and Wikström, 1978], but for a long time the stoichiometric ratio between translocated protons and transported electrons (H^+/e^- stoichiometry) remained a matter of discussion. In fact, H^+/e^- stoichiometries between zero [Moyle and Mitchell, 1978; Papa et al., 1980], 1.0 [Casey et al., 1979; Sigel and Carafoli, 1980; Prochaska et al., 1981; Proteau et al., 1983], and 2.0 [Azzone et al., 1979; Reynafarje et al., 1982; Beavis and Lehninger, 1986; Beavis, 1987; de Virville and Moreau, 1990] were determined. It was only recently accepted that the H^+/e^- stoichiometry in cytochrome c oxidase of bacteria [Haltia et al., 1991] and mitochondria [Babcock and Wikström, 1992] is 1.0 and invariable. The different H^+/e^- stoichiometries of previous studies were explained by application of unsuitable methods to determine the correct value. Recent data and the results of this dissertation clearly demonstrate, however, that the H^+/e^- stoichiometry is variable (Table 2).

A H^+/e^- stoichiometry of 0.5 instead of 1.0 was measured in cytochrome c oxidase of the ba_3-type from *Thermus thermophilus* under standard conditions [Kannt et al., 1998]. Decreased H^+/e^- stoichiometries in mammalian cytochrome c oxidase are obtained after removal of subunit III (review in [Prochaska and Fink, 1987; Murphy, 1989]), and after chemical modification with reagents specific for carboxyl groups in hydrophobic environs like DCCD (dicyclohexylcarbodiimide) [Prochaska and Fink, 1987; Murphy, 1989] and EEDQ (N-ethoxycarbonyl-2-ethoxy-1,2-dihydrochinolin) [Steverding and Kadenbach, 1991]. A decrease of H^+/e^- stoichiometry with increasing mitochondrial membrane potential $\Delta\psi_m$ (140-180 mV) was found for cytochrome c oxidase, but not for cytochrome c reductase, by Murphy and Brand [1988]. With isolated mitochondria [Papa et al., 1991], as well as with the reconstituted enzyme [Capitanio et al., 1991 and 1996], a decrease of H^+/e^- stoichiometry with increasing flow rate, i.e. increasing pH-gradient, was found with cytochrome c oxidase, but not with cytochrome c reductase.

Table 2. Variable H^+/e^- stoichiometries of cytochrome c oxidase (COX) were described in the literature.

Species	Tissue	Object	H^+/e^- stoichiometry	Reference
Rat	liver	mitochondria	0.8	Sigel and Carafoli, 1978
Rat	liver	mitochondria	2.0	Azzone et al., 1979
Rat	liver	mitochondria	0.6 - 0.7	Wikström and Pentillä, 1982
Rat	liver	mitoplasts	1.5 - 2.0	Reynafarje et al., 1982
Rat	liver	mitoplasts	1.9 - 2.1	Reynafarje et al., 1986
Rat	liver	mitochondria	1.5	Beavis, 1987
Potato	tuber	mitochondria	1.5 - 2.0	de Virville and Moreau, 1990
Rat	liver	mitochondria	1.0 → 0.0 [$\Delta\psi$ = 120 →180 mV]	Murphy and Brand, 1988
Rat	liver	mitochondria	1.0 → 0.0 [ΔpH = low → high]	Capitanio et al., 1991
Bovine	heart	reconstituted COX	0.6 - 0.75	Sigel and Carafoli, 1980
Bovine	heart	reconstituted COX	1.0 → 0.0 [ΔpH = low → high]	Papa et al., 1991
Bovine	heart	reconstituted COX	1.0 → 0.5 [intraliposomal ADP → ATP]	Frank and Kadenbach, 1996
Bovine	liver, kidney	reconstituted COX	0.5 [intraliposomal ATP or ADP]	Hüttemann et al., 1999
Bovine	kidney	reconstituted COX	1.0 [+ 1 % cardiolipin (CL)]	Lee and Kadenbach, 2001
Bovine	kidney	reconstituted COX	1.0 → 0.5 [+ 1% CL → 1% CL + palmitate]	Lee and Kadenbach, 2001

1.2.7. High ATP/ADP-ratios decrease the H^+/e^- stoichiometry of only heart cytochrome c oxidase

By increasing the intraliposomal ATP/ADP-ratio, a decrease of the H^+/e^- ratio from 1.0 to 0.5 was measured with the reconstituted enzyme from bovine heart [Frank and Kadenbach, 1996; Hüttemann et al., 1999]. The decrease was half-maximal at an ATP/ADP-ratio of 100, and occurred by the exchange of bound ADP by ATP at the matrix domain of subunit VIaH, since preincubation of the enzyme with a monoclonal antibody against subunit VIaH prevented the decrease of the H^+/e^- ratio. The suggested nucleotide binding site at the matrix domain of subunit VIaH [Anthony et al., 1993; Frank and Kadenbach, 1996] was verified in the crystal structure of the enzyme by identification of a cholate molecule at this site, since cholate is structurally very similar to ADP [Tsukihara et al., 1996]. With reconstituted cytochrome c oxidase from bovine liver and kidney, H^+/e^- ratios of 0.5 were measured which were not influenced by the intraliposomal ATP/ADP-ratio or by a monoclonal antibody against subunit VIaH (and subunit VIc) [Frank and Kadenbach, 1996]. It was postulated that the decrease of H^+/e^- ratio of the enzyme from skeletal muscle (subunit VIaH) at high ATP/ADP-ratios participates in thermogenesis (e.g. during sleep) [Kadenbach et al., 1998].

A third type of subunit VIa was identified in fish (trout, carp and tuna), which differs equally from the mammalian isoforms VIaH and VIaL [Hüttemann et al., 2000; Hüttemann et al., 1997]. This suggests that subunit VIa has evolutionary diverged in different species into three different isoforms according to the demands of thermogenesis. It is possible that cytochrome c oxidase from fish, which are poikilotherm animals, have a permanent high H^+/e^- ratio, to prevent wasting of energy by heat production.

1.3. Regulation of cell respiration

1.3.1. The first mechanism of respiratory control

Respiratory control was originally defined as stimulation of oxygen uptake of isolated mitochondria after addition of ADP (active, state 3 respiration) and subsequent inhibition due to transformation of ADP into ATP (controlled, state 4 respiration) [Lardy and Wellman, 1952; Chance and Williams, 1955]. The chemiosmotic hypothesis [Mitchell, 1961 and 1966] postulates that the proton motive force, Δp is the energy-rich intermediate of oxidative phosphorylation which allows the interpretation of respiratory control as follows; ADP uptake by

the ATP/ADP carrier into the mitochondria accelerates ATP synthase activity, accompanied by a decrease of Δp, which consequently stimulates the activity of the three proton pumps of the respiratory chain, and therefore mitochondrial respiration [Nicholls and Ferguson, 1992]. The uncoupling of oxidative phosphorylation in mitochondria by protonophores (uncouplers) [Loomis and Lipman, 1948; Weinbach and Garbus, 1969] strongly supported the chemiosmotic hypothesis which is well established.

Even though the chemiosmotic hypothesis could explain the control of respiration in bacteria [Wilson et al., 1976] and chloroplasts [Hauska and Trebst, 1977], many results with mitochondria were reported which could not be interpretated by, and even conflicted with, the regulation of respiration by Δp [Ghazi, 1985; Slater 1987; Ernster et al., 1959; DiMauro et al., 1976; Weinbach and Garbus, 1965 and 1969; Padan and Rottenberg, 1973; Ho and Wang, 1981; Wilson and Forman, 1982; Zoratti et al., 1982; Mandolino et al., 1983; Starkov et al., 1997a and 1997b]. The relationship between the extent of Δp and the rate of respiration or ATP synthesis is lacking in intact mitochondria, but was found only under restricted conditions, e.g. with aged or uncoupled mitochondria or mitochondrial particles.

1.3.2. The second mechanism of respiratory control

A new mechanism to interpretate the regulation of cell respiration was recently found, the so called "second mechanism of respiratory control", which is based on the inhibition of cytochrome c oxidase activity at high intramitochondrial ATP/ADP-ratios [Kadenbach and Arnold, 1999], as described above. The binding of ATP on subunit IV was already identified by photoaffinity labelling [Montecucco et al., 1986; Bisson et al., 1987; Rieger et al., 1995]. Somehow the sigmoidal relationship between the intraliposomal ATP/ADP-ratio, or intraliposomal [ADP], and the rate of reconstituted cytochrome c oxidase from bovine heart, is very similar to the relationship between the ADP concentration and the rate of respiration measured by ^{31}P-NMR in the human arm *in vivo* [Jeneson et al., 1996]. The allosteric ATP-inhibition of cytochrome c oxidase is sensitive to several experimental and physiological conditions that "uncouple" the second mechanism of respiratory control, thus high ATP/ADP-ratios no longer induce allosteric inhibition of enzyme activity. These parameters include (1) dodecylmaltoside as a detergent which monomerizes the enzyme [Suarez et al., 1984], (2) TMPD (N,N,N′,N′-tetramethyl-p-phenylenediamine) which is frequently used as an electron carrier between ascorbate and cytochrome c, (3) micromolar concentrations of 3, 5-diiodo-L-thyronine which binds specifically to the matrix-oriented subunit Va [Arnold et al., 1998], (4)

submicromolar concentration of palmitate [Shahla Hammerschmidt (1998) Diplomarbeit, Fachbereich Chemie, Universität Marburg], (5) insufficient amounts of cardiolipin in the enzyme [Arnold and Kadenbach, 1997], as well as (6) Ca^{2+}-dependent dephosphorylation of cytochrome c oxidase [Bender and Kadenbach, 2000]. By these parameters the allosteric ATP-inhibition is easily released and the second mechanism of respiratory control is abolished.

1.4. Aims of the present dissertation

The aim of this dissertation was to investigate the regulation of cytochrome c oxidase by isoform expression and by allosteric effectors. Applying molecular biological methods, one aim was to search for the cDNA of the isoform of subunit IV (IV-2) in mammals and birds. Subunit IV-2 had already been identified in yeast and tuna.

In further studies the reversibility of allosteric ATP-inhibition of cytochrome c oxidase by cAMP-dependent phosphorylation and dephosphorylation was investigated. In particular, the location of the phosphorylation site in the enzyme was expected to be identified.

Finally the different H^+/e^- stoichiometries of the isozymes from bovine heart, containing subunit VIaH, and from bovine kidney, containing subunit VIaL, was investigated. In particular the reason for the lower H^+/e^- stoichiometry of the subunit ViaL containing isozyme, which had been measured with the reconstituted enzymes from bovine liver and kidney, compared to the subunit VIaH-containing isozyme from bovine heart, was investigated.

2. Materials

2.1. Tissues

bovine heart, kidney, lung, and testis	slaughterhouse, Marburg
turkey heart, liver, and muscle	from a butcher shop

2.2. Bacteria and vector

E. coli XL1 Blue	Stratagene, Heidelberg
pBluescript KS-	Stratagene, Heidelberg

2.3. Primers

The primers were produced by MWG-Biotec (Ebersberg).

Q_T: 5´-CCA GTG AGC AGA GTG ACG AGG ACT CGA GCT CAA GC$(T)_{17}$-3´

Q_O: 5´-CCA GTG AGC AGA GTG ACG-3´

Q_I: 5´-GAG GAC TCG AGC TCA AGC-3´

IV+1: 5´-GAT TAY CCN YTN CCN GA-3´

IV+3.3: 5´-GAG TST TTY GCN GAR ATG AA-3´

IV+4.8: 5´-AAA GMG TAT GYT NGA YAT G-3´

sequencing primer: 5´-CGC TCT AGA ACT AGT GGA TCC-3´

M [A,C]
N [A,G,C,T]
R [A,G]
Y [C,T]

2.4. Apparatuses

ABI PRISM 301 Genetic Analyzer	Perkin Elmer, Weierstadt
Amicon™ Microcon Filter 100 (blue)	Amicon, Danvers, USA
Clark-oxygen electrode	Yellow Springs Instruments
nanoampere meter and amplifier for Clack-oxygen electrode	institute workshop of Philipps-Universität, Marburg
Biofuge 13	Heraeus Christ, Osterode
cooling centrifuge RC2-B	Sorvall, Stuttgart
JA-10 rotor	Sorvall, Stuttgart
JA-20 rotor	Beckmann, Frankfurt
ultracentrifuge L5-65	Beckmann, Frankfurt
60 Ti rotor	Beckmann, Frankfurt
Digital pH meter pH525 and pH electrode	WTW, Weilheim
DNA Thermal Cycler	Perkin Elmer Cetus, Norwalk, USA
Gene pulser ®II, electroporator	Bio-rad, München
gel electrophoresis apparatus	institute workshop of Philipps-Universität, Marburg
Pegasus Semi-Dry-Blot, blotting apparatus	Phase, Moelln
pH electrode, InLab®423	Mettler Toledo, Gießen
pH meter, Expandomatic IV	Beckmann, Frankfurt
Photometer 1101 M	Eppendorf, Hamburg
Speed-vac concentrator	Bachhofer, Reutlingen
table top microcentrifuge	Eppendorf, Hamburg

UV transilluminator	H. Saur, Reutlingen
Uvikon 940, 2-rays spectrophotometer	Kontron Instruments, Offenbach
Videocopy processor and attached camera	Mitsubishi, Japan

2.5. Kits

ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit	Perkin Elmer, Weiterstadt
Easy Pure, DNA Purification Kit	Biozym, Olendorf
Expand™ High Fidelty PCR System	Boehringer, Mannheim

2.6. Chromatographic materials

Amberlite XAD-2	Serva, Heidelberg
DEAE-Sephacel	Pharmacia, Freiburg
Sephadex G-25	Pharmacia, Freiburg

2.7. Membranes

electromembrane (high sensitivity) and electrolyte for Clark-oxygen electrode	Yellow Springs Instruments
Nitrocellulose Protran Cellulosenitrat	Schleicher und Schuell, Dassel
ProBlot™ PVDF-Membran	Applied Biosystems, Weiterstadt

2.8. Chemicals

All substances were of analytical grade and purchased from Fluka (Deisenhofen), Sigma (München), Roth (Karlsruhe), Merk (Darmstadt) and Riedle-de-Haen, (Seelze). D.W.(deionized water) was obtained from "Reinstwasser-System Seralpur 90 CN" with a macromolecule filter (pore size 0.2µm), SERAL, Ramsbach-Baumbach.

acrylamide	Serva, Heidelberg
agarose, ultrapure	Gibco BRL, Karlsruhe
ascorbate, free form	Merck, Darmstadt
bacto agar	Gibco BRL, Karlsruhe
BSA, fraction V	Sigma, München
choline-Cl	Sigma, München
DEPC	Fluka, Deisenhofen
DNA-BioLadder™ 100	AGS, Heidelberg
guanidinium thiocyanate	Fluka, Deisenhofen
heparin	Roth, Karlsruhe
N-laurylsarcosin, sodium salt	Fluka, Deisenhofen
N,N'-methylenbisacrylamide	Serva, Heidelberg
PEG 6000	Serva, Heidelberg
Pepton 140	Gibco BRL, Karlsruhe
TEMED	Sigma, München
TMPD	Fluka, Deisenhofen
yeast extract	Gibco BRL, Karlsruhe

2.9. Lipids, fatty acids, and derivatives

asolectin (L-α-phosphatidylcholine), type IV-S from soyabean	Sigma, München
cardiolipin, sodium salt in ethanol from bovine heart	Fluka, Deisenhofen
cetylalchol	Sigma, München

laurate, sodium salt	Sigma, München
myristate, sodium salt	Sigma, München
palmitate, sodium salt	Sigma, München
oleate, sodium salt	Sigma, München
palmitoyl CoA	Sigma, München
3-palmitoyl-sn-glycerol	Sigma, München
stearate, sodium salt	Sigma, München

2.10. Detergents

Brij 35	Fluka, Deisenhofen
cholate, sodium salt	Serva, Heidelberg
deoxycholate, sodium salt	Serva, Heidelberg
dodecylmaltoside (laurylmaltoside)	Fluka, Deisenhofen
SDS	Fluka, Deisenhofen
Triton X-100 and Triton X-114	Serva, Heidelberg
Tween 20	Fluka, Deisenhofen

2.11. Dyes

amidoblack 10 B	Roth, Karlsruhe
bromophenol blue	Serva, Heidelberg
Coomassie Brilliant Blue R250	Serva, Heidelberg
DACM	Sigma, München
ethidium bromide	Sigma, München
xylenecyanol FF	Sigma, München

2.12. Antibodies

anti-mouse IgG (whole molecule), alkaline phosphatase conjugated, from goat	Sigma, München
anti-phosphoserine, monoclonal, from mouse	Calbiochem, Darmstadt
anti-phosphothreonine, monoclonal, from mouse	Calbiochem, Darmstadt

2.13. Nucleotides

ADP, potassium salt (98-100 % purity)	Boehinger, Mannheim
ATP, disodium salt (grade I: 99 % purity)	Sigma, München
cAMP, sodium salt (grade I:99 % purity)	Sigma, München
dATP, dCTP, dGTP, dTTP, each PCR-grade (≥ 99 % purity)	Boehringer, Mannheim

2.14. Substrates

BCIP	Sigma, München
cytochrome c, type VI form horse heart	Sigma, München
IPTG	Sigma, München
PEP, potassium salt	Fluka, Deisenhofen
NBT	Sigma, München
X-Gal	Sigma, München

2.15. Uncouplers and reductants

CCCP	Sigma, München
DTE	Sigma, München
DTT	Roth, Karlsruhe
ß-mercaptoethanol	Roth, Karlsruhe

valinomycin	Sigma, München

2.16. Enzymes

Pvu II (*CAG↓CTG)*	Amersham, Braunschweig
Klenow DNA polymerase	Amersham, Braunschweig
RNase H	Amersham, Braunschweig
M-MuLV reverse transcriptase	System (AGS), Heidelberg
phosphatase from shrimp	Amersham, Braunschweig
T4 polynucleotide kinase	Amersham, Braunschweig
T4 DNA ligase	Amersham, Braunschweig
Taq/Pwo DNA polymerase	Boehringer, Mannheim
PK, from rabbit muscle	Merk, Darmstadt
PKA, from bovine heart	Sigma, München
PP1, catalytic subunit, α-isoform from rabbit, recombinant expressed in *E. coli*	Sigma, München

2.17. Antibiotic and inhibitor

ampicillin	Serva, Heidelberg
RNase block (ribonuclease inhibitor)	Stratagene, Heidelberg

3. Methods

3.1. Molecular biological methods

All equipments and D.W. were sterilized by autoclaving at 121 °C for 20 min. Buffers were autoclaved after preparation or prepared with sterilized D.W.

3.1.1. General methods

Preparation of LB medium

Mediums for bacterial culture were sterilized by autoclaving.

LB medium:	10 g	NaCl
	10 g	Pepton 140
	5 g	yeast extract
	10 ml	1 M $MgSO_4$ solution
	5 ml	1 M NaOH
	in 1 l	
LB agar medium:	15 g	bacto agar
	1 l	LB medium

After autoclaving the medium was cooled down to ca. 50 °C and 50 µg/ml ampicillin and X-Gal/IPTG (2.5 ml X-Gal and 0.5 ml IPTG solution per 1 l of medium) were added. LB agar medium was molded in Petri dishes (87 mm diameter). To remove air bubbles in the medium the surface of the plates were shortly exposed to a flame. After molding, the plates were kept at 4 °C for 3 months.

ampicillin stock solution:	50 mg/ml	ampicillin
	dissolved with NaOH and kept at 4 °C	
IPTG stock solution*:	100 mM	IPTG
X-Gal stock solution*:	4 %	X-Gal in DMF (w/v)
	*sterile-filtered and kept at 20 °C in the dark	

Agarose gel electrophoresis of DNA

About 100-200 ml of gel bed volume were applied for an electrophoresis. 1 % agarose in 1 x TBE buffer was melted by boiling. 1.5 % ethidium bromide ($1/10^{-4}$ of gel bed volume), fluorescence dye, was added when agarose got cooled down to ca. 50 °C. The agarose suspension was poured into a tray and a comb was positioned in it for the sample wells. After gelatination of agarose the comb was taken out of and the gel was soaked in 1x TBE buffer. The DNA sample was mixed with 1/6 volume of 6 x sample buffer and loaded in a well of the gel. 0.05-2 µg molecular weight standard DNA ladder (DNA-BioLadder™ 100) was used as a standard. The agarose gel with big wells (size of 2-4 combined wells) was recommended for the DNA elution from the gel. Electrophoresis was performed at 100-150 mA. Separated DNA bands were detected under a transilluminator (at 302 nm).

10 x	TBE buffer:	890 mM	Tris-Cl
		890 mM	boric acid
6 x	sample buffer:	50 %	glycerol (v/v)
		0.25 %	bromophenol blue
		20 mM	EDTA, pH 8.3
		0.25 %	xylenecyanol FF
ethidium bromide solution		10 mg/ml	ethidium bromide stored at 4 °C in the dark

DNA precipitation by Na-acetate and ethanol

To precipitate DNA, 1/10 volume of 3 M Na-acetate, pH 4.5-5 and 3 volumes of ethanol were added to the DNA containing solution. The suspension was incubated at -20 °C overnight or at –80 °C for 1 h and centrifuged for 20 min in a table top centrifuge at maximal speed. The supernatant was discarded and the nucleic acid pellet was briefly washed with cold 70 % ethanol. The pellet was dried by standing at room temperature for 15 min or with a Speed-vac concentrator connected with an oil vacuum pump for 1-2 min. The DNA pellet was dissolved in TE buffer and stored at -20 °C.

TE buffer:	10 mM	Tris-Cl, pH 7.0-8.5
	1 mM	EDTA

Photometric quantitation of DNA and RNA

The concentrations of DNA and RNA were determined spectrophotometrically at the wave length of 260 nm. The absorbance difference at 260 nm and 300 nm was recommended for the accurate determination of concentration. The basal absorbances of TE buffer and D.W., where DNA and RNA were dissolved, were used as baseline of the spectra. The concentrations of different nucleotides were estimated by following molar absorbance coefficients.

$E_{260} = 1 \equiv 50$ µg/ml for double stranded DNA
$E_{260} = 1 \equiv 40$ µg/ml for single stranded DNA or RNA
$E_{260} = 1 \equiv 33$ µg/ml for oligonucleotides or primer

The ratio E_{260}/E_{280} indicated the purity of nucleotides (e.g. a pure sample has in general a value between 1.8 and 2.0).

3.1.2. RNA preparation

General instruction for RNA preparation

Ribonucleases (RNases) do not require any cofactor for their activity and are stable from heat compared to deoxyribonucleases (DNases). To avoid contamination by RNases, careful attention was necessary for each procedure. Glasswares were backed at 240 °C for 3 h and other equipments and buffers including water were autoclaved. In addition 0.1 % DEPC was added to the buffers and water, because DEPC deactivates RNases by cleavage of functional S-S bonds. Sterilization of the working bench with 70 % ethanol and wearing of hand gloves were required during the whole procedures.

Purification of total RNA

Excised tissues from a rat and a turkey were immediately soaked in liquid nitrogen. Total RNA was isolated from these tissues with guanidinium thiocyanate, a strong inhibitor of ribonucleases [Chomczymski and Sacchi, 1987]. 50 mg tissues were homogenized with 500 µl buffer D at room temperature (D-buffer might be kept at room temperature for 3 months in the absence of ß-mercaptoethanol). 50 µl of 2 M Na-acetate, pH 4.0, 100 µl of chloroform/isoamylalcohol (49:1, v/v) and 500 µl of water-equilibrated phenol were added to the homogenate. The mixture was shaken at 4 °C for 10-20 min, incubated on ice for 30-45 min and

then centrifuged at 15,000 g. After centrifugation 3 phases were obtained: an upper phase, an interphase, and a lower phase containing RNA, DNA and aggregated proteins, and phenol/chloroform/isoamylalcohol, respectively. The upper phase was transferred into a new cup carefully, not to be contaminated from the interphase. To precipitate RNA the upper phase solution was mixed with 1 ml of isopropanol and incubated at -20 °C overnight or at -80 °C for 2 h. A RNA pellet was obtained after centrifugation at 15,000 rpm for 30 min. The pellet was dried under the vacuum pump and dissolved in 300 µl of buffer D. The RNA was reprecipitated by addition of 300 µl isopropanol and by incubation as described above. The obtained RNA pellet was rinsed 2 times with 500 µl of 70 % ethanol and dried. The RNA was dissolved in DEPC treated water and kept at - 20 °C.

buffer D:	50 g	guanidinium thiocyanate
(100 ml)	63.4 ml	DEPC treated D.W.
	3,5 ml	0.75 M Na-citrate, pH 7.0
	1.7 ml	30 % N-laurylsarcosin
	720 µl	ß-mercaptoethanol*
		* added directly before the addition of buffer D to the sample

3.1.3. Complementary DNA (cDNA) preparation

Reverse transcription (RT)

Complementary DNA was synthesized from mRNA (in total RNA) by a RT-PCR (reverse transcription-polymerase chain reaction).

Reaction mixture:	1 µl	Q_T primer (10 pmol/µl)
	0.4 µl	DTT
	0.5 µl	RNase Block
	5 µl	10 x dNTP solution (each 2 mM dATP, dCTP, dGTP, and dTTP)
	x µl	2.5-5 µg total RNA
	14,1- x µl	D.W.

The reaction mixture was heated at 80 °C for 3 min to denature mRNA and the primer. Complete denaturation of mRNA and the primer is the key point to get a full length cDNA. Since slow cooling allows the denatured RNA to reform a thermodynamically stable secondary structure, after denaturation the reaction mixture was immediately chilled on ice for 2 min. Then the reverse transcriptase was added together with RT buffer into the mixture because transcriptases

are instable from heat. The mixture was incubated at 37 °C for 90 min. Desired cDNA was synthesized from mRNA with a gene specific primer during incubation.

2.5 µl	10 x incubation buffer for the reverse transcriptase
1.5 µl	M-MuLV reverse transcriptase

10 x incubation buffer for: the reverse transcriptase	500 mM	Tris-HCl, pH 8.3
	60 mM	$MgCl_2$
	400 mM	KCl
	0.5 %	BSA (w/v)

To obtain a complete length cDNA, mRNA was redenatured during the RT reaction. The incubated suspension was heated at 75 °C, chilled on ice for 2 min. The RT reaction was continued after addition of 2 µl of fresh reverse transcriptase to the mixture at 37 °C for 30 min. To stop the RT reaction, the mixture was heated at 70 °C for 15 min.

Purification of cDNA after RT-PCR

The single strand cDNA was obtained from mRNA-cDNA hetero double strands after RT-PCR. The RT mixture was incubated with RNase H at 37 °C for 20 min to eliminate the mRNA.

0.3 µl	RNase H
2.5 µl	5 x incubation buffer for RNase H
22 µl	D.W.

5 x incubation buffer: for RNase H	100 mM	Tris-HCl, pH 7.5
	100 mM	KCl
	50 mM	EDTA
	0.5 mM	DTT

The cDNA was further purified with "Amicon™ Microcon-Filter 100 (blue)" to remove the remaining salts, the primers, and the nucleotides from RT-PCR. This microconcentrator is suitable for 300 bp of single stranded and 125 bp of double stranded polynucleotides. The volume of cDNA containing solution was adjusted to 200 µl with TE buffer and transferred to the filter of the microconcentrator. By centrifugation at 300 g for 15 min other components in the mixture went through out of the filter and the cDNA was left on it. The filter was washed 2 times with 200 µl TE buffer and centrifuged again. The microconcentrator was placed upside down into a new cup and the cDNA was obtained by centrifugation at 600 g for 10-30 min.

3.1.4. Polymerase Chain Reaction (PCR)

A desired region of the prepared cDNA (see in 3.1.3.) was amplified by PCR. Several combined polymerase chain reaction (PCR) techniques, Hot-Start-PCR, Touch-Down-PCR, and RACE-PCR were applied in this dissertation.

Standard-PCR

Both Taq and Pwo DNA polymerases of "Expand^{TM} High Fidelity PCR System" from Boehringer, Mannheim were used for the amplification. The combination of Pwo DNA polymerase which has a 3′,5′-exonuclease activity, and of Taq DNA polymerase resulted in 3 times higher accuracy of DNA synthesis. This combined system might have an error of only ca. 8.5×10^{-6} frequency in contrast to Taq polymerase itself which has an error of ca. 2.6×10^{-5} frequency. PCR mixture with Taq/Pwo DNA polymerase was prepared as following.

PCR mixture:	1-2 µl	0.5-50 ng DNA template
	5 µl	10 x dNTP solution (each 2 mM dATP, dCTP, dGTP and dTTP)
	5 µl	10 x PCR buffer I
	1-1.5 µl	10-50 pmol/µl forward primer
	1-1.5 µl	10-50 pmol/µl reverse primer
	0.5 µl	1 U Taq/Pwo DNA polymerase

Concentrations of 10 pmol/µl for the specific primers (Q_O, Q_I primers, and sequencing primer) and of 50 pmol/µl for the degenerative primers were used. The PCR oil was unnecessary since the thermal cycler had a heating cover. The following 3 steps were repeated for 30-35 times (cycles) in the "DNA Thermal Cycler" from Perkin Elmer.

denaturation:	at 94 °C for 1 min
annealing:	at 50-65 °C for 30 s
elongation:	at 72 °C for 30-60 s

The annealing temperature depends on the melting temperature of the applied primers. The melting temperature was determined by the following equation:

$$T_{annealing} = (4 \cdot (G, C) + 2 \cdot (T, A)) \text{ °C}$$

The elongation time was varied by the length of template, in general 1 kb requires ca. 1 min elongation time.

Hot-Start-PCR

PCR is started at 94 °C in the preheated thermal cycler to denature templates and primers. The denaturation of the sample at 94 °C for 5 min avoids an error which is easily caused by misannealing during the first amplification cycle.

Touch-Down-PCR

This PCR is suitable for amplifications with degenerated primers, because these primers contain different polynucleotides of variable melting temperatures. Most of the amplification steps are identical to Standard-PCR except the annealing step. To optimize annealing of the primers on the template the temperature was continuously decreased every 2 °C during the amplification cycles. i.e. 4 cycles at 58 °C, 6 cycles at 56 °C and 16 cycles at 50 °C.

3′-rapid amplification of cDNA ends-PCR (3′-RACE-PCR)

3′- and 5′-RACE-PCR is the specialized PCR to amplify the 3′- and 5′-region of cDNA respectively [Frohmann, 1995]. In this dissertation, 3-RACE-PCR was applied to search for the cDNA of cytochrome c oxidase subunit IV isoform (IV-2), because 5′-RACE-PCR requires a gene specific primer. A general scheme of 3′-RACE-PCR is shown in Fig. 3. cDNA was synthesized from mRNA with Q_T primer by RT-PCR as described in 3.1.3. Q_T primer (5′-Q_O-Q_I-dT_{17}-3′) consists of 17 bp of poly T sequences, 18 bp of Q_I, and 18 bp of Q_O. These poly T sequences are hybridized with the poly A tail of mRNA. The 3′-end region was amplified from the whole synthesized cDNA with Q_O primer and a degenerated primer (outer-PCR) by Touch-down-PCR. Because a degenerated primer anneals nonspecifically on a template many fragments were obtained after outer-PCR in general. The outer-PCR products were electrophoresed on an agarose gel and desired DNA fragments were extracted (see in 3.1.5.) for a nested-PCR. Nested-PCR was performed by Touch-Down-PCR with Q_I primer and another degenerated primer. This degenerate primer had the sequence which is closer to the 3′-end of cDNA than the sequence of the primer for the outer-PCR. The amplified DNA was electrophoresed and extracted from the gel for cloning.

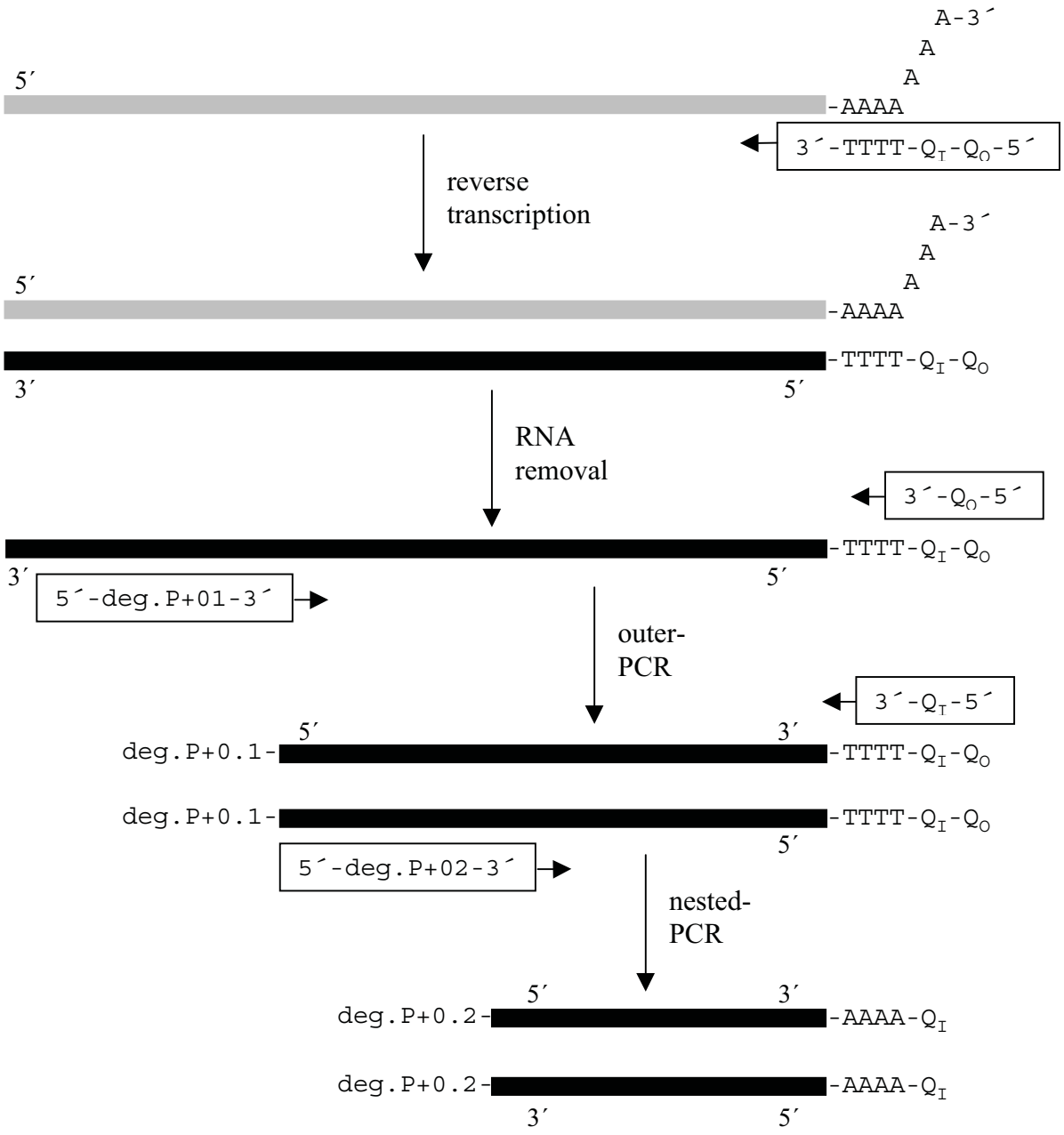


Fig. 3. Scheme of RT-reaction and 3´-RACE-PCR.
RNA is illustrated in gray and DNA is in black. The primers are boxed. deg.P+0.1 and deg.P+0.2 are the degenerated primers for outer-PCR and nested-PCR respectively to amplify the 3´-cDNA end.

3.1.5. Preparation of DNA for cloning

Elution of DNA from agarose gel

DNA was eluted from an agarose gel by "Easy Pure, DNA Purification Kit" from Biozym. After PCR the amplified DNA was electrophoresed on an agarose gel. Desired DNA was selected by calculation of the molecular size. The DNA band was cut out as precise as possible. 50 µl MELT solution and 450 µl SALT solution from the kit were added to 100 mg of the cut gel fragment. The gel was completely melted by incubation at 55 °C. 7-9 µl of well vortexed DNA-binding silicamatrix BIND was added to the melted gel suspension and the mixture was incubated at room temperature for 5-15 min. During incubation the mixture was frequently vortexed. The DNA-silicamatrix was obtained by centrifugation for 5 s and washed 2-3 times with 1 ml WASH solution. The DNA-matrix pellet was dried up with a Speed-vac connected with an oil vacuum pump. To elute DNA from the silicamatrix, 25 µl TE buffer was added to the pellet and incubated at 55 °C for ca. 30 min. The DNA containing supernatant was obtained after centrifugation at room temperature for 10 min. For the elution of the rest DNA from the matrix, the last step could be repeated.

Refilling of dsDNA-ends with Klenow DNA polymerase

About 50 % of the amplified DNA with Taq/Pwo polymerase had an incomplete paired 3´-end with adenosine. This incomplete end of DNA was paired by incubation with Klenow DNA polymerase and nucleotide triphosphates at 37 °C for 30 min. The reaction was stopped by incubation at 72 °C for 10 min.

0.5 µl	2 mM dNTP solution
0.5 µl	4 U/µl Klenow DNA polymerase
2.5 µl	10 x incubation buffer for Klenow enzyme
22 µl	0.5-20 ng DNA

10 x incubation buffer: for Klenow enzyme	0.5 M	Tris-Cl, pH 7.5
	6.7 mM	$MgCl_2$
	10 mM	DTT
	0.5 %	BSA (w/v)

Phosphorylation of DNA

The 5´-end of DNA was phosphorylated to be ligated into a plasmid vector.

10-50 ng	DNA
3.5 µl	10 x incubation buffer for T4 polynucleotide kinase
2.63 µl	0.75 mM ATP
5.27 µl	1:10 diluted T4 polynucleotide kinase

in total 23.7 µl

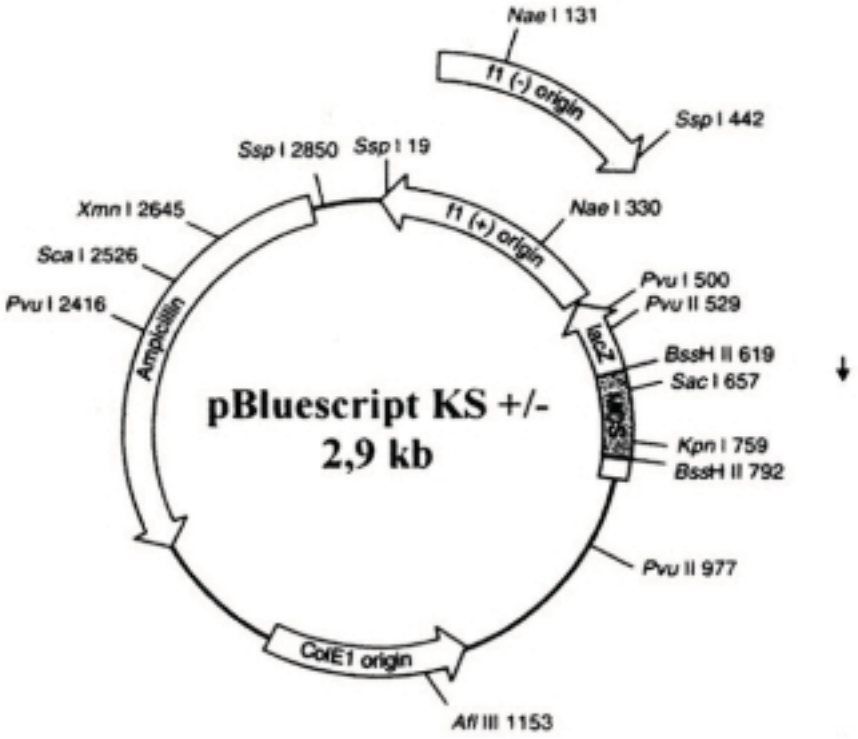
10 x incubation buffer for:	500 mM	Tris-Cl, pH 8.3
T4 polynucleotide kinase	100 mM	$MgCl_2$
	400 mM	KCl
	0.5 %	BSA (w/v)

The phosphorylation was performed by incubation with T4 polynucleotide kinase at 37 °C for 1 hr. The reaction was stopped by incubation at 70 °C for 10 min. The phosphorylated DNA was precipitated with ethanol and dissolved in TE buffer (see in 3.1.1.).

3.1.6. Preparation of vector plasmid for cloning

The plasmid, pBluescript KS- was used as vector. The simplified genetic map and the sequence of multiple cloning site (MCS) of the pBluescript KS +/- vector are presented in Fig. 4. Sites for variable restriction enzymes and for the sequencing primer (see 3.1.12) are indicated.

A



B

```
5′- GGAAACAGCTATGACCATGATTACGCCAAGCTCGAAATTAACCCTCACTAAAGGGAAC
3′- CCTTTGTCGATACTGGTACTAATGCGGTTCGAGCTTTAATTGGGAGTGATTTCCCTTG

                                                          Eco RI
AAAAGCTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCG
TTTTCGACCCATGGCCCGGGGGGGAGCTCCAGCTGCCATAGCTATTCGAACTATAGCTTAA
         Kpn I          Xho I

                                                      Sac I
AATTCCTGCAGCCCGGGGGATCCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTC
    GGACGTCGGGCCCCCTAGGTGATCAAGATCTCGCCGGCGGTGGCGCCACCTCGAG
              3′-CCTAGGTGATCAAGATCTCGC-5′
                  ◄──────────
                  sequencing primer

CAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAA -3′
GTTAAGCGGGATATCACTCAGCATAATGTTAAGTGACCGGCAGCAAAATGTT -5′
```

Fig. 4. The simplified genetic map of pBluescript KS +/- vector (A) and the sequence of its multiple cloning site (MCS) (B).
The sites for several restriction enzymes are presented in B. Site for the sequencing primer which was used in this dissertation (see in 3.1.12) is marked as arrows in A and B.

Dephosphorylation with shrimp alkaline phosphatase

The 5′-region of a vector DNA was dephosphorylated with shrimp phosphatase. The reaction mixture was incubated at 37 °C for 2 h.

2 µl	10 x incubation buffer for shrimp phosphatase
1U	shrimp phosphatase
10 pmol	plasmid DNA
in total 20 µl	

10 x incubation buffer:	200 mM	Tris-Cl, pH 8.0
for shrimp phosphatase	100 mM	$MgCl_2$

The reaction was stopped by incubation at 65 °C for 10 min. The dephosphorylated plasmid was used for the ligation with inserted DNA.

3.1.7. Preparation of competent cells for electrotransformation

E. coli XL-1-Blue was cultivated in LB medium overnight. 4 ml of cultivated *E. coli* suspension was transferred in 400 ml of new LB medium and continuously grown up under shaking (250-350 rpm) in the absence of ampicillin till an $O.D._{590}$ = 0.5-1.0. The cultivated bacterial suspension was transferred to a sterilized Falcon tube and cooled down on ice for 10 min. The bacterial pellet was obtained by centrifugation (6,000 rpm) at 4 °C for 10 min. The precipitated cells were suspended in 50 ml cold and sterilized D.W. and centrifuged at 4 °C for 10 min. The precipitated bacteria were cooled down to 0 °C and suspended in 25 ml cold D.W. *E. coli* was suspended in 1 ml of 10 % glycerol after centrifugation and transferred to Eppendorf cups in aliquots of 40 µl. After shock freezing by liquid nitrogen the cells were kept at -80 °C for several months.

3.1.8. Ligation of DNA with plasmid

The phosphorylated DNA (see in 3.1.5) and the dephosphorylated plasmid (see 3.1.6.) were ligated with T4 DNA ligase. The ratio of DNA:plasmid DNA in the ligation mixture was 1:2 (mol:mol).

ligation mixture:	x µl (150 ng)	DNA (vector and inserted DNA)
	2 µl	10 x incubation buffer for T4 DNA ligase
	1 µl	1 U T4 DNA ligase
	1.5 µl	50 % PEG 6000
	15.5-x µl	D.W.

10 x incubation buffer:	660 mM	Tris-Cl, pH 8.0
for T4 DNA ligase	0.1 M	$MgCl_2$
	100 mM	DTT
	600 µM	ATP

The mixture was incubated at 16 °C for 16 h or at room temperature for 5-6 h.

3.1.9. Transfer of plasmid DNA into *E. coli* by electroporation

Electroporation was performed with "Gene pulser® II" from Bio-rad. The plates of LB agar medium (see in 3.1.1.) were warmed up to room temperature before using. An aliquot of frozen electrocompetent *E. coli* XL-1-Blue cells (see in 3.1.7.) was thawed up carefully on ice and mixed with 1.5 µl ligated plasmid (see in 3.1.8). The mixture was transferred in the middle of metal contact plates of an electroporation cuvette which was precooled on ice. The cuvette was placed in the electroporation apparatus. 2.5 kV electric tension was applied to the cuvette. Statistically each one cell contains one plasmid. After the electric pulse, the *E. coli* was immediately transferred from the cuvette to 1 ml pre-warmed (37 °C) LB medium. The cell suspension was cultivated at 37 °C for 1 h in a roller. 150-450 µl of cultivated bacterial suspension was spreaded on a plate of LB agar medium by a spreader. The plate was incubated at 37 °C overnight. The cells containing plasmids grew as white colonies on the agar medium, in contrast cells which did not contain plasmids grew as blue colonies. The grown bacterial colonies were directly further cultivated in LB medium for the plasmid preparation (3.1.10.) or stored at 4 °C in the medium plate after tight sealing.

3.1.10. Rapid preparation of plasmid DNA by the TENS method

Plasmids were isolated from cultivated *E. coli* (see in 3.1.9.) by the TENS method. A white bacterial colony which contained plasmids was picked up from the agar medium plate and grown in 2 ml liquid medium at 37 °C overnight under vigorous shaking. 1.5 ml of cultivated cell

suspension was centrifuged for 15 min at 14,000 rpm. The supernatant was discarded after autoclaving. To lyse the cells the pellet was vortexed with 300 µl TENS buffer for 2-5 s.

TENS buffer:	10 mM	Tris
	1 mM	EDTA
	0.1 M	NaOH
	0.5 %	SDS

150 µl of 3 M Na-acetate, pH 5.2 was added to the viscous lysed suspension. The time interval between the lysation with TENS buffer and the addition of Na-acetate to the cell pellet should not be longer than 10 min, because long incubation with alkaline TENS buffer causes denaturation of plasmid DNA. The mixture was vortexed for 2-5 s and then centrifuged. The supernatant was transferred into a new cup and incubated with 900 µl of 100 % ethanol which was stored at -20 °C. The precipitated plasmids were obtained after centrifugation for 30 min at 14,000 rpm. The pellet was rinsed 2 times with 1 ml of 70 % ethanol and dried up by a Speed-vac. The plasmid was dissolved in 30 µl TE buffer and kept at – 20 °C.

3.1.11. Digestion of plasmid DNA with Pvu II

After purification, plasmids were digested with the restriction endonuclease Pvu II. Plasmids were incubated with Pvu II (0.5-1 µg DNA/ 1 U enzyme) at 37 °C for 1-2 h.

2 µl	10 x incubation buffer for Pvu II
2 µl	10 U/µl Pvu II
x µl (20 µg DNA)	0.1-1.0 µg/µl DNA solution
16-x µl	D.W.

10 x incubation buffer:	100 mM	Tris-Cl, pH 7.5
for Pvu II:	100 mM	$MgSO_4$
	500 mM	NaCl
	10 mM	DTT

The reaction was stopped by heat-denaturation at 72 °C for 15 min or by addition of 10 mM EDTA.

3.1.12. DNA sequencing

The plasmids, incubated with Pvu II, were electrophoresed on an agarose gel. The plasmids, ligated with desired DNA, were selected and used for sequencing PCR as templates.

To anneal the primer to the template plasmid DNA, plasmids were chemically denatured by incubation with NaOH at room temperature for 15 min and then neutralized with 7 µl of 3 M Na-acetate, pH 4.8.

x µl (3.5 µg)	plasmid DNA
8 µl	2 M NaOH
32-x µl	D.W.

4 µl of D.W. was added to the denatured plasmids. The plasmids were precipitated by overnight incubation with 120 µl of ethanol at –20 °C. After centrifugation the precipitated plasmids were dried and dissolved in 4 µl of D.W.

Sequencing PCR was performed with "ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit" from Perkin Elmer and the sequencing primer (see in Material): denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and elongation at 60 °C for 3 min, with 25 cycles. With dNTP and ddNTP, tagged with base specific fluorescence markers, DNA fragments of variable length were obtained, which were labeled at the 3´-end.

x µl	400-800 ng DNA
1 µl	5 pmol primer
2.5 µl	termination mixture
6.5-x µl	D.W.

For the precipitation of the amplified DNA the PCR mixture was mixed with 10 µl TE buffer, and incubated with 50 µl of 100 % ethanol and 2 µl of 3 M Na-acetate, pH 5.2. After centrifugation at 14,000 rpm at 4 °C for 30 min, the pellet was washed 2 times with 70 % ethanol. The pellet was dried and dissolved in 30 µl of Template Suppression Reagent (TSR) from the kit. The DNA in TSR was denatured at 95 °C for 5 min and analysed by "ABI PRISM 301 Genetic Analyzer" from Perkin Elmer.

3.2. Protein chemical methods

Cytochrome c oxidase was isolated either from mitochondria by the Triton X-100 method [Kadenbach et al., 1986] or from mitochondrial particles (Keilin-Hartree particles) by the cholate method [Yoshikawa et al., 1977 and 1988]. Purification was performed in a cold room. Equipments and buffers were placed on ice (0-4 °C) during the whole isolation procedure. To obtain phosphorylated cytochrome c oxidase, 10 mM KF (nonspecific inhibitor of protein

phosphatases) and 2 mM EGTA were included in all isolation buffers. In most cases the isolation of cytochrome c oxidase was performed by standard procedures (without KF and EGTA).

3.2.1. Isolation of mitochondria

isolation buffer for :	20 mM	Tris-HCl, pH 7.4
heart, kidney, testis,	250 mM	D(+)-saccharose
and lung	2 mM	EDTA
isolation buffer for:	10 mM	Tris-HCl, pH 7.2
skeletal muscle	110 mM	D(-)-mannitol
	70 mM	D(+) saccharose
	100 mM	KCl
	0.2 mM	EDTA
	0.2 mM	EGTA
	50 U/ml	heparin

Bovine heart, kidney, testis, and lung were obtained from a slaughterhouse in Marburg and turkey heart, liver, and muscle were purchased from a butcher shop. The tissues were used directly for the isolation of mitochondria or kept at -80 °C for further preparation. In the latter cases, the frozen tissues were thawed up on ice overnight in a cold room for the isolation of mitochondria. The connective tissue and fat were removed from the tissue sample. The sample was cut into cubes (each about 5 cm) and minced by a meat grinder. The minced tissue was homogenized 3 times for 15 s (with 30 s intervals) with 5 volumes of isolation buffer by a commercial blender (from Krups). During homogenization, the pH of the homogenate was adjusted to pH 7.4 with unbuffered 2 M Tris. The homogenate was centrifuged at 650 g (2000 rpm, JA-10 rotor) for 10 min in a cooling centrifuge. The mitochondria containing supernatant was filtered through a cheese cloth to remove connective tissue. To obtain more mitochondria, the cell sediment was rehomogenized with isolation buffer and centrifuged as described above. After centrifugation of the combined supernatant at 16,300 g (10,000 rpm) for 20 min (Table 3), the mitochondrial pellet was obtained. The pellet was homogenized by a potter homogenizer with isolation buffer. To remove actin and myosin, which are easily coprecipitated with muscle mitochondria, the mitochondrial suspension was centrifuged at 370 g (1,500 rpm, JA-10 rotor) for 5 min. The pellet was discarded and the supernatant was transferred into a new tube. Mitochondria were obtained after centrifugation at 16,300 g. The mitochondrial fraction was washed 2-3 times by centrifugation with isolation buffer. The mitochondrial pellet was homogenized in a small amount of isolation buffer. The mitochondrial suspension was either

directly used for purification of cytochrome c oxidase by a Triton X-100 method or stored at -80 °C.

Table 3. Centrifugation conditions for isolation of mitochondria from various tissues (RCF = relative centrifugal force).

Tissues	Sediment	RCF (g)	Speed (rpm)	t (min)
Heart	cell fragments	650	2,000	10
	mitochondria	16,300	10,000	20
Muscle	cell fragments	370	1,500	10
	mitochondria	19,700	11,000	20
Liver	cell fragments	650	2,000	10
	mitochondria	16,300	10,000	20

3.2.2. Purification of cytochrome c oxidase by the Triton X-100 method

Cytochrome c oxidase was purified from isolated mitochondria using the nonionic detergents Triton X-114 and Triton X-100, followed by DEAE-Sephacel chromatography, exchange of the nonionic detergent by cholate, and fractionated precipitation with ammonium sulfate ($AmSO_4$) [Kadenbach et al., 1986].

phosphate-stock solution:	1 M	KPi, pH 7.4
Triton X-114:	20 %	Triton X-114 (w/v)
washing buffer:	200 mM	KPi, pH 7.4
extraction buffer:	200 mM 5 %	KPi, pH 7.4 Triton X-100 (w/v)
equilibration buffer:	50 mM 0.1 %	KPi, pH 7.4 Triton X-100 (w/v)
elution buffer:	200 mM 0.1 %	KPi, pH 7.4 Triton X-100 (w/v)
$AmSO_4$ solution:	saturated and buffered to pH 7.4 with concentrated NH_4OH at 4°C	
anion column chromatography:	DEAE-Sephacel	

Extraction by Triton X-114 and Triton X-100

To the mitochondrial suspension (20 mg protein/ml) the phosphate stock solution was added to a final concentration of 200 mM under stirring. Heart mitochondria were solubilized by adding 2 ml of 20 % Triton X-114 and the suspension was incubated for 15 min under stirring. For the mitochondria from liver, kidney, testis, and lung 1 ml of Triton X-114 was applied. Solubilized mitochondria were ultracentrifuged at 250,000 g (48,000 rpm, 60 Ti rotor) for 30 min. The heme aa_3 content of the supernatant was determined with a spectrophotometer (difference spectrum, 3.2.1.4.). Normally no heme aa_3 was detected in the supernatant after Triton X-114 treatment. The cytochrome c oxidase pellet was washed with washing buffer using a potter homogenizer and centrifuged. Cytochrome c oxidase in the pellet was dissolved with Triton X-100 extraction buffer. After centrifugation at 250,000 g (48,000 rpm, 60 Ti rotor) for 20 min a green supernatant (dark brown at high enzyme concentration) was obtained. The heme aa_3 content of the supernatant was determined spectrophotometrically. The extraction step was repeated until all heme aa_3 was extracted from the sediment.

DEAE-Sephacel anion exchange chromatography

The enzyme containing supernatant was 1:4 (v/v) diluted with D.W. and loaded on a pre-equilibrated DEAE-Sephacel column. Unbound proteins were washed out with two column volumes of equilibration buffer. Cytochrome c oxidase was eluted with elution buffer and fractionated into portions of ca. 2 ml. The green fractions, containing enzyme were combined.

Purification of cytochrome c oxidase by fractionated ammonium sulfate precipitation

1.5 % (w/v) Na-cholate was dissolved in the combined green column fractions and the pH was adjusted to pH 7.4 with 1 N NaOH by pH paper. Saturated ammonium sulfate ($AmSO_4$) solution was added drop by drop under stirring until a saturation of 28 % was reached and the pH was readjusted. Readjustment of pH was required whenever $AmSO_4$ was added. The solution was stirred overnight (at least longer than 14 h) and centrifuged for 15 min at 27,000 g (15,000 rpm, JA-20 rotor). $AmSO_4$ was added to the supernatant to 37 % saturation, stirred for 30 min and centrifuged. Further fractionations were done with 42 % and 46 % $AmSO_4$ (and 55 % for the lung enzyme). The precipitated proteins (at 28 %, 37 %, 42 %, 46 %, and 55 % $AmSO_4$ saturation) were dissolved in small amounts of mitochondrial isolation buffer and kept at -80 °C.

3.2.3. Isolation of cytochrome c oxidase by the cholate method

Cytochrome c oxidase from bovine heart was also isolated from Keilin-Hartree particles in the presence of cholate according to Yoshikawa et al. [1977 and 1988].

Isolation of Keilin-Hartree particles

800 g of fresh bovine heart from the slaughterhouse, or of frozen heart which was kept at -80 °C, was cut into 6 pieces. One piece (ca. 130 g) of tissue was homogenized with 100 ml buffer I and 750 ml water/ice by a commercial blender at maximal speed for 10 min.

buffer I:	200 mM	NaPi, pH 7.4
buffer II:	20 mM	NaPi, pH 7.4
buffer III:	100 mM	NaPi, pH 7.4

The homogenate was centrifuged at 4,500 rpm (JA-10 rotor) for 10 min. The supernatants were collected and the pellet was resuspended with buffer II in a final volume of 750 ml and centrifuged at 4,500 rpm for 10 min. The supernatants were combined and the pH was adjusted to pH 5.15-5.2 with 30 % acetic acid. After centrifugation at 4,500 rpm for 25 min, precipitated mitochondrial fragments (Keilin-Hartree particles) were obtained. The pellet was washed with 3 l of D.W. and centrifuged at 55,000 rpm (JA-10 rotor) for 20 min. The mitochondrial fragments were suspended in buffer III to a protein concentration of 30 mg/ml. In general, a suspension of 340 ml mitochondrial fragments (30 mg/ml) was obtained from 800 g bovine heart.

Isolation of cytochrome c oxidase

Whenever proteins were precipitated by addition of ammonium sulfate, the pH of the suspension was adjusted to pH 7.2-7.4 with NaOH using a pH electrode under continuous stirring.

buffer IV:	40 %	Na-cholate (w/v)
	100 mM	NaPi, pH 7.4
buffer V:	2 %	Na-cholate (w/v)
	100 mM	NaPi, pH 7.4

buffer VI:	0.5 %	Na-cholate (w/v)
	100 mM	NaPi, pH 7.4
buffer VII:	0.34 %	Brij 35 (w/v)
	100 mM	NaPi, pH 7.4
buffer VIII:	0.21 %	Brij 35 (w/v)
	100 mM	NaPi, pH 7.4
buffer VIIII:	10 mM	NaPi, pH 7.4

29.6 ml of buffer IV (3.2 % final concentration of cholate) was added to 340 ml of the mitochondrial fragment suspension and $AmSO_4$ (powder) was immediately added to a final saturation of 33 %. After 30 min incubation, the suspension was centrifuged at 11,000 rpm (JA-20 rotor) for 10 min. $AmSO_4$ was added to the supernatant to 50 % saturation, incubated for 10 min, and centrifuged for 10 min at 11,000 rpm. The pellet from 50 % $AmSO_4$ precipitation was dissolved in buffer V to a final volume of 200 ml and fractionated again with $AmSO_4$. The suspension was incubated with $AmSO_4$ for 10 min (for 35 % and 55 % $AmSO_4$ saturation) and for 30 min (for 25 % $AmSO_4$ saturation). The scheme of fractionation with $AmSO_4$ is presented below (COX = cytochrome c oxidase).

Detergent (buffer)	Final concentration of $AmSO_4$		Total volume
2 % Na-cholate (buffer IV)			200 ml
	25 % COX in supernatant		
		50 % COX in pellet	
0.5 % Na-cholate (buffer V)			200 ml
	25 % COX in supernatant		
		35 % COX in pellet	
0.5 % Na-cholate (buffer V)			135 ml
	25 % COX in supernatant		
		35 % COX in pellet	
0.34 % Brij 35 (buffer VI)			200 ml
	25 % COX in supernatant		
		35 % COX in pellet	
0.21 % Brij 35 (buffer VII)			200 ml

Standing overnight at 4 °C

	25 % COX in supernatant		
		35 % COX in pellet	
0.21 % Brij 35 (buffer VIII)			135 ml
	25 % COX in supernatant		
		33 % COX in pellet	

The final precipitated pellet was dissolved in buffer VIIII as concentrated as possible and dialysed overnight against the same buffer at 4 °C to remove excess detergent. The dialysed cytochrome c oxidase was centrifuged at 19,000 rpm for 20 min and stored at -80 °C after shock freezing with liquid nitrogen.

3.2.4. Spectrophotometric determination of cytochrome concentrations

Difference spectrum

The concentration of heme aa_3 in turbid enzyme solutions or in solubilized mitochondria was calculated from the difference spectrum between air-oxidized and dithionite-reduced enzyme with a spectrophotometer (Uvikon 940, Kontron Instruments, Offenbach). The applied extinction coefficient for cytochrome aa_3 was $\varepsilon_{605\text{-}630\ nm\ (red\text{-}ox)} = 24\ mM^{-1} \cdot cm^{-1}$ [von Jagow and Klingenberg, 1972; Büge and Kadenbach, 1986].

measuring buffer:	200 mM	KPi, pH 7.2
	5 %	Triton X-100 (w/v)
		stored at 4 °C

50 µl sample was dissolved in the measuring buffer and incubated for ca 3 min. To remove turbid particles a brief centrifugation was followed. The supernatant was distributed in two quartz cuvettes and the baseline was recorded. A few grains of sodium dithionite were added to the sample cuvette and mixed well. After 2-3 min the difference spectrum was recorded.

Absolute spectrum

The purity and concentration of isolated cytochrome c oxidase were determined from the absolute spectra of air-oxidized and sodium dithionite-reduced enzyme. The value of the ratio E_{280}/E_{420} of the oxidized enzyme was a parameter of its purity [Kadenbach et al., 1986]. The lower value means the purer enzyme.

measuring buffer:	200 mM	KPi, pH 7.2
	2 %	Na-cholate (w/v)
	0.5 %	Na-deoxycholate (w/v)
		kept at 4 °C

The baseline of the spectrophotometer was measured. The air-oxidized spectrum of 1:100 diluted cytochrome c oxidase was measured from 250 to 650 nm against the buffer in the reference cuvette. A few grains of sodium dithionite were added to the sample cuvette, and the reduced spectrum was measured after 2-3 min incubation. The following extinction coefficients were applied.

cytochrome aa_3 : $\varepsilon_{605\text{-}630\ nm\ (red\text{-}ox)} = 24\ mM^{-1} \cdot cm^{-1}$
[von Jagow and Klingenberg, 1972]

$\varepsilon_{605\text{-}650\ nm\ (red)} = 40\ mM^{-1} \cdot cm^{-1}$

$\varepsilon_{421\text{-}490\ mm\ (red)} = 140\ mM^{-1} \cdot cm^{-1}$

$\varepsilon_{443\text{-}490\ mm\ (red\text{-}ox)} = 204\ mM^{-1} \cdot cm^{-1}$
[Wikström et al., 1981]

cytochrome c: $\varepsilon_{550\ nm\ (red\text{-}ox)} = 21\ mM^{-1} \cdot cm^{-1}$
[Papa et al., 1983]

3.2.5. Determination of protein concentration

A modified biuret method [Kadenbach, 1966] was applied to determine high amounts of protein (from 0.1 to 5 mg), in particular for mitochondrial suspensions.

biuret reagent :

1.5 g	$CuSO_4 \cdot 5\ H_2O$
4.5 g	potassium sodium tartrate
4.0 g	NaOH
2.5 g	KI

dissolved in 500 ml D.W. and stored at room temperature in the dark

The protein sample was diluted with D.W. to a final volume of 2 ml and 200 µl 3 M trichloroacetic acid (TCA) was added. After incubation for at least 30 min at room temperature, the suspension was centrifuged at 250 g (1500 rpm, Biofuge 13 from Heraeus Christ) for 10 min. The pellet was dissolved in 2 ml biuret reagent and incubated for further 15 min. After dilution with 2 ml D.W. the absorbance of the sample was measured at 546 nm before (E_0) and after (E_{KCN}) addition of solid KCN in an Eppendorf photometer (Photometer 1101M) with a 2 cm cuvette. The protein concentration was calculated as follows:

$$(E_0 - E_{KCN}) \cdot 7 = \text{mg protein / 200 µl sample}$$

3.2.6. SDS-polyacrylamide gel electrophoresis (SDS –PAGE)

The polypeptide pattern of cytochrome c oxidase was examined by SDS-PAGE according to Kadenbach et al. [1983]. The applied system uses 18 % acrylamide and high amounts of urea (6 M) in the separation gel, like that for DNA sequencing.

<buffers>

acrylamide solution:	50 %	acrylamide (w/v)
	1.3 %	N, N‘-methylenbisacrylamide (w/v)
separation gel buffer:	0.75 M	Tris-HCl, pH 8.3
	7.2 M	urea
	0.2 %	SDS
stacking gel buffer:	0.125 M	Tris-HCl, pH 6.8
	0.125 %	SDS (w/v)
electrophoresis buffer:	25 mM	Tris
	192 mM	glycine
	0.1 %	SDS (w/v)
staining solution:	0.25 %	Coomassie Brilliant Blue R250 (w/v)
	10 %	acetic acid (v/v)
	30 %	isopropanol (v/v)
destaining solution:	7.5 %	acetic acid (v/v)
	5 %	ethanol (v/v)

<gels>

separation gel:	2.9 g	urea
18 % acrylamide (w/v)	7.5 ml	acrylamide solution
	10 ml	separation gel buffer
	50 µl	8.3 % ammonium peroxidisulfate solution
	10 µl	TEMED
stacking gel:	2 ml	acrylamide solution
10 % acrylamide (w/v)	4 ml	stacking gel buffer
	25 µl	8.3 % ammonium peroxidisulfate solution
	5 µl	TEMED
sample buffer for COX:	62.5 mM	Tris-Cl, pH 6.8
	20 %	glycerol (w/v)
	2-8 %	SDS (w/v)
	0.01 %	bromophenol blue (w/v)
	5 %	β-mercaptoethanol (v/v)

20-40 µg enzyme was denatured with 10-20 µl of sample buffer at room temperature for 2 h under shaking. The readjustment of pH of separation gel buffer was necessary for each gel preparation to get a good resolution. A gel size of 230 x 160 x 0.75 mm (length x width x thickness) was used, because longer gels have in general a better resolution power. Polymerization of the acrylamide gel was started by addition of ammonium peroxidisulfate and TEMED. The stacking gel was molded on the separation gel and a comb was positioned in it. After polymerization (for ca. 90 min) the comb was removed from the gel and wells were rinsed with electrophoresis buffer. The denatured enzyme samples were loaded in the wells and the electrophoresis was run. For better separation electrophoresis was first run for 45 min at low voltage (90 V), followed by separation overnight at 235 V.

Staining of protein bands with Coomassie Blue

Staining and destaining of gels was done at room temperature under shaking. Gels were taken from the apparatus and gently rinsed with D.W. to remove remaining urea. They were soaked in staining buffer and stained for 1 h. Then gels were carefully washed with D.W. and destained overnight under slow shaking in destaining buffer until the protein bands were clearly distinguished from the background.

staining buffer:	10 %	acetic acid (v/v)
	30 %	isopropanol (v/v)
	0.25 %	Coomassie Brilliant Blue R250 (w/v) stored after filtration
destaining buffer:	5 %	ethanol (v/v)
	4.5 %	acetic acid (v/v)

3.2.7. Labelling of SH-groups in cytochrome c oxidase with DACM

With the fluorescence thiol reagent DACM (N-(7-dimethylamino-4-methyl-3-coumarinyl) maleinimide) SH-groups in proteins can be detected in a pmol range (Yamamoto et al., 1977). The adduct of DACM to cysteine residues of proteins exhibits a fluorescence if illuminated under UV light (Fig. 5). Cytochrome c oxidase was solubilized in 1.5 % Na-cholate, 50 mM KPi, pH 7.4, and reduced with 25 mM DTE at room temperature for 1 h. The reduced enzyme was precipitated with $AmSO_4$. The enzyme pellet was dissolved in 20 µl sample buffer for electrophoresis at a final concentration of 20 µM. 1 mM DACM, dissolved in aceton, was added (50 times excess to enzyme) to the enzyme suspension. The enzyme was denatured at

room temperature for 2 h under shaking and electrophoresed. After SDS-PAGE, the gel was fixed with destaining buffer for SDS-PAGE and photographed under UV light (at 302 nm).

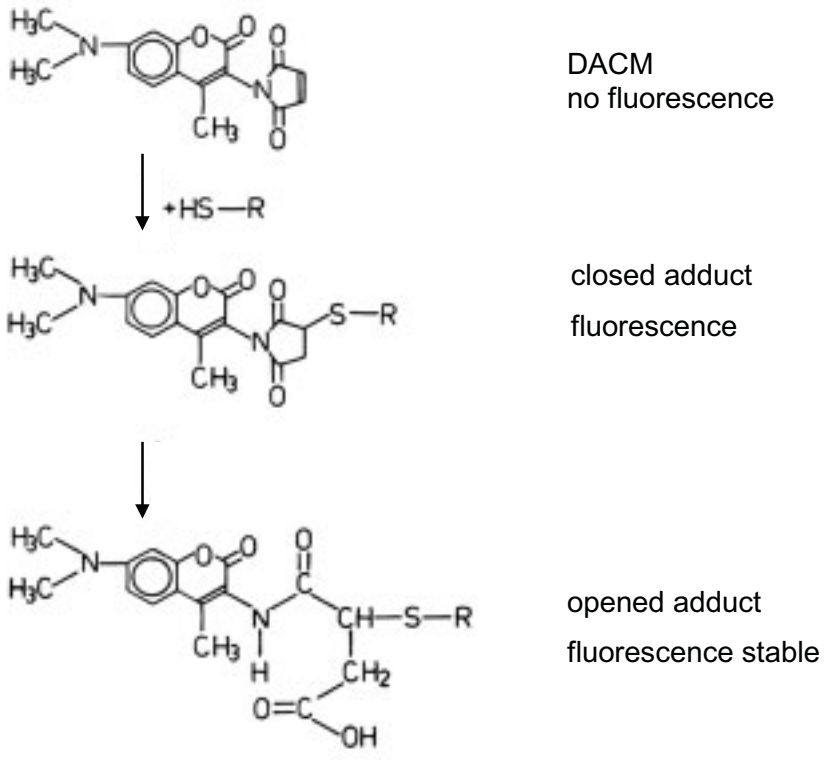


Fig. 5. Chemical structures of DACM and its adducts

3.2.8. Electroblotting of proteins onto nitrocellulose and PVDF membrane

For N-terminal amino acid sequencing and Western Blot analysis, the polypeptides of a gel separated by SDS-PAGE, were transferred onto PVDF or nitrocellulose (Nitrocellulose Protran Cellulosenitrat) membranes by "semi-dry-blotting" at room temperature [Eckerskorn et al., 1988]. For N-terminal amino acid sequencing special care had to be taken to avoid contamination by other proteins and amino acids like glycine which exists everywhere. PVDF membranes (Problot^{TM} PVDF-Membran, Applied Biosystems, Weiterstadt) were prefered for N-terminal amino acid sequencing of small amounts of protein, since its hydrophobicity prevented blotting-through of proteins, and because more protein is bound after prolonged transfer time,

compared to nitrocellulose. For electroblotting the apparatus "Pegasus Semi-Dry-Blot" with plate electrodes from glass carbon was used [Kyhse-Andersen, 1984].

<buffers for nitrocellulose membranes>		
cathode buffer:	25 mM	Tris
	40 mM	α-amino caproic acid
	20 %	methanol (v/v)
anode buffer I:	30 mM	Tris
	20 %	methanol (v/v)
anode buffer II:	300 mM	Tris
	20 %	methanol (v/v)
<buffers for PVDF membranes>		
anode buffer:	50 mM	Na-borate, pH 9.0
	20 %	methanol (v/v)
cathode buffer	50 mM	Na-borate, pH 9.0
	5 %	methanol (v/v)

PVDF membranes were washed in methanol for a short time (20 s) to obtain complete wetting of the membrane for soaking with buffer. The remaining methanol was removed by 20 min washing with anode buffer including 3 times change of the buffer. The nitrocellulose membrane was wetted in anode buffer I. The electrophoresed gel was cut into the size of the membrane and shortly washed with D.W., as was done with the plate electrodes of the blotting apparatus. In the so-called "sandwich" procedure was placed above the cathode plate successively: 5 blotting papers, soaked in cathode buffer I, the gel, the nitrocellulose membrane, soaked in anode buffer I, and finally 5 papers soaked in anode buffer II. For blotting onto PVDF membranes only one anode buffer was used. Air bubbles in the "sandwich" were removed by rolling a glass rod under pressure. Finally the cathode plate was put on top of the sandwich and a weight of 2 kg was added. The transfer time was 30-60 min for nitrocellulose membranes and about 5 h for PVDF membranes at 370-410 A/gel (ca. 1 mA/cm^2) [Eckerskorn et al., 1988].

Staining with amido black and Coomassie Blue

After blotting, the transfer of proteins on nitrocellulose membranes was controlled by reversible staining with amido black [Kadenbach et al., 1990]. The membranes were stained for 3-5 min in staining buffer I. Destaining was done with D.W. The membranes were directly used for immunodetection with antibodies or stored at 4 °C in wet. For permanent staining of PVDF

membranes with Coomassie Blue, the membranes were put for 1-2 min into staining buffer II after blotting. For destaining the membranes were first washed with D.W. and then several times with 50 % methanol (v/v) until the bands were clearly distinct from the background. In order to remove remnants of urea and glycine, the stained membranes were washed at 4 °C for 24 h in excess D.W. Afterwords the interested bands were cut out and sent for N-terminal amino acid sequencing.

staining solution I:	0.025 %	amido black 10B(w/v)
	22 %	ethanol (v/v)
	7.5 %	acetic acid (v/v)
staining solution II:	0.1 %	Coomassie Brillant Blue R250 (w/v)
	30 %	isopropanol (v/v)
	10 %	acetic acid (v/v)
destaining solution:	50 %	methanol (v/v)

3.2.9. N-terminal and internal amino acid sequencing

To determine the primary structure of a protein the "Edman-degradation" with an automatic protein sequencer was performed [Edman and Begg, 1967]. The N-terminal amino acid is reacted with phenylisothiocyanate. From the formed phenylthiocarbamoyl derivative the cyclic phenylthiohydantoin- (PTH) derivative of the N-terminal amino acid is cleaved off under mildly acidic conditions. The one amino acid shortened peptide is then reacted for the same cycle. To assure no loss of the peptide during the reagent changes, the peptide is immobilized on a hydrophobic membrane. After each cycle of about 20 min the cleaved PTH-amino acid is washed off and identified by its retention time during combined HPLC, so that the sequence of cycles and the registration of the HPLC chromatogram is computer programmed. For N-terminal sequencing of cytochrome c oxidase subunits, blotted on PVDF membranes, the amino acid sequencer model 477 A (Applied Biosystems, Weiterstadt) was used and performed by Dr. D. Linder, Biochemisches Institut, Klinikum, Universität Gießen and by Prof. F. Lottspeich, MPI für Biochemie, Martinsried.

For internal amino acid sequencing, the gel after SDS-PAGE was stained with Coomassie Brilliant Blue (staining buffer II). Destaining was done only with 10 % acetic acid (v/v), since methanol inhibits the analysis of internal amino acid sequences. The desired protein band was excised from the gel and soaked in D.W. to remove urea and excess glycine. The gel band was

sent to and analyzed by Prof. F. Lottspeich, MPI für Biochemie, Martinsried. The gel band was incubated with trypsin which cleaves a protein at the carboxyl end of lysine and arginine residues. The cleaved peptide fragments were eluted from the gel and separated by HPLC, and either the mass of individual peptides was determined by MALDI-TOF, or the N-terminal amino acid sequence was determined [Eckerskorn and Lottspeich, 1989].

3.3. Measurement of cytochrome c oxidase activity

3.3.1. Preparation of ferri- and ferro-cytochrome c solutions

Ferrocytochrome c solutions were prepared for proton translocation measurements. 40-50 mg of cytochrome c from horse heart (Sigma) were dissolved in measuring buffer for proton translocation and reduced with sodium dithionite. Excess reductant was removed by gel chromatography on Sephadex G-25 (Pharmacia) which was equilibrated with the same buffer.

measuring buffer:	1 mM	K-Hepes, pH 7.2
for proton translocation	100 mM	choline-Cl
	5 mM	KCl

The concentration of ferrocytochrome c was determined by the difference spectrum at 550 nm before and after addition of K-hexacyanoferrat-(III). $\varepsilon_{550\ nm\ (red\text{-}ox)} = 21\ mM^{-1} \cdot cm^{-1}$. The reduced cytochrome c solution was freshly prepared for each measurement.

For respiration measurements of solubilized and reconstituted cytochrome c oxidase, ferri-cytochrome c solutions were prepared. Cytochrome c was dissolved in D.W. and the exact concentration was determined by a spectrophotometer.

3.3.2. Purification of asolectin and XAD-2

Asolection (L-α-phosphatidylcholine, type IV-S from soyabean, Sigma) was purified to eliminate oxidized products [Kagawa and Racker, 1971]. 5 g asolectin were dissolved in 100 ml pure aceton, stirred at 4 °C for 48 h and filtrated. The undissolved remnant was washed with aceton and dissolved in 20 ml diethylether. The suspension was filtrated again. The solvent of the clear solution was evaporated by a rotavapor and the asolectin was obtained in solid form. The purified asolectin was kept at -20 °C under argon gas.

XAD-2 was purified before use. 5 g Amberlite XAD-2 from Serva was mixed with 100 ml of 10 mM NaCl, 100 mM Na_2CO_3 and stirred at room temperature for 15 min at low speed [Müller et al., 1986]. XAD-2 beads were filtrated and washed with 1 l of D.W. The beads were further stirred in 100 ml methanol for 15 min. Again the XAD-2 was filtrated and washed with 1 l D.W. This washing step was repeated. The purified XAD-2 was kept at 4 °C in D.W.

3.3.3. Preparation of cytochrome c oxidase with regulatory properties

For the preparation of cytochrome c oxidase with regulatory properties, the 10 bound cholate molecules of the isolated enzyme [Napiwotzki et al., 1997 and 1998] had to be exchanged by nucleotides and cardiolipin had to be added [Arnold and Kadenbach, 1997]. The ethanol of cardiolipin (5 mg/ml in ethanol, Fluka) was evaporated by N_2 gas. 1 % cardiolipin (w/v) was dissolved in solubilization buffer containing 0.5 mM ATP. 3 µM cytochrome c oxidase were dissolved in cardiolipin containing solubilization buffer.

solubilization buffer:	50 mM	KPi, pH 7.4
	1 %	Tween 20 (w/v)
	1 mM	EDTA

To remove the bound cholate from the nucleotide binding sites of cytochrome c oxidase the enzyme suspension was dialysed against 400 volumes of solubilization buffer in the presence of 0.5 mM ATP at 4 °C for 4 h. The buffer was changed and dialysis was continued overnight.

3.3.4. Reconstitution of cytochrome c oxidase for activity measurements

reconstitution buffer I:	10 mM	K-Hepes, pH 7.4
	40 mM	KCl
	1.5 %	Na-cholate (w/v)

Cytochrome c oxidase was reconstituted in asolectin vesicles using the hydrophobic Amberlite XAD-2. 40 mg/ml asolectin lipids, containing 5 % cardiolipin, were sonicated with reconstitution buffer I several times for 1 min with 2 min intervals (ca. 25 % output, Branson Sonifier) until the asolectin was dissolved, i.e. the suspension became clear. The asolectin suspension was put in an ice bath during sonication to avoid heating up. Then 5 µM cytochrome c oxidase and 5 mM ATP or 5 mM ADP were mixed with the asolectin suspension. The

detergent (cholate) was removed by incubation with XAD-2 (100 mg/ml) at 4 °C for 22 h under shaking [Shechter and Bloch, 1971].

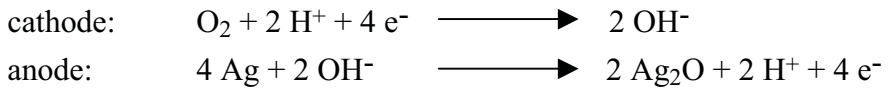
3.3.5. Phosphorylation and dephosphorylation of cytochrome c oxidase

To investigate the effect of phosphorylation on the enzyme activity, cytochrome c oxidase was incubated with cAMP-dependent protein kinase (PKA) and ATP. The solubilized and reconstituted enzyme were incubated at 30 °C for 20 min and at 37 °C for 40 min, respectively, with 50 µM cAMP, 300 U/ml PKA, and an ATP-regenerating system (10 mM K-phosphoenolpyruvate (PEP), 2 U/ml pyruvate kinase (PK), 5 mM $MgSO_4$). For the time dependency of phosphorylation on enzyme activity, the solubilized enzyme was incubated at 30 °C for 0-60 min.

Solubilized cytochrome c oxidase (final concentration 2.5 µM) was dephosphorylated by incubation with 500 U/ml catalytic subunit of protein phosphatase 1 (PP1) and 100 mM $MnCl_2$. Dephosphorylation was performed either with the enzyme isolated in the presence of 10 mM KF and 2 mM EGTA, or with enzymes isolated in the absence of KF and EGTA but afterward phosphorylated by incubation with PKA.

3.3.6. Measurement of enzyme activity by the polarographical method

Oxygen uptake was measured polarographically with a Clark-type electrode [Kielly, 1963; Ferguson-Miller et al., 1978] in a closed system at 25 °C. At 700 mV the following chemical reactions occur at the electrodes:



The measured current corresponds to the oxygen concentration in the measuring cell. To calibrate the electrode, the measuring cell was filled with oxygen saturated deionized water of 25 °C. Corresponding calibration was done with measuring buffer. The calculation of oxygen content in water was based on Gmelin [1958], that of the measuring buffer by correlation of the currents measured in water and measuring buffer. The time dependent changes of the current was documented by a recorder. The turnover number (TN) of cytochrome c oxidase was defined as:

$$TN = \frac{\text{consumed oxygen (1/4 } O_2\text{) (}\mu\text{M)}}{\text{concentration of heme } aa_3\text{, (}\mu\text{M)} \cdot \text{time (s)}}$$

The ascorbate respiration of cytochrome c oxidase (final concentration 50-150 nM heme aa_3) was measured at increasing cytochrome c concentrations (0-20 µM). Respiratory control ratios (RCR) were determined at a constant concentration of cytochrome c (10 µM). The calculation of all kinetic measurements and the graphical presentation were done using the program "Origin 5.0".

Activity measurement of cytochrome c oxidase with regulatory properties

The activity of cytochrome c oxidase with regulatory properties (solubilized enzyme) (final concentration 150 nM), was measured with 18 mM K-ascorbate, 5 mM ATP or 5 mM ADP in 50 mM KPi, pH 7.4, 1 % Tween-20, 1 mM EDTA at increasing concentrations of cytochrome c. If indicated, an ATP-regenerating system (10 mM PEP, 2 U/ml PK, and 5 mM $MgSO_4$) was included for keeping maximal ATP/ADP-ratios, since already 2 % of ADP completely released the allosteric ATP-inhibition of cytochrome c oxidase [Arnold and Kadenbach, 1997]. If mentioned, 0.7 mM N,N,N',N'-tetramethyl-p-phenylenediamine-hydrochloride (TMPD), an electron carrier between ascorbate and cytochrome c, was added during measurement.

Activity measurement of reconstituted cytochrome c oxidase

Proteoliposomes (50 nM heme aa_3) were suspended in 10 mM K-Hepes, pH 7.4, 40 mM KCl. With 18 mM K-ascorbate, and 5 mM ATP or 5 mM ADP, the oxygen consumption was measured at increasing concentrations of cytochrome c. For the uncoupled respiration, 1 µM valinomycin and 3 µM CCCP were added into the measuring vessel before titration with cytochrome c.

Determination of respiratory control ratio (RCR)

RCRs of proteoliposomes were calculated from the ratios of respiration rates under uncoupled and coupled conditions. Proteoliposomes (50 nM heme aa_3) were suspended in 10 mM K-Hepes, pH 7.4, 40 mM KCl with 18 mM K-ascorbate, 5 mM ATP or 5 mM ADP. The oxygen consumption was measured at 10 µM cytochrome c in the presence (uncoupled) and

absence (coupled) of 1 µM valinomycin and 3 µM CCCP (carbonylcyanide m-chlorophenylhydrazone).

$$RCR = \frac{\text{respiration in the presence of uncoupler - basal respiration}}{\text{respiration in the absence of uncoupler - basal respiration}}$$

3.4. Measurement of proton translocation

3.4.1. Reconstitution of cytochrome c oxidase for proton translocation measurements

For measurement of proton translocation in cytochrome c oxidase proteoliposomes, high buffer capacity inside and low buffer capacity outside of the vesicles are required.

reconstitution buffer II:	100 mM	K-Hepes, pH 7.2
	1.5 %	Na-cholate
	10 %	DTE
dialysis buffer I:	10 mM	K-Hepes, pH 7.2
	27 mM	KCl
	73 mM	saccharose
	0.5 %	DTE
dialysis buffer II:	1 mM	K-Hepes, pH 7.2
	30 mM	KCl
	79 mM	saccharose
	0.5 mM	DTE

The enzyme was reconstituted in asolectin vesicles according to Frank and Kadenbach [1996]. 3 µM cytochrome c oxidase containing proteoliposomes were prepared with reconstitution buffer II as described under 3.3.4. If not otherwise indicated, 1 % (0.4 mg/ml) cardiolipin was added before sonication. To investigate its effect on proton translocation of cytochrome c oxidase, 0-1.5 % 3-palmitoyl-sn-glycerol was added before sonication in the presence and absence of 1 % cardiolipin. After reconstitution the proteoliposomes were dialysed against 400 volumes of dialysis buffer I at 4 °C for 4 h, followed by overnight dialysis against 400 volumes of dialysis buffer II, in order to decrease the buffer capacity outside of the proteoliposomes.

3.4.2. Proton translocation measurements using a pH electrode

The H^+/e^- stoichiometry of reconstituted cytochrome c oxidase was measured by the reductant-pulse method. Into a thermostatic (25 °C) open vessel, stirred mechanically from the top, was given 1.4 ml 1 mM K-Hepes, pH 7.2, 100 mM choline-Cl, 5 mM KCl. After addition of 80 µl proteoliposomes (0.2 µM heme aa_3) and 1 µg/ml valinomycin, the pH of the measuring system was adjusted to the pH of the ferrocytochrome c solution with a microcombination pH electrode (InLab®423, Mettler Toledo) connected to a Beckman Expandometric IV pH meter. After addition of 4.9-7.6 nmol ferrocytochrome c (8 enzyme turnover), the pH changes were recorded. The redox-linked pH changes, elicited by pulses of ferrocytochrome c, were calibrated with small aliquots (2-3 µl) of 10 mM HCl. The alkalinization due to water formation was measured in the presence of 3 µM CCCP. For calculation of H^+/e^- stoichiometries, the peak of acidification after addition of ferrocytochrome c was used [Frank and Kadenbach, 1996].

For investigation of the effects of fatty acids and their derivates on proton translocation of cytochrome c oxidase, they were preincubated at 4 °C for 30 min (0-75 min for palmitate) with the proteoliposomal suspension. Before measurement, the temperature of the mixture was adjusted for 5 min at 25 °C. Because of their low solubilities, the Na-salts of fatty acids were dissolved in alkaline solvent at high temperature. Laurate (C12), myristate (C14), palmitate (C16), stearate (C18), oleate (C18-1, Δ^9) were dissolved in hot 1 mM NaOH. The solutions of cetylalcohol (1-hexadecanol) and palmitoyl CoA were prepared in ethanol and in D.W., respectively.

3.4.3. Proton translocation measurement using a stopped-flow spectrophotometer

Proton pumping experiments using a stopped-flow spectrophotometer [Sarti et al., 1985a] were performed in collaboration with Maarten Ruitenberg in the group of Prof. K. Fendler, MPI of Biophysics, Department of Biophysical Chemistry, Frankfurt/Main. The proteoliposome suspension (750 nM heme aa_3, 12.5 µM valinomycin, and 85.7 µM phenol red) was mixed with 31.25 µM reduced cytochrome c (ca. 10 TN) by pressure in a thermostatic chamber. The absorbance changes of phenol red were measured in the presence and absence of 6 µM CCCP at 555.6 nm, a wavelength that was strictly isosbestic for the respective cytochromes. As a control, the change of the absorbance with oxidized cytochrome c was measured. The pH of all solutions used in the experiments was carefully adjusted to pH 7.300, not to give any absorbance changes unconnected with the enzymatic reduction. Calibration of the observed signal was performed by

the change in absorbance of trypsin-catalyzed hydrolysis of N-α-tosyl-L-arginyl-O-methyl ester (TAME, Sigma) as described by Sarti et al. [1985b]. TAME is hydrolyzed by trypsin as follows:

$$RCOOCH_3 + 2\,H_2O \xrightarrow{\text{trypsin}} RCOO^- + CH_3OH + H_2O + H^+$$

3.4.4. Proton conductivity measurement of proteoliposomes

The K^+ diffusion potential-driven proton conductivity of liposomes was measured with a pH electrode as described [Papa et al., 1987; Steverding and Kadenbach, 1990]. 80 µl of cytochrome c oxidase vesicles containing inside 100 mM K-Hepes were suspended in measuring buffer for proton translocation (1 mM K-Hepes, pH 7.2, 100 mM choline-Cl and 5 mM KCl) to a final volume of 1.4 ml. This resulted in a potassium ion gradient of about 100 mM inside and 8 mM outside of the vesicles. By addition of 1 µM valinomycin potassium ions move from the inside of the vesicles to the outside driven by the concentration gradient. A steeper increase of pH change after addition of valinomycin indicated a higher proton conductivity. The system was calibrated with 2 µl of 10 mM HCl.

3.5. Immunochemical methods

Colorimetric detection of immunoreactivity in Western Blots using alkaline phosphatase

The sequence of manipulations for immunochemical detection with alkaline phosphatase [DeBlas and Cherwinski, 1983; Blake et al., 1984] is the following:

1. separation of protein samples by SDS-PAGE
2. protein transfer on nitrocellulose
3. blocking of the membrane with 1 % BSA (w/v)
4. incubation with primary antibody
5. incubation with alkaline phosphatase conjugated secondary antibody
6. color detection by NBT and BCIP

Western Blot analysis is based on an antigen-antibody reaction. The primary antibody and the secondary antibody recognize and bind to the antigen protein and the primary antibody, respectively.

PBS:	150 mM	NaCl
	20 mM	NaPi, pH 7.4
washing buffer:	0.5 %	Tween 20 (w/v)
(PBS-T)		in PBS
blocking reagent:	1 %	BSA (w/v)
		in PBS
coloring buffer:	0.1 M	Tris-HCl, pH 9.5
	0.1 M	NaCl
	0.05 M	$MgCl_2$
stock solution :	100 mg	NBT/1.5 ml 70 % DMF (v/v)
for the color detection	50 mg	BCIP/1 ml DMSO
		kept at -20 °C in aliquots
coloring reagent:	75 ml	coloring buffer
	330 µl	NBT
	250 µl	BCIP

To avoid contamination of other proteins, careful treatments in the whole procedure were required. The subunits of cytochrome c oxidase were separated by SDS-PAGE and blotted onto nitrocellulose (see in 3.2.8.). After electroblotting, the membrane was stained with staining buffer I, to confirm that all proteins were transferred onto the membrane. The membrane was immediately destained with D.W. after the blotted bands became visible. The membrane was incubated with blocking reagent at room temperature for 2 h or at 4 °C overnight. Covering of the membrane surface with bovine serum albumin (BSA) reduced nonspecific interactions of antibodies and other non-antigenic proteins. The membrane was washed 2 times for 10 min with PBS-T buffer and incubated with the primary antibody. 1 µg/ml monoclonal antibodies against phosphoserines and against phosphothreonines raised from mice (Calbiochem), were incubated with the membranes in PBS buffer at room temperature for 1-2 h under shaking. Nonspecifically bound antibodies on the membrane were removed by washing 2 times for 5 min with PBS-T and 2 times with PBS. For 1-2 h the secondary antibody, anti-mouse IgG conjugated with alkaline phosphatase, was incubated with the membranes in 1:1000 dilution with PBS. The membrane was washed again as above. By incubation of the membrane with freshly prepared coloring reagent the protein bands appeared in color on the membrane. To stop the color reaction, the membrane was washed with D.W.

4. Results

4.1. Search for the cDNA of a new isoform of subunit IV (IV-2)

The expression of two isoforms of subunit V in yeast, (Va and Vb), which are homologous to mammalian subunit IV, is known [Poyton et al., 1988]. The expression of two isoforms is regulated by the oxygen concentration in the growth medium [Allen et al., 1995]. Recently two isoforms of subunit IV were identified in cytochrome c oxidase from tuna fish [Hüttemann, 2000], which has the same 13 subunit composition like the mammalian enzyme [Arnold et al., 1997]. The presence of isoforms in yeast and fish, as well as indirect studies by immunohistochemistry in rat [Kuhn-Nentwig and Kadenbach, 1985] and human tissues [Nakagawa and Miranda, 1987; Romero et al., 1990] strongly suggest the existence of subunit IV isoforms also in mammals and birds.

Table 4. Expected cDNA sizes after the amplification of 3′-regions with variable primers.

Primer	Sequence	Expected 3′-region cDNA size
IV+1	5′-GATTAYCCNYTNCCNGA-3′	ca. 520 bp
IV+3.3	5′-GAGTSTTTYGCNGARATGAA-3′	ca. 350 bp
IV+4.8	5′-AAAGMGTATGYTNGAYATG-3′	ca. 240 bp

In order to seek for the cDNA of a second subunit IV (IV-2) in mammals and birds, 3'-RACE-PCR screening with multiple degenerated primer sets was performed. The primers were designed from the conserved regions of the known human, bovine, and rat subunit IV (Fig. 6). 3'-untranslated regions (ca 110 bp), poly-A tails (ca 20 bp), and the appended sequences of Q_I primers (17 bp) were expected to be amplified by PCR together with the 3'-coding region of subunit IV. The expected size of 3'-region cDNA after amplification with different primers are presented in table 4.

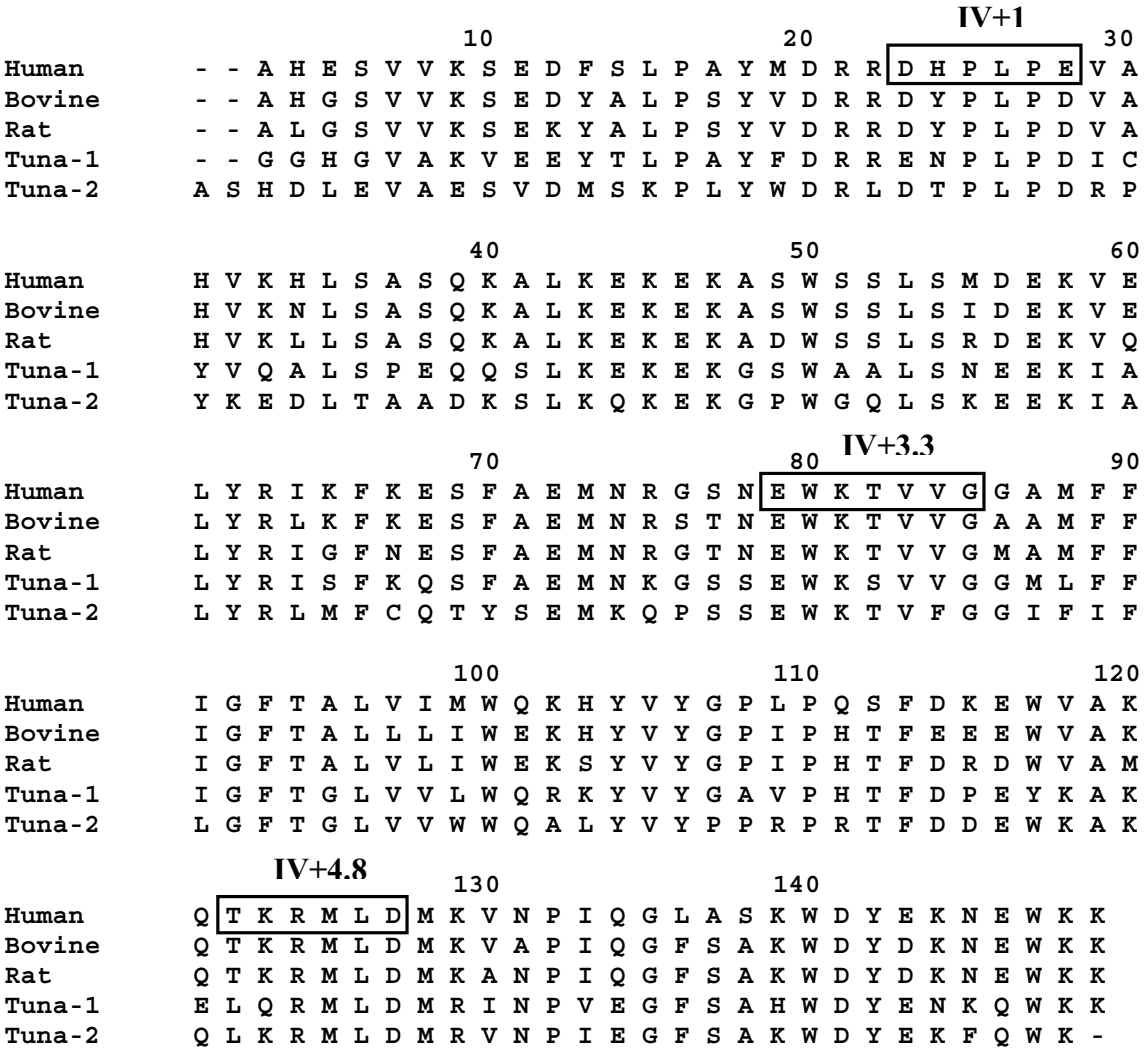


Fig. 6. Amino acid sequence alignment of matured subunit IV from human [Zeviani et al., 1987], bovine [Sacher et al., 1979], rat [Gopalan et al., 1989; Goto et al., 1989], and tuna [Hüttemann, 2000].
Designed degenerated primers IV+1, IV+3.3 and IV+4.8 were deduced from boxed amino acid sequences.

4.1.1. Cloning and sequencing of subunit IV cDNA from rat

cDNAs were prepared from mRNAs of rat liver, heart, and muscle by RT-PCR. To get the 3'-region of subunit IV-2 cDNA, mRNA was amplified by Touch-down-PCR with primers IV+1 and Q_O. Fig. 7 shows the products of the outer-PCR from liver (lane 8), heart (lane 9), and muscle (lane 10). The ca. 350 bp cDNAs were extracted from the gel and amplified with primers IV+4.8 and Q_I by a following nested-PCR.

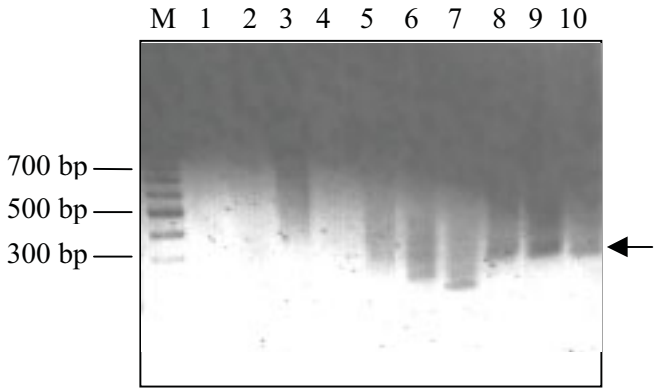


Fig. 7. Outer-PCR for the amplification of cytochrome c oxidase subunit IV from rat liver, heart, and muscle.
cDNAs were amplified with primers IV+3.3 (75 pmol) and Q_O (15 pmol) in 50µl volume by Touch-down-PCR: at 94 °C for 45 s (denaturation) with 1 cycle; for 45 s at 60 °C down to 48 °C for every 2 cycles with 2 °C decrease (annealing); at 72 °C for 50 s with 35 cycles (elongation). Each 20 µl of amplified PCR products were electrophoresed on an agarose gel containing 1.2 % ethidium bromide. The fractions from lane 8, 9, and 10, indicated by an arrow, were extracted and amplified by a following nested-PCR. Lane 8: liver; lane 9: heart; lane 10: muscle; lane 1-7: products from 3´-RACE-PCR of subunit VIa of cytochrome c oxidase from turkey tissues. M: DNA molecular size ladder.

The amplified DNA from the nested-PCR is presented in Fig. 8. Since the part of 3´-untranslated regions between IV-1 and IV-2 cDNA was expected to vary, different sized fragments were extracted. The fragments of 400 bp and 350 bp from muscle, 350 bp, 300 bp, and 250 bp from liver and 300 bp, 250 bp, and 220 bp from heart were selected. The extracted DNAs were cloned into pBluescript KS- plasmids and the plasmids were transferred into *E. coli* XL-1-Blue by electroporation (see in 3.1.9.). The transformed cells were cultivated on the LB agar medium at 37 °C overnight.

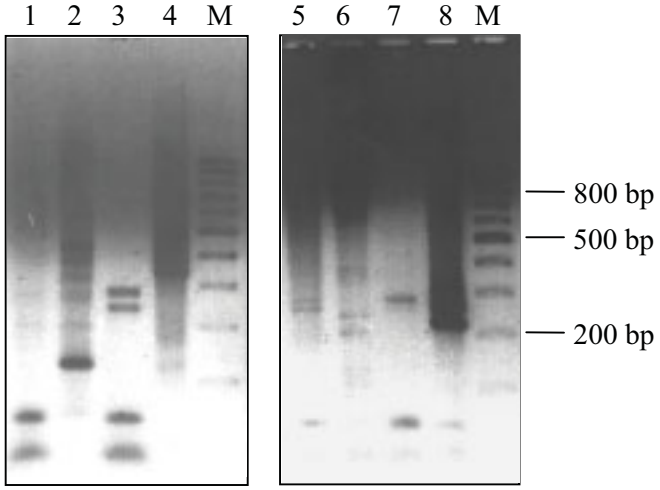


Fig. 8. Nested-PCR for the amplification of cytochrome c oxidase subunit IV cDNA from rat muscle, liver and heart.
1µl of 1:100 diluted outer-PCR product (Fig. 7) was amplified with primers IV+4.8 and Q_I by Touch-down-PCR as described in Fig. 7. The amplified products were electrophoresed on agarose gels. The selected cDNA fractions were cloned into pBluescript KS- plasmids. Fraction 1-2: 400 and 350 bp from lane 2; fraction 3-4: 300 and 250 bp from lane 3; fraction 5: 350 bp from lane 4; fraction 6: 250 bp from lane 6; fraction 7: 300 bp from lane 7; fraction 8: 220 bp from lane 8; lane 1-2: muscle; lane 3-4: liver; lane 5-8: heart; M: DNA molecular size ladder.

10-20 bacterial colonies were selected from each transformed cell which was grown on the agar plate. The selected cells were further cultivated in LB medium. Plasmids were isolated from the cultivated *E. coli* by the TENS method (see in 3.1.10) and incubated with the restriction enzyme Pvu II (see in 3.1.11.). The digested plasmids were electrophoresed on agarose gels. Among the obtained fragments after digestion some which are expected to contain a subunit IV-2 cDNA (as inserted DNA) were selected. The plasmids containing the selected fragments were used directly for sequencing-PCR with "ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit". The sequences were analyzed by the "ABI prism 310 Genetic Analyzer". Most fragments did not contain cDNAs of subunit IV-2, since their sequences were not homologous to subunit IV-1 cDNA except fragments A to E. The fragments A to E turned out to be subunit IV-1 cDNA (Fig. 14).

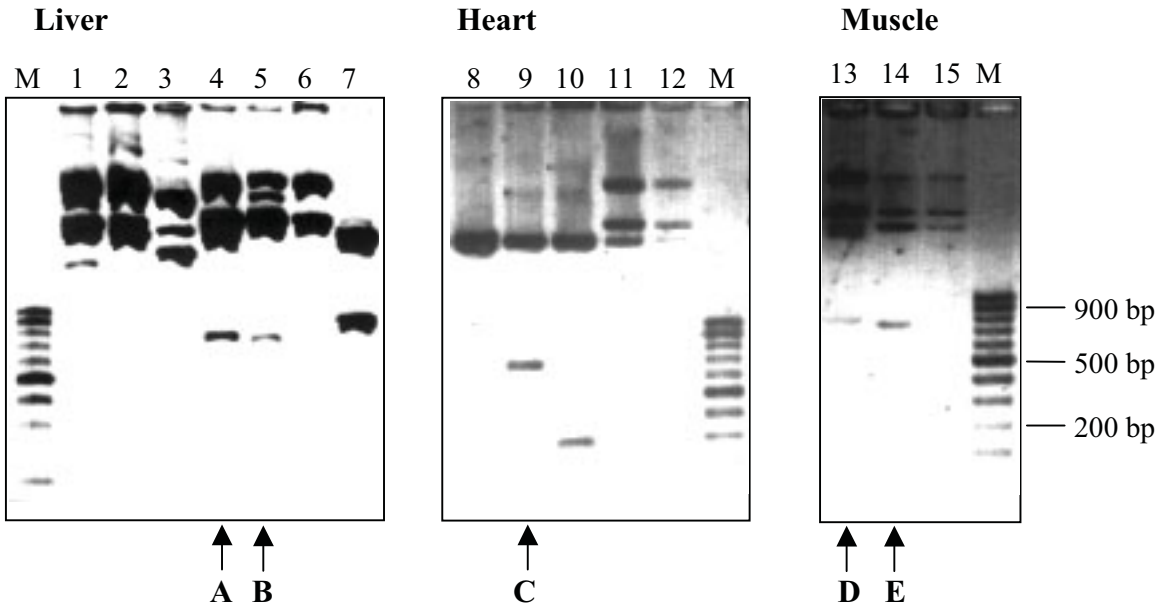


Fig. 9. Characterization of the cloned plasmids of 3'-region subunit IV cDNA from rat liver (C) heart (A) and muscle (B) by incubation with Pvu II.
Plasmid DNAs were prepared by the TENS method. The inserted DNA and the 450 bp of vector DNA were cleaved by Pvu II (Fig. 3). The digested plasmids were seperated on agarose gels. Fragments (A to E) indicated by arrows are corresponding to the subunit IV-1 cDNA. Lanes 1-5: fraction 4 from nested-PCR (Fig. 8); lanes 6-7: fraction 3; lanes 8-11: fraction 8; lane 12: fraction 7; lanes 13-15: fraction 2; M: DNA molecular size ladder.

4.1.2. Cloning and sequencing of subunit IV cDNA from turkey

cDNAs were prepared from mRNAs of turkey liver, heart, and muscle by RT-PCR and amplified by Touch-down-PCR with primers IV+1 and Q_O to get the 3'-region of subunit IV-2 cDNA. Fig. 10 presents the products of the outer-PCR from heart (lane 1), muscle (lane 2), and liver (lane 3). The ca. 600-800 bp cDNAs were extracted from the gel and amplified by the following 1^{st} nested-PCR with primers IV+3.3 and Q_I (Fig. 11).

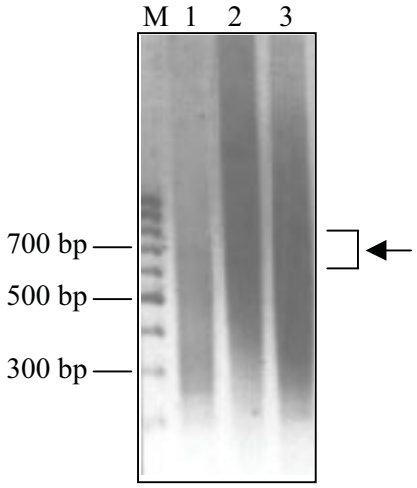


Fig. 10. Outer-PCR for the amplification of cytochrome c oxidase subunit IV cDNA from turkey heart, muscle, and liver.
cDNAs were amplified with primers IV+1 and Q_O by Touch-down-PCR (described in detail in Fig. 7). The amplified PCR products were electrophoresed on an agarose gel. The fractions from 600 to 800 bp regions, indicated by an arrow, were extracted and amplified by the following 1st nested-PCR. Lane 1: heart; lane 2: muscle; lane 3: liver; M: DNA molecular size ladder.

Fig. 11 presents the amplified DNAs from the 1st nested-PCR which was performed with IV+3.3 and Q_I primers. The regions of ca 300-350 bp from liver, heart and muscle were extracted from the gel and further amplified by the 2nd nested-PCR with primers IV+4.8 and Q_I .

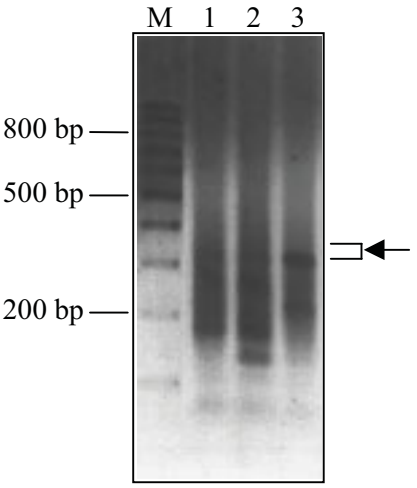


Fig. 11. 1st nested-PCR for the amplification of cytochrome c oxidase subunit IV cDNA from turkey liver, muscle, and heart.
The outer-PCR products (Fig. 10) were amplified with primers IV+3.3 and Q_I by Touch-down-PCR (described in detail in Fig. 7). The amplified PCR products were electrophoresed on an agarose gel. The fraction of ca 300-350 bp from each lane marked by an arrow, were extracted and amplified by the following 2nd nested-PCR. Lane 1: liver; lane 2: muscle; lane 3: heart; M: DNA molecular size ladder.

The amplified DNAs from the 2nd nested PCR are shown in Fig. 12. Since the part of 3´-untranslated regions between IV-1 and IV-2 cDNA was expected to vary, different sized fragments were extracted. The fragments of 200 bp, 210 bp, and 250 bp from liver, 250 bp and 300 bp from muscle and two of 280 bp from heart were selected. The extracted DNAs were cloned into pBluescript KS- plasmids and the plasmids were transferred into *E. coli* XL-1-Blue by electroporation (see 3.1.9.). The transformed cells were cultivated on the LB agar medium at 37 °C overnight.

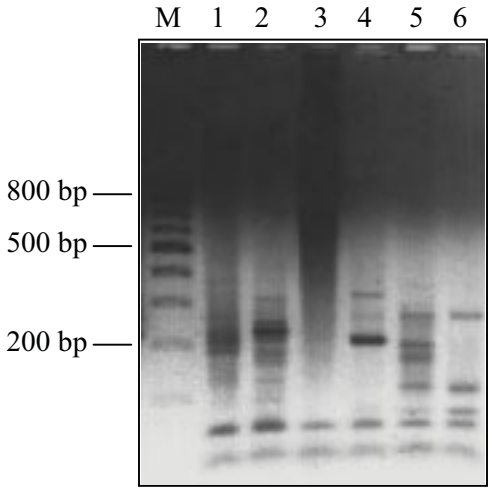


Fig. 12. 2nd nested-PCR for the amplification of cytochrome c oxidase subunit IV cDNA from turkey liver, muscle, and heart.
The outer-PCR products (Fig. 11) were amplified with primers IV+4.8 and Q_I by Touch-down-PCR (described in detail in Fig. 7). The amplified products were electrophoresed on an agarose gel. The selected DNA fractions are cloned into pBluescript KS-. Fraction 1: 200 bp from lane 1; fraction 2-3: 250 and 210 bp from lane 2; fraction 4-5: 300 and 250 bp from lane 4; fraction 6: 280 bp from lane 5; fraction 7: 280 bp from lane 6. Lanes 1-2: liver; lanes 3-4: muscle; lanes 5-6: heart; M: DNA molecular size ladder.

10-20 bacterial colonies were selected from each transformed cell which was grown on the agar plate. The selected cells were further cultivated in LB medium. Plasmids were isolated from the cultivated *E. coli* by the TENS method (see in 3.1.10) and incubated with the restriction enzyme Pvu II (see in 3.1.11.). The digested plasmids were electrophoresed on agarose gels (Fig. 13). Among the fragments obtained after digestion some which are expected to contain a subunit IV-2 cDNA (as inserted DNA) were selected. The plasmids containing the selected fragments were used directly for sequencing-PCR with "ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit". The sequences were analyzed by the "ABI prism 310 Genetic Analyzer". Most fragments did not contain cDNAs of subunit IV-2, since their sequences were not homologous to the subunit IV-1 cDNA except fragments F to I turned out to be the subunit IV-1 cDNA (Fig. 14).

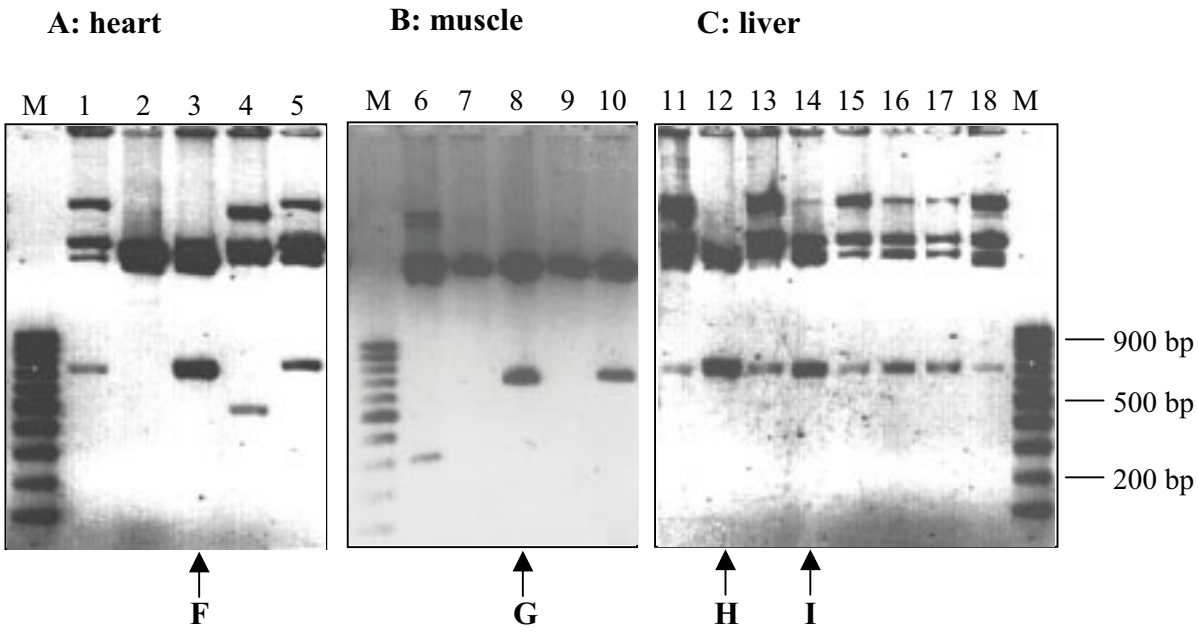
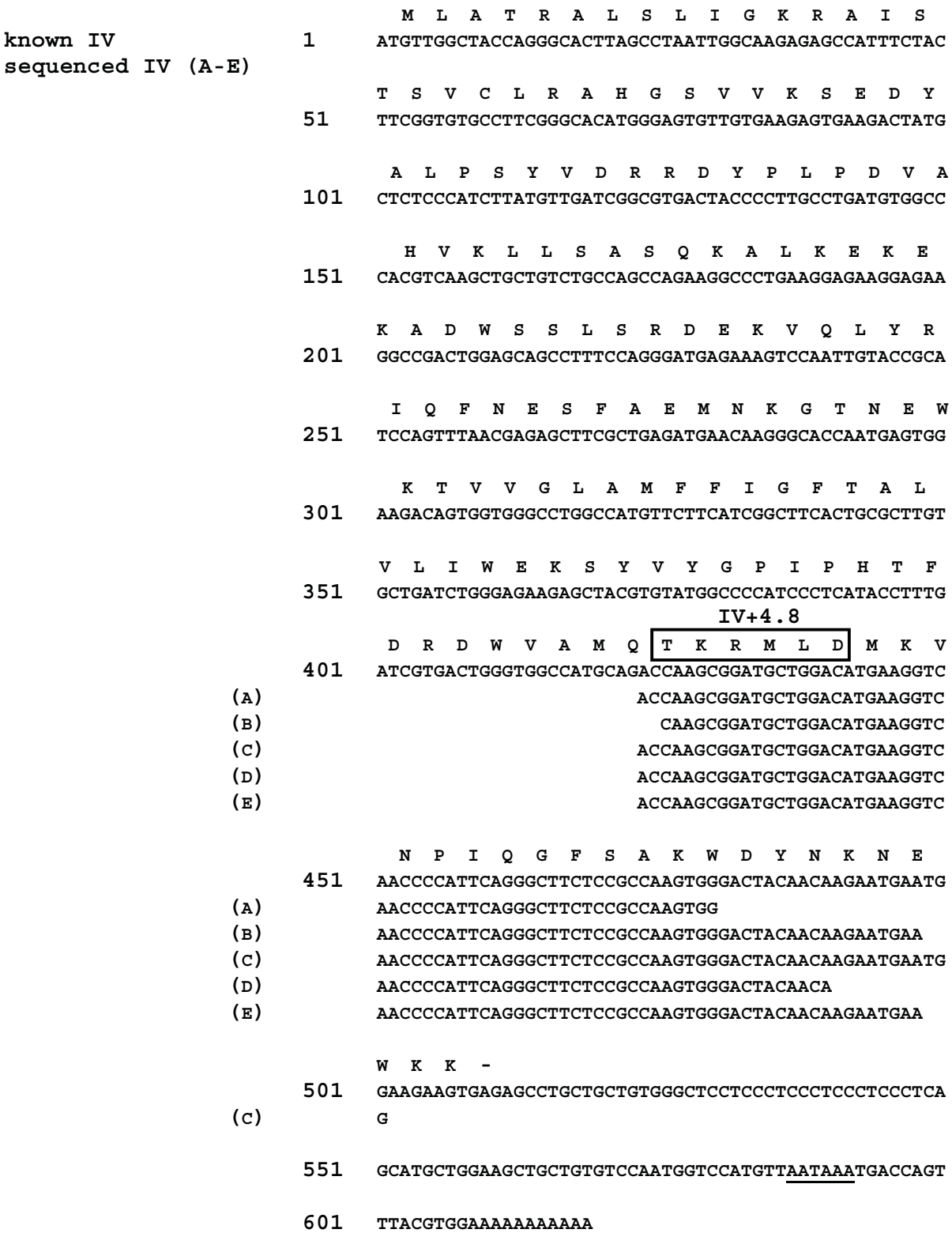


Fig. 13. Characterization of the cloned plasmids of 3′-region subunit IV cDNA from turkey heart (A), muscle (B), and liver (C) by incubation with Pvu II.
Plasmid DNAs were prepared by the TENS method. The inserted DNA and the 450 bp of vector DNA were cleaved by Pvu II (Fig. 3). The digested plasmids were seperated on agarose gels. Fragments (F to I) marked by arrows correspond to the subunit IV-1 cDNA. Lanes 1-5: fraction 6 from the 2nd nested-PCR (Fig. 12); lanes 6-9: fraction 4; lane 10: fraction 5; lanes 11-15: fraction 2; lanes 16-18: fraction 3; M: DNA molecular size ladder.

The cDNA sequences of fragments A to I (from Fig. 9 and 13) are presented with the known subunit IV (IV-1) sequences in Fig. 14. In most cases, the sequences of the fragments start from the primer IV+4.8 site except the fragment B. Two nucleotides at 5′-region are missing in the fragment B. Fragments F and G from turkey heart and muscle, respectively, have the full length sequences of the 3′-coding and 3′-untranslated regions including the poly A tail. The fragments H and I (from turkey liver) have also the full 3′-coding region of subunit IV-1, but not the poly A tail and the poly A adenylation site. In contrast, all fragments from rat (A to E) contain the incomplete 3′-coding regions of subunit IV-1 cDNA. This incompletion of cDNA from turkey and specially from rat might be caused by the improper preparation and/or isolation (TENS method) of the vector plasmids.

[Rat]


M L A T R A L S L I G K R A I S
known IV 1 ATGTTGGCTACCAGGGCACTTAGCCTAATTGGCAAGAGAGCCATTTCTAC
sequenced IV (A-E)
T S V C L R A H G S V V K S E D Y
51 TTCGGTGTGCCTTCGGGCACATGGGAGTGTTGTGAAGAGTGAAGACTATG
A L P S Y V D R R D Y P L P D V A
101 CTCTCCCATCTTATGTTGATCGGCGTGACTACCCCTTGCCTGATGTGGCC
H V K L L S A S Q K A L K E K E
151 CACGTCAAGCTGCTGTCTGCCAGCCAGAAGGCCCTGAAGGAGAAGGAGAA
K A D W S S L S R D E K V Q L Y R
201 GGCCGACTGGAGCAGCCTTTCCAGGGATGAGAAAGTCCAATTGTACCGCA
I Q F N E S F A E M N K G T N E W
251 TCCAGTTTAACGAGAGCTTCGCTGAGATGAACAAGGGCACCAATGAGTGG
K T V V G L A M F F I G F T A L
301 AAGACAGTGGTGGGCCTGGCCATGTTCTTCATCGGCTTCACTGCGCTTGT
V L I W E K S Y V Y G P I P H T F
351 GCTGATCTGGGAGAAGAGCTACGTGTATGGCCCCATCCCTCATACCTTTG
IV+4.8
D R D W V A M Q T K R M L D M K V
401 ATCGTGACTGGGTGGCCATGCAGACCAAGCGGATGCTGGACATGAAGGTC
(A) ACCAAGCGGATGCTGGACATGAAGGTC
(B) CAAGCGGATGCTGGACATGAAGGTC
(C) ACCAAGCGGATGCTGGACATGAAGGTC
(D) ACCAAGCGGATGCTGGACATGAAGGTC
(E) ACCAAGCGGATGCTGGACATGAAGGTC
N P I Q G F S A K W D Y N K N E
451 AACCCCATTCAGGGCTTCTCCGCCAAGTGGGACTACAACAAGAATGAATG
(A) AACCCCATTCAGGGCTTCTCCGCCAAGTGG
(B) AACCCCATTCAGGGCTTCTCCGCCAAGTGGGACTACAACAAGAATGAA
(C) AACCCCATTCAGGGCTTCTCCGCCAAGTGGGACTACAACAAGAATGAATG
(D) AACCCCATTCAGGGCTTCTCCGCCAAGTGGGACTACAACA
(E) AACCCCATTCAGGGCTTCTCCGCCAAGTGGGACTACAACAAGAATGAA
W K K -
501 GAAGAAGTGAGAGCCTGCTGCTGTGGGCTCCTCCCTCCCTCCCTCCCTCA
(C) G
551 GCATGCTGGAAGCTGCTGTGTCCAATGGTCCATGTTAATAAATGACCAGT
601 TTACGTGGAAAAAAAAAAA

[Turkey]

```
                          M  L  A  S  R  A  F  S  L  I  G  R  R  A  L  S
known IV-1            1   ATGTTGGCTTCAAGAGCATTCAGCCTCATTGGGAGAAGAGCCCTTTCCAC
sequenced IV (F-I)
                          T  S  I  C  V  R  A  H  G  H  A  G  V  V  K  A  E
                     51   CTCCATCTGTGTGAGGGCACATGGGCATGCTGGTGTTGTCAAAGCAGAGG

                          D  F  S  H  P  A  Y  V  D  R  R  D  V  P  L  P  E
                    101   ATTTCAGCCATCCAGCATACGTTGATCGCCGTGATGTTCCCCTGCCAGAA

                          A  A  R  V  K  E  L  S  A  Q  Q  K  A  L  K  E
                    151   GCGGCCTTTGTAAAGGAGCTCTCTGCTCAGCAGAAGGCTCTGAAAGAAAA

                          K  E  K  P  S  W  T  A  L  S  V  D  E  K  V  E  L
                    201   GGAAAAGCCATCTTGGACCGCTTTGTCTGTTGATGAGAAAGTTGAATTGT

                          Y  P  I  K  F  N  E  S  Y  A  E  M  N  K  G  T  N
                    251   ACCGTATCAAATTCAATGAGAGCTATGCAGAAATGAACAAAGGAACAAAT

                          E  W  K  T  V  L  G  G  V  L  G  G  L  G  V  S
                    301   GAGTGGAAGACCGTCCTTGGTGGAGTGCTTTTCTTTCTTGGCGTATCTGG

                          G  L  I  L  I  W  Q  K  M  Y  M  Y  G  P  I  P  H
                    351   CCTCATCCTCATTTGGCAGAAAATGTATATGTACGGCCCTATTCCGCACA
                                                                IV+4.8
                          T  F  S  D  E  W  L  S  M  Q [T  K  R  M  L  D] M
                    401   CCTTCTCTGATGAGTGGCTGTCAATGCAGACAAAGAGAATGTTGGACATG
(F)                                                   ACAAAGAGAATGTTGGACATG
(G)                                                   ACAAAGAGAATGTTGGACATG
(H)                                                   ACAAAGAGAATGTTGGACATG
(I)                                                   ACAAAGAGAATGTTGGACATG

                          R  I  N  P  V  Q  G  I  S  S  Q  W  D  Y  E  K
                    451   CGAATTAATCCCGTCCAGGGCATCTCTTCCCAGTGGGATTACGAGAAGAA
(F)                       CGAATTAATCCCGTCCAGGGCATCTCTTCCCAGTGGGATTACGAGAAGAA
(G)                       CGAATTAATCCCGTCCAGGGCATCTCTTCCCAGTGGGATTACGAGAAGAA
(H)                       CGAATTAATCCCGTCCAGGGCATCTCTTCCCAGTGGGATTACGAGAAGAA
(I)                       CGAATTAATCCCGTCCAGGGCATCTCTTCCCAGTGGGATTACGAGAAGAA

                          N  E  W  K  K  -
                    501   CGAGTGGAAGAAATGAAGCAGCTCAGCGGAACCTGCTCTGCTTGAATATG
(F)                       CGAGTGGAAGAAATGAAGCAGCTCAGCGGAACCTGCTCTGCTTGAATATG
(G)                       CGAGTGGAAGAAATGAAGCAGCTCAGCGGAACCTGCTCTGCTTGAATATG
(H)                       CGAGTGGAAGAAATGAAGCAGC
(I)                       CGAGTGGAAGAAATGAAGCAGCTCAGCGGAACCTGCTCTGCTTGAATA

                    551   AATGATTCCATCACCTGTGTGTGACCTCGTTGCTTATGTACTGGAACAAT
(F)                       AATGATTCCATCACCTGTGTGTGACCTCGTTGCTTATGTACTGGAACAAT
(G)                       AATGATTCCATCACCTGTGTGTGACCTCGTTGCTTATGTACTGGAACAAT

                    601   CTCTTCACCTAAATAGATGAAAATAAACATCTGGTTAACTTGAAAAAAAA
(F)                       CTCTTCACCTAAATAGATGAAAATAAACATCTGGTTAACTTGAAAAA
(G)                       CTCTTCACCTAAATAGATGAAAATAAACATCTGGTTAACTTGAAAAAAAA
```

```
          651   AAAAAAAAAA
(G)             AA
```

Fig. 14. Comparison of the cDNA sequences of screened subunit IV with the known subunit IV (IV-1) from rat [Goto et al., 1989] and turkey [Hüttemann, 2000].
The sequences of the mature proteins are presented above the cDNA sequences. The polyadenylation sites are underlined. Most screened sequences except that of fragment B, start from the primer IV+4.8 site (in boxes) which was used for the nested-PCR.

The failure in finding the subunit IV-2 cDNA might have the following reasons: (1) The designed primers, IV+1, IV+3.3, and IV+4.8 for the amplifications were not proper (little homology to cDNA of subunit IV-2), (2) The subunit IV-2 cDNA was not synthesized during the RT-reaction, because of secondary structures of subunit IV-2 mRNA, and/or (3) the subunit IV-2 of cytochrome c oxidase does not exist in mammals and birds.

Later, however, the cDNA of the new isoform of subunit IV (IV-2) was found in human, rat, mouse [Hüttemann et al., 2001], and cow [Hüttemann et al., in preparation] by Dr. Maik Hüttemann. Deduced amino acid sequences of subunits IV-2 from human, mouse, rat, and cow are compared with the amino acid sequences of subunit IV-1 in Fig. 15 [Hüttemann, personal communication]. The cDNA sequences of subunit IV-2 among human, rat, and mouse are 63-78 % identical, which is lower than the identity among the subunit IV-1 isoforms (69-88 %). These low homologies for the mammalian sequences of subunit IV-2 cDNA might be the reason for the failure of the above performed screening. Interestingly three conserved cysteine residues are present in subunit IV-2 in human, rat, and mouse and two cysteine residues in cow, but no cysteine is present in the mature subunit IV-1. The expression of subunit IV-2 transcripts was observed in the lung of adult and fetal human and adult rat, as well as in the muscle of fetal human by Northern Blot analysis [Hüttemann et al., 2001]. Other adult tissues showed only weak or undetectable transcription levels. In contrast to the IV-2 isoform, subunit IV-1 was ubiquitously transcribed in all tissues, including lung.

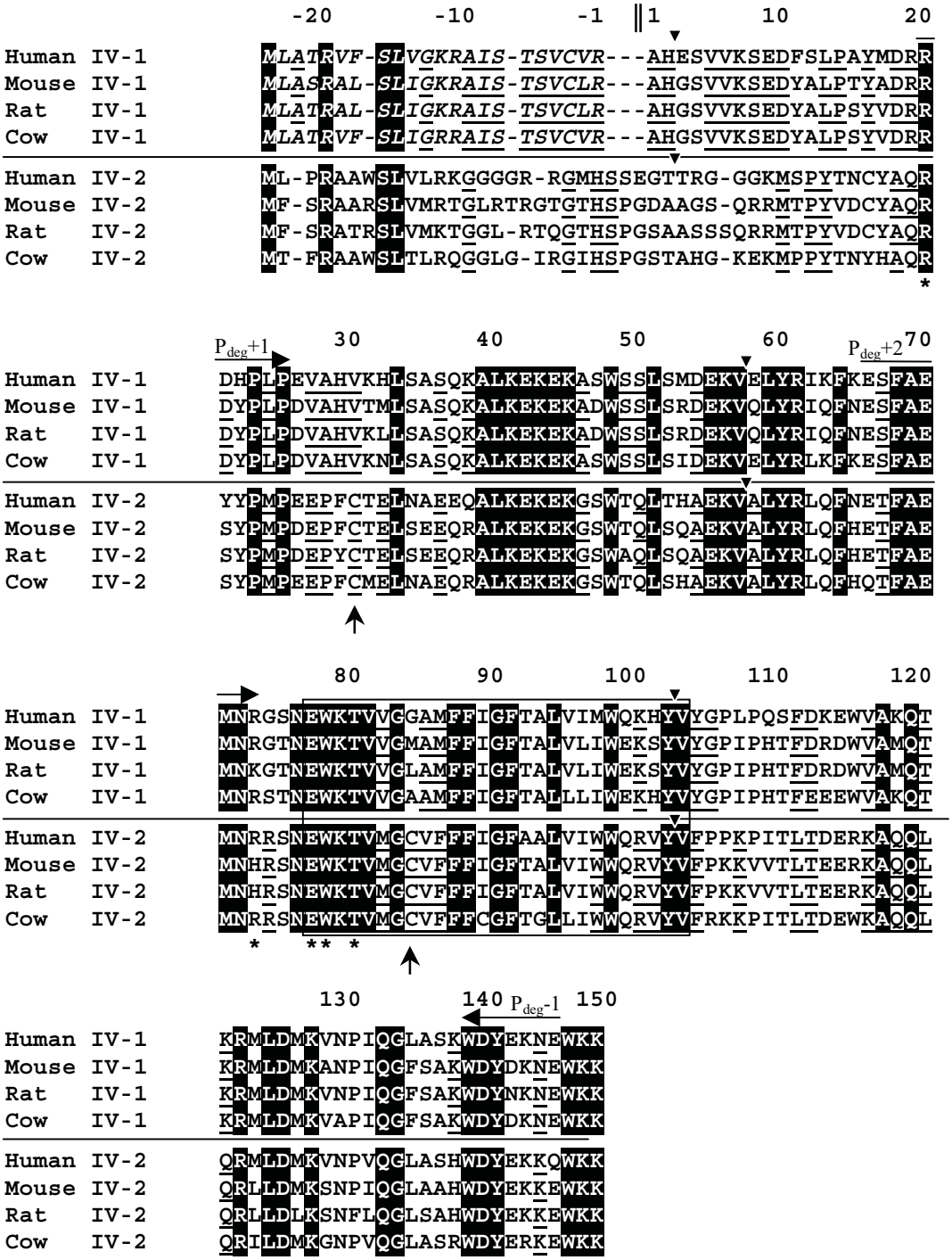


Fig. 15. Alignment of cytochrome c oxidase subunit IV-1 precursor proteins from human [Zeviani et al., 1987], mouse [Grossman and Akamatsu, 1990], rat [Gopalan et al., 1989], and cow [Sacher et al., 1979] with the new subunit IV-2 isoform from human, mouse, rat [Hüttemann et al., 2001], and cow [kindly offered by Maik Hüttemann].
Identical amino acids in both isoforms are shown in white on black. The beginning of mature subunits IV is indicated only for the IV-1 isoform. Underlined amino acids are identical in only one isoform and do not occur in the other one. Arrows indicate the positions and directions of degenerate primers that yielded the correct PCR product in rat. Vertical arrows show the highly conserved cysteine residues present only in the new isoforms. Triangles show the exon-exon junctions for both human sequences. The boxed sequence represents the transmembraneous region [Tsukihara et al., 1996]. Amino acids proposed to be involved in ATP/ADP binding at the matrix side are marked with an asterisk.

4.2. Search for cytochrome c oxidase subunit isoforms in bovine and turkey

4.2.1. Comparison of bovine cytochrome c oxidase subunit patterns

To get more informations about isoforms of cytochrome c oxidase, the enzymes were isolated from bovine heart, kidney, lung, and testis, and the subunit compositions were analysed by SDS polyacrylamide gel electrophoresis. The Coomassie Blue stained polypeptide pattern of the enzymes from bovine tissues which were isolated by the Triton X-100 method are presented in Fig. 16. With subunits VIa and VIII, different isoforms are clearly identified in different tissues by electrophoretic mobilities. Subunit VIa of the bovine heart enzyme (VIaH) has an apparent higher molecular mass (lower electrophoretic mobility) than that of bovine kidney (VIaL). The enzymes from bovine testis and lung contain mainly the liver type of subunit VIa (VIaL). The identity of the double band of the testis enzyme in the region of the heart-type of VIa is not known. Subunit VIII of the testis enzyme consists of both, heart type (VIIIH) and liver type of subunit VIII (VIIIL), occurring at similar amounts. In contrast, the enzyme from bovine heart contains only subunit VIIIH, and the enzymes from kidney and lung contain only subunit VIIIL, as previously described for the isozymes from chicken [Merle and Kadenbach, 1980], bovine [Merle and Kadenbach, 1982], and pig [Kuhn-Nentwig and Kadenbach, 1985].

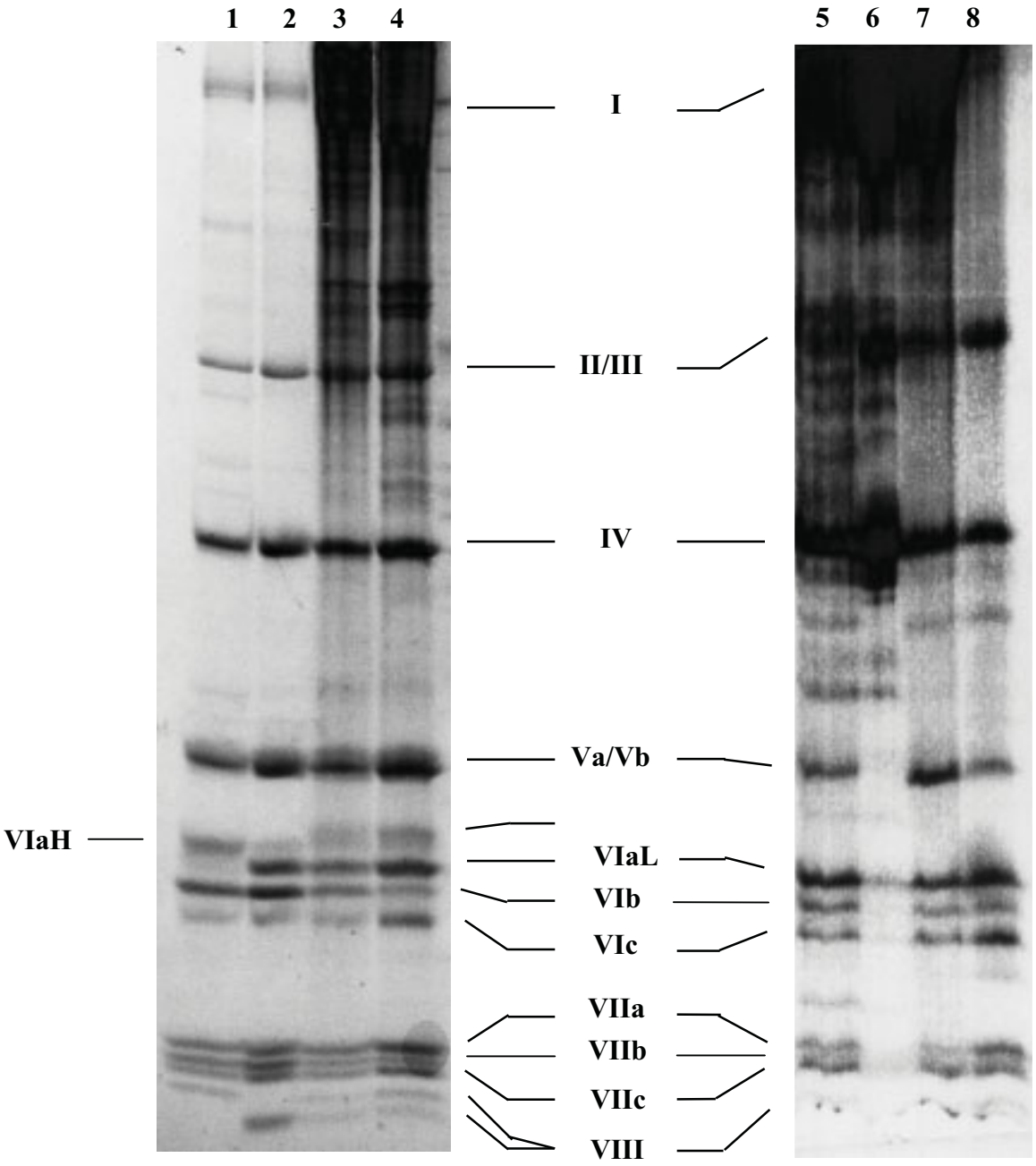


Fig. 16. Comparison of the subunit pattern of isolated cytochrome c oxidases from bovine heart, kidney, testis, and lung.

SDS-PAGE and Coomassie Blue staining were performed as described in 3.2.6.

Lane 1: heart, 48 % $AmSO_4$
Lane 2: kidney, 48 % $AmSO_4$
Lane 3: testis, 48 % $AmSO_4$
Lane 4: testis, 37 % $AmSO_4$
Lane 5: lung, 37 % $AmSO_4$
Lane 6: lung, 28 % $AmSO_4$
Lane 7: lung, 46 % $AmSO_4$
Lane 8: lung, 55 % $AmSO_4$

4.2.2. Searching for subunit IV-2 in bovine lung cytochrome c oxidase

The cDNA of the second isoform of subunit IV (IV-2) in animals was first identified in tuna tissues [Hüttemann, 2000]. In further studies, the expression of subunit IV-2 mRNA was detected mainly in rat lung by Northern Blot analysis and by *in situ* hybridization [Hüttemann et al., 2001]. To prove the expression of subunit IV-2 in bovine tissues and to investigate its regulatory properties, cytochrome c oxidase was isolated from bovine lung, using the Triton X-100 method. The subunits of the bovine lung enzyme were separated by SDS-PAGE and blotted on PVDF membrane. After staining with Coomassie Blue, subunit IV was excised from the membrane and the N-terminal amino acid was sequenced by Prof. F. Lottspeich (MPI für Biochemie, Martinsried). As shown in Fig. 17, very similar N-terminal amino acid sequences were found for subunit IV from bovine lung and for the known bovine subunit IV (IV-1). In some positions different amino acids were found, which might have originated from contaminating impurities. In order to exclude that subunit IV-2 from lung is N-terminally blocked, internal amino acid sequences were determined from proteolytic fragments of the lung subunit IV by Prof. F. Lottspeich. The molecular masses of the digested fragments, determined by MALDI-TOF, were identical to those of the expected proteolytic fragments of bovine heart subunit IV (data not shown). These results indicate that either cytochrome c oxidase subunit IV-2 is not expressed in bovine lung at the protein level, or its amount in the isolated enzyme is too low to be detected by amino acid sequencing methods.

```
          1                  10                  20            27
Lung      A E G S V V K S E D Y G L G Q Y V F T F(A)Y P ?(P)V F
                    (Q W P)A   A P(P)S M   D   D   D    (E)  V
            *                                * * *     *   *
IV-1      A H G S V V K S E D Y A L P S Y V D R R D Y P L P D V
IV-2      A H G - - - K E K M P P Y T N Y H A Q R S Y P M P E E
```

Fig. 17. Comparison of N-terminal amino acid sequences of cytochrome c oxidase subunit IV from bovine lung with subunits IV-1 and IV-2 from bovine.
When two amino acids were detected at the same Edman degradation step, the second is presented on the lower line with minor amino acids in parentheses. Amino acids of subunit IV-1, not found in the sequence of subunit IV from lung, are indicated by asterisks.

The protein sequence of subunit IV-2 deduced from the cDNA sequence of bovine, have two cysteines, which do not occur in subunit IV-1 [Hüttemann et al., 2001] (Figure 15). Therefore the possible occurrence of subunit IV-2 in the isolated enzyme from bovine lung was investigated by using an SH-reagent. The fluorescent reagent N-(7-Dimethylamino-4-

methyl-3-cumarinyl)-maleinmide (DACM) reacts with free –SH groups of proteins, which are visualized by illumination with UV light [Yamamoto et al., 1977]. The heart, kidney, and lung enzymes were solubilized with Na-cholate and reduced by incubation with dithiothreitol (DTE) (see in 3.2.7.). The reduced enzymes were precipitated, resolubilized in electrophoresis sample buffer with 1 mM DACM, and subunits were separated by SDS-PAGE. The polypeptides which reacted with their free –SH bonds were visualized under UV light, as presented in Fig. 18. Fluorescence at the position of subunit IV-2 is detected in all enzymes with decreasing intensity from lung, kidney, to heart. This suggests that subunit IV-2 exists mainly in the isolated lung enzyme and to a lower extent in kidney and heart. The less pure enzyme (lane 9, 28 % $AmSO_4$ precipitate) illuminates more strongly. Subunit IV-2 seems to have been gradually eliminated during the purification steps. The final purified lung enzyme appears to contain too small amounts of subunit IV-2 to be detected by amino acid sequencing.

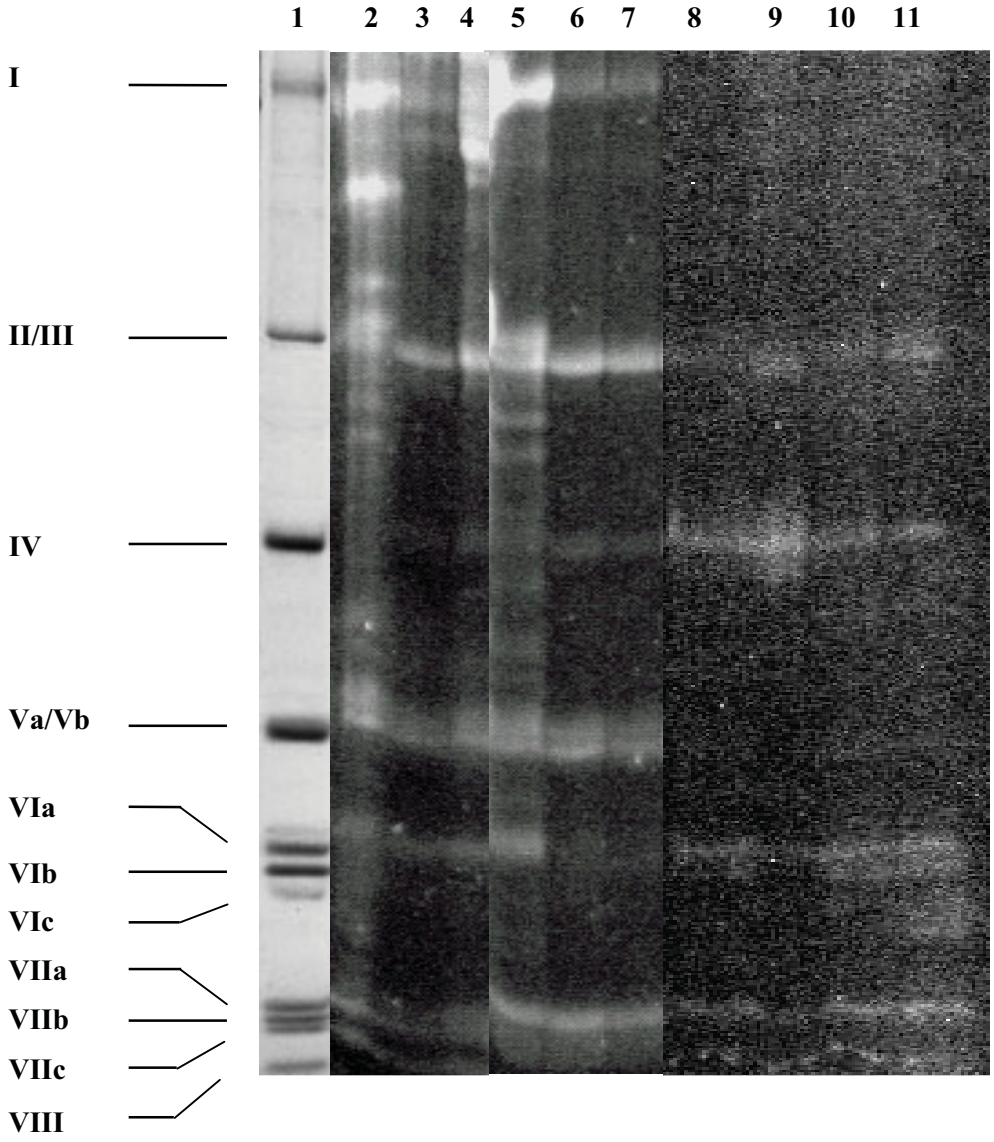


Fig. 18. Detection of fluorescent subunit IV-2 of bovine heart, kidney, and lung cytochrome c oxidases by DACM.

Cytochrome c oxidases from bovine tissues isolated by the Triton X-100 method, were solubilized in 1.5 % of Na-cholate and incubated with 50 mM DTE at room temperature for 1 h. The enzymes were precipitated at 35 % $AmSO_4$ saturation and centrifuged. 20 µg of precipitated enzymes were solubilized in electrophoresis sample buffer with 1 mM DACM. After SDS-PAGE the fluorescent polypeptides were visualized under UV light (at 302 nm).

Lane 1: Coomassie Blue staining of lane 4

Lane 2: kidney, 37 % $AmSO_4$
Lane 3: kidney, 42 % $AmSO_4$
Lane 4: kidney, 46 % $AmSO_4$
Lane 5: heart, 37 % $AmSO_4$
Lane 6: heart, 42 % $AmSO_4$
Lane 7: heart, 46 % $AmSO_4$
Lane 8: lung, 37 % $AmSO_4$
Lane 9: lung, 28 % $AmSO_4$
Lane 10: lung, 46 % $AmSO_4$
Lane 11: lung, 55 % $AmSO_4$

4.2.3. Comparison of subunit patterns of turkey cytochrome c oxidases

Cytochrome c oxidases were purified from turkey liver, heart, and skeletal muscle by the Triton X-100 method and their subunit compositions were analysed by SDS-PAGE. The patterns of Coomassie Blue stained polypeptides are presented in Fig. 19. Subunit VIa of cytochrome c oxidases from all turkey tissues shows apparently the same molecular mass. This is in contrast to mammalian cytochrome c oxidase isozymes, which always have different subunits VIa in skeletal muscle (VlaH) and non-skeletal muscle tissues (VIaL) [Linder et al., 1995]. The band marked with an asterisk (VIa*) is the cleaved subunit VIa of the bovine heart enzyme which was isolated by the cholate method [Yoshikawa et al., 1977 and 1988]. The enzyme isolated by the cholate method usually has two bands of subunit VIaH, due to cleavage of 3 N-terminal amino acids (Arnold, Kadenbach, and Lottspeich, unpublished results). Subunit VIII has a lower apparent molecular mass in turkey liver (VIIIL) compared to turkey muscle (VIIIH). In turkey heart mainly subunit VIIIH occurs but a small amount of the liver type is also visible.

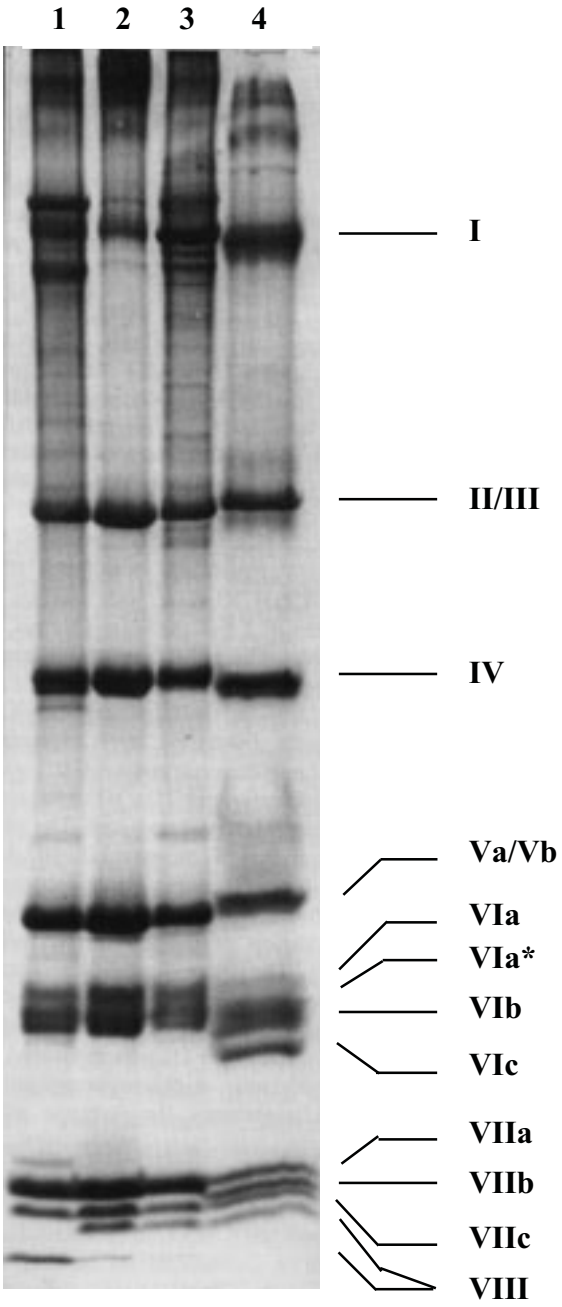


Fig. 19. Subunit patterns of isolated cytochrome c oxidases from turkey liver, heart, and skeletal muscle and from bovine heart.
SDS-PAGE and Coomassie Blue staining were performed as described under 3.2.6.
Lane 1: turkey liver, 46 % $AmSO_4$, Triton X-100 method.
Lane 2: turkey heart, 46 % $AmSO_4$, Triton X-100 method.
Lane 3: turkey skeletal muscle, 46 % $AmSO_4$, Triton X-100 method.
Lane 4: bovine heart enzyme, Cholate method.

4.2.4. Identification of subunit VIaL isoform in turkey liver, heart, and skeletal muscle

In order to prove the identity of cytochrome c oxidase subunit VIa in turkey liver, heart, and skeletal muscle, their amino acid sequences were determined. The gel bands, separated by SDS-PAGE were blotted onto a PVDF membrane and subunit VIa was excised. The N-terminal amino acids of subunit VIa were sequenced by Dr. D. Linder (Gießen) (Fig. 20). For internal amino acid sequencing, subunit VIa was excised from Coomassie Blue stained gels and sequenced directly (liver and heart), or after digestion with endoproteinase Lys-C, and separation of fragments by HPLC by Prof. F. Lottspeich. Identical N-terminal amino acid sequences were found for subunit VIa from all three turkey tissues. Internal amino acid sequences were also determined from proteolytic fragments of subunit VIa of the skeletal muscle enzyme (Fig. 20). These sequences were identical with the deduced amino acid sequence of the turkey cDNA for subunit VIa, determined by Maik Hüttemann [Hüttemann et al., 2000]. Comparison of the sequenced data with the amino acid sequences of subunits VIaL and VIaH from mammals (Fig. 21) clearly demonstrates that subunit VIa from turkey liver, heart, and skeletal muscle, corresponds to the mammalian subunit VIaL (liver type). The average identity between turkey subunit VIa and mammalian subunit VIaL is 71 % and between turkey subunit VIa and mammalian subunit VIaH is 63 %.

```
<N-terminal sequences>

                1                  10                  20
Liver           A A A A A H E G G G A R L W K T L ? F V . . .
Heart           A A A A A H E G G G A R L W K T L ? F V . . .
Muscle          A A A A A H E G G G A R L W K . . .


<Internal sequences>

               44          50        55
Muscle      . . H E R P E F I R Y A H L R I R . . .
               59          65
            . . T K P F P W G D G N K . . .*
               70        75        80        85
            . . T L F H N P H T N A L P T G Y E D E N . . .
```

Fig. 20. N-terminal and internal amino acid sequences of cytochrome c oxidase subunit VIa from turkey liver, heart, and skeletal muscle.
The amino acid sequences were determined directly from bands of subunit VIa, separated by SDS-PAGE and blotted onto PVDF membranes (liver and heart), or after digestion of gel bands with endoproteinase Lys-C, separation by HPLC and sequencing (muscle). One fragment was identified by mass (*).

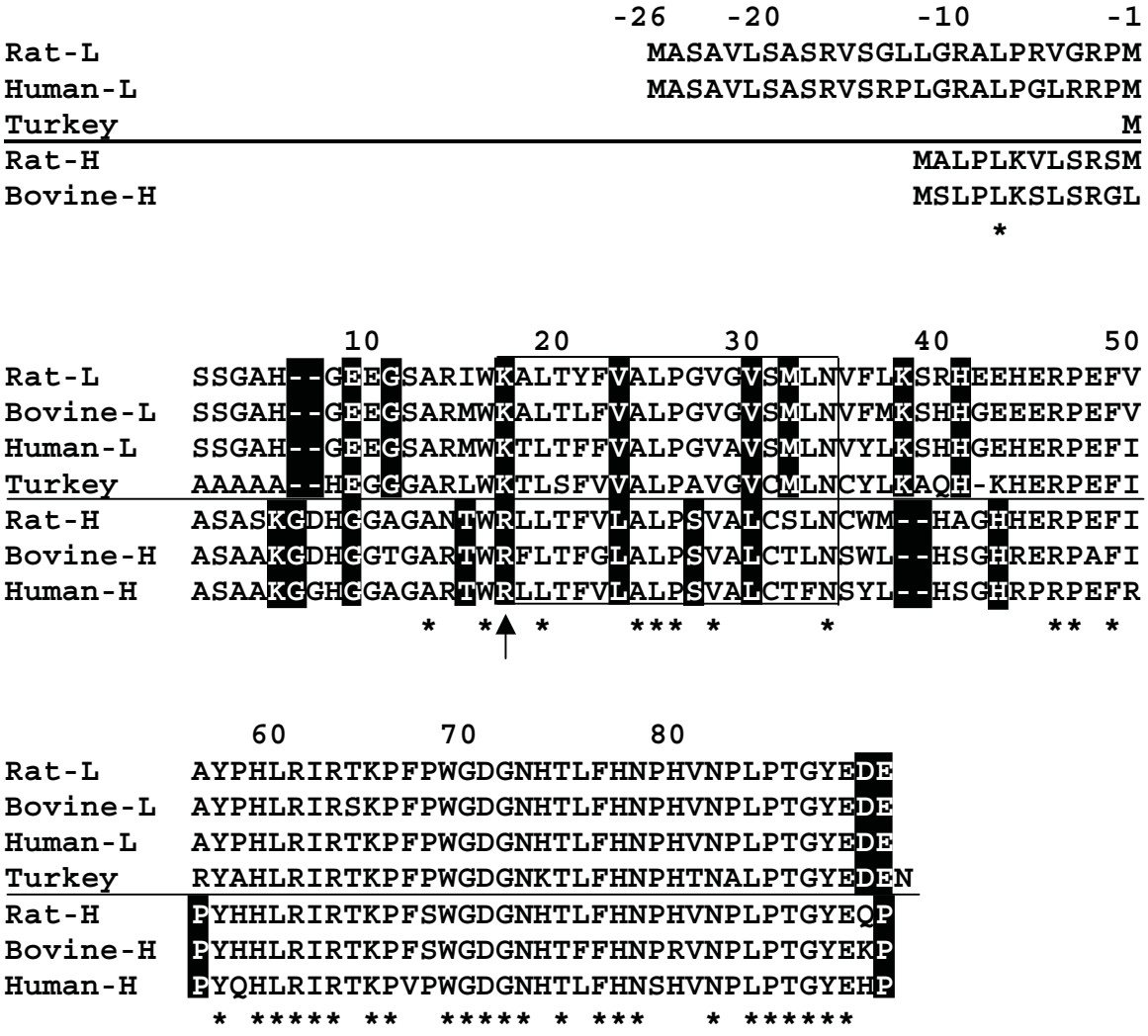


Fig. 21. Comparison of the amino acid sequences of cytochrome c oxidase subunit VIa (L: liver type, H: heart type) from different species [taken from Hüttemann et al., 2000]. Identical amino acids in all tissues and species are marked by asterisks. Identical amino acids of mature proteins in all liver type or heart type, which do not occur in the other isotype, including the two amino acid gaps in VIaL and VIaH, are indicated white on black. The transmembraneous region is boxed. The conserved Arg^{17} in VIaH, representing one of three amino acids of the bovine heart subunit involved in the binding of ADP [Tsukihara et al., 1996], is indicated by an arrow and differs from a conserved lysine in VIaL.

4.3. Phosphorylation of cytochrome c oxidase subunits

4.3.1. Identification of phosphorylated subunits of bovine heart cytochrome c oxidase with antibodies against phosphoserine and phosphothreonine

Previous studies indicated that the allosteric ATP-inhibition of cytochrome c oxidase occurs only in the cAMP-dependent phosphorylated enzyme [Bender and Kadenbach, 2000]. Therefore it was of interest to investigate phosphorylated sites in the enzyme using antibodies against phosphoserine and phosphothreonine. Cytochrome c oxidases from bovine heart were isolated in the presence and absence of KF (an nonspecific inhibitor of protein phosphatases) and EGTA by the Triton X-100 method as well as by the cholate method. After SDS-PAGE, Western Blot analysis was performed with the antibodies. As shown in Fig. 22, different enzyme preparations show different staining of subunits with the two antibodies. Most subunits except Va and Vb react with both antibodies. The staining of subunits II/III, IV, and VIIa with both antibodies is stronger in enzymes which were isolated with KF and EGTA, either by the Triton X-100 or the cholate method, than in enzymes which were isolated without KF and EGTA. Very intensive staining with phosphothreonine antibody of subunit IV in the enzymes purified with KF and EGTA, is remarkable. Subunit VIb is only stained in the enzyme isolated by the Triton X-100 method in the absence of KF and EGTA. Except the enzyme prepared with KF and EGTA by the Triton X-100 method, all other preparations show almost the same staining intensity of subunits VIc, VIIa and VIIb. The N-terminal cleaved subunit VIa (VIa*) of the enzymes isolated by the cholate method, are shown by staining with Coomassie Blue on the gel (lane 1) and also by staining with antibodies on the membranes. Interestingly, the degradation of VIa seems to be blocked partially by addition of KF and EGTA during the purification. Some cross reactivity seems to exist between the two antibodies.

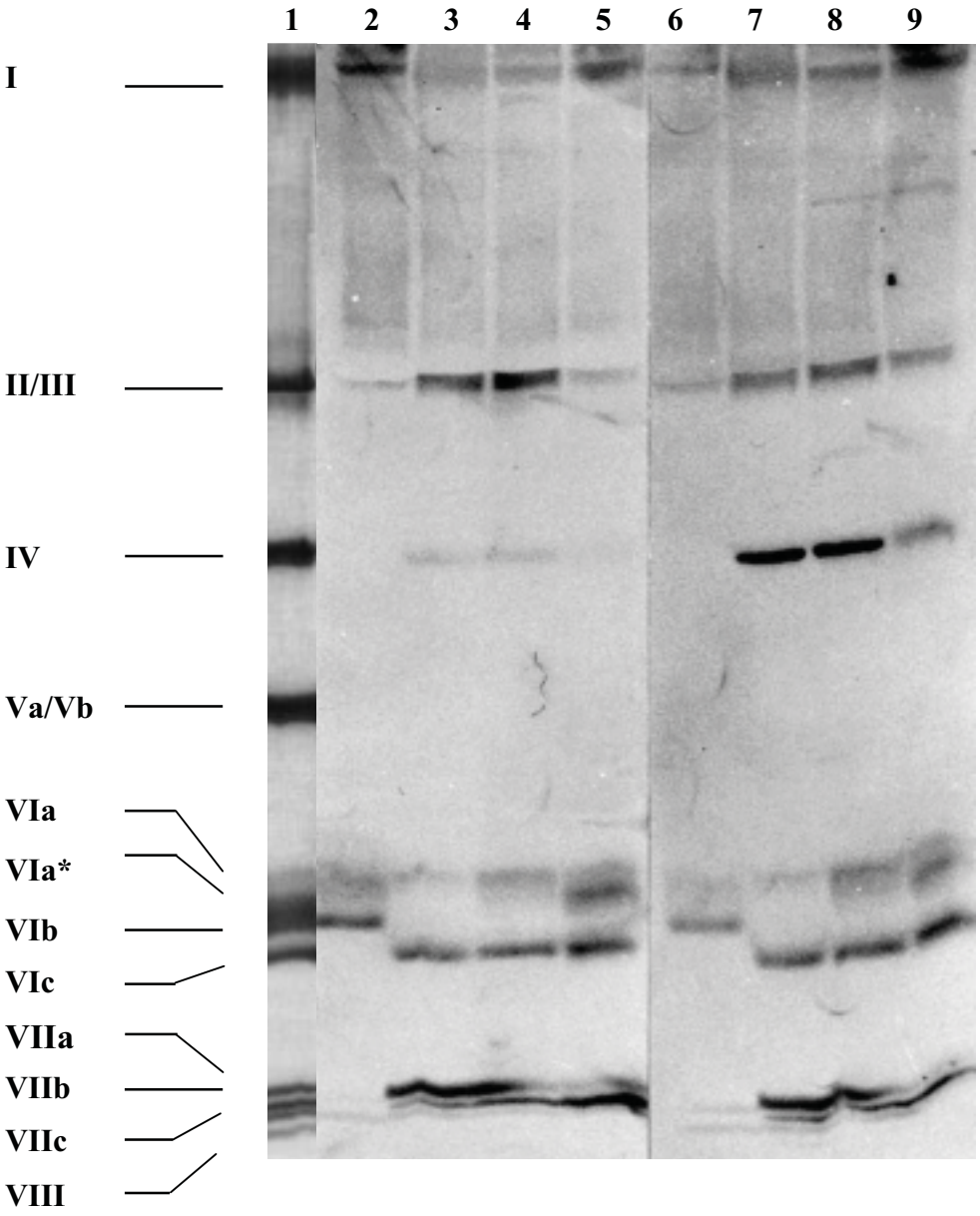


Fig. 22. Western Blot analysis of different bovine heart cytochrome c oxidase preparations with antibodies against phosphoserine (lane 2-5) and phosphothreonine (lane 6-9).

Immunodetection of the subunits with antibodies was performed as described in 3.5.
Lane 1: Coomassie Blue staining, cholate method, without EGTA and KF
Lanes 2-9: Immunostaining. Conditions of enzyme isolation:
Lane 2 and 6: Triton X-100 method, without EGTA and KF
Lane 3 and 7: Triton X-100 method, with EGTA and KF
Lane 4 and 8: cholate method, with EGTA and KF
Lane 5 and 9: cholate method, without EGTA and KF

4.3.2. Possible phosphorylation sites in the subunits of bovine heart cytochrome c oxidase

Fig. 22 suggests the existence of phosphorylated serines and threonines in the subunits of cytochrome c oxidase *in vivo*. The sites in subunits of bovine heart cytochrome c oxidase which could be phosphorylated by protein kinases [Pearson and Kemp, 1991] were picked out. Many possible phosphorylation sites were identified in all subunits, as shown in Fig. 23. The consensus sequences for cAMP-dependent protein kinases (PKAs) are boxed, and for cGMP-dependent protein kinases and calcium activated protein kinases (PKCs) are underlined and overlined. The consensus sequences for cGMP-dependent protein kinases and PKCs are not distinguished in Fig. 23, because they are almost identical. The phosphorylated amino acids are denoted by asterisks. Only three consensus sequences for cAMP-dependent phosphorylation could be found in bovine heart cytochrome c oxidase, namely in subunits I, III, and Vb, contrasting many sites for cGMP-dependent protein kinases and PKCs.

Subunit I

```
                10                  20                  30                  40
M F I N R W L F S T N H K D I G T L Y L L F G A W A G M V G T A L S L L I R A E
          *                   *
                50                  60                  70                  80
L G Q P G T L L G D D Q I Y N V V V T A H A F V M I F F M V M P I M I G G F G N
                90                  100                 110                 120
W L V P L M I G A P D M A F P R M N N M S F W L L P P S F L L L L A S S M V E A
                130                 140                 150                 160
G A G T G W T V Y P P L A G N L A H A G A S V D L T I F S L H L A G V S S I L G
                170                 180                 190                 200
A I N F I T T I I N M K P P A M S Q Y Q T P L F V W S V M I T A V L L L L S L P
                210                 220                 230                 240
                          *
V L A A G I T M L L T D R N L N T T F F D P A G G G D P I L Y Q H L F W F F G H
                *
                250                 260                 270                 280
P E V Y I L I L P G F G M I S H I V T Y Y S G K K E P F G Y M G M V W A M M S I
                                    *
                290                 300                 310                 320
G F L G F I V W A H H M F T V G M D V D T R A Y F T S A T M I I A I P T G V K V
                                              *
                330                 340                 350                 360
F S W L A T L H G G N I K W S P A M M W A L G F I F L F T V G G L T G I V L A N
  *                   *
                370                 380                 390                 400
S S L D I V L H D T Y Y V V A H F H Y V L S M G A V F A I M G G F V H W F P L F
                410                 420                 430                 440
S G Y T L N D T W A K I H F A I M F V G V N M T F F P Q H F L G L S G M P R R Y
                450                 460                 470                 480
                                                                        _
S D Y P D A Y T M W N T I S S M G S F I S L T A V M L M V F I I W E A F A S K R
*                                                                       *
```

```
                490                 500                 510     514
        *
E V L T V D L T T T N L E W L N G C P P P Y H T F E E P T Y V N L K
```

Subunit II

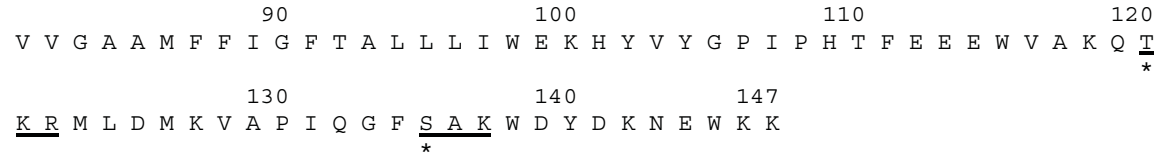
```
                10                  20                  30                  40
M A Y P M Q L G F Q D A T S P I M E E L L H F H D H T L M I V F L I S S L V L Y

                50                  60                  70                  80
            *
I I S L M L T T K L T H T S T M D A Q E V E T I W T I L P A I I L I L I A L P S
          *           *                                                     *

                90                  100                 110                 120
L R I L Y M M D E I N N P S L T V K T M G H Q W Y W S Y E Y T D Y E D L S F D S
                          *

                130                 140                 150                 160
                                                                *
Y M I P T S E L K P G E L R L L E V D N R V V L P M E M T I R M L V S S E D V L
                                                    *
                170                 180                 190                 200
H S W A V P S L G L K T D A I P G R L N Q T T L M S S R P G L Y Y G Q C S E I C
                                  *       *
                210                 220         227
                                            *
G S N H S F M P I V L E L V P L K Y F E K W S A S M L
                                      *
```

Subunit III

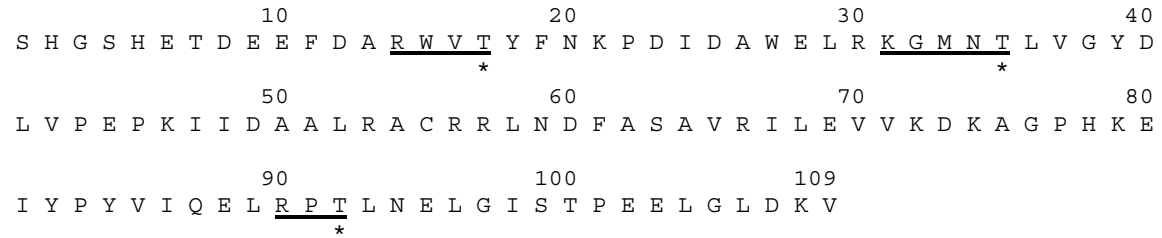
```
                10                  20                  30                  40
M T H Q T H A Y H M V N P S P W P L T G A L S A L L M T S G L T M W F H F N S M

                50                  60                  70                  80
T L L M I G L T T N M L T M Y Q W W R D V I R E S T F Q G H H T P A V Q K G L R
                                        * *
                90                  100                 110                 120
Y G M I L F I I S E V L F F T G F F W A F Y H S S L A P T P E L G G C W P P T G

                130                 140                 150                 160
I H P L N P L E V P L L N T S V L L A S G V S I T W A H H S L M E G D R K H M L

                170                 180                 190                 200
Q A L F I T I T L G V Y F T L L Q A S E Y Y E A P F T I S D G V Y G S T F F V A

                210                 220                 230                 240
T G F H G L H V I I G S T F L I V C F F R Q L K F H F T S N H H F G F E A G A W
                                              *
                250                 260
Y W H F V D V V W L F L Y V S I Y W W G S
```

Subunit IV

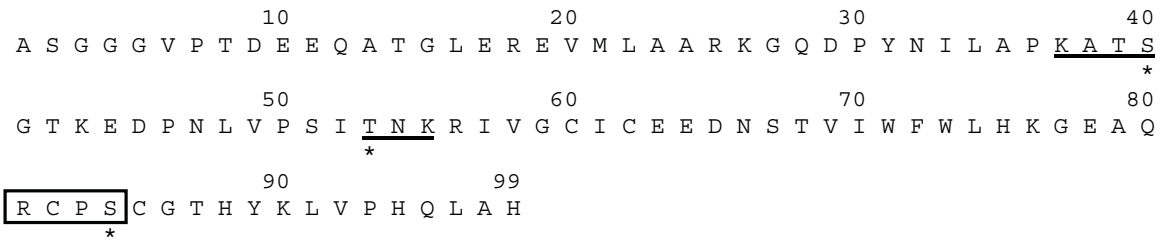
```
                10                  20                  30                  40
A H G S V V K S E D Y A L P S Y V D R R D Y P L P D V A H V K N L S A S Q K A L
                                                        *   *
                50                  60                  70                  80
K E K E K A D W S S L S R D E K V Q L Y R I Q F N E S F A E M N K G T N E W K T
              *                                                 *
```



Subunit Va

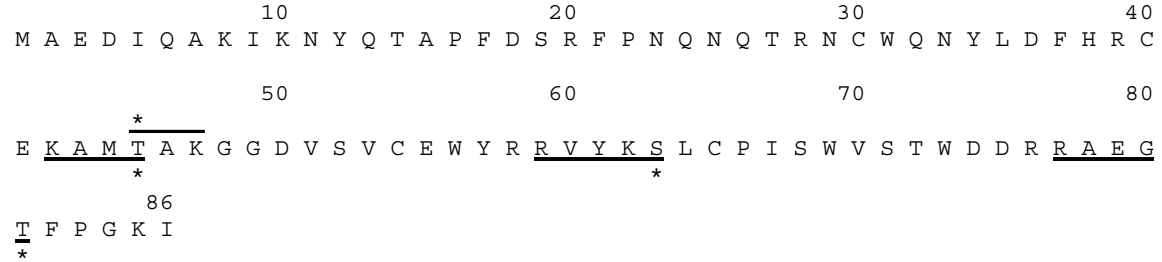


Subunit Vb



Subunit VIa

Subunit VIb



Subunit VIc

```
                10                  20                  30                  40
S T A L A K P Q M R G L L A R R L R F H I V G A F M V S L G F A T F Y K F A V A

                50                  60                  70    73
E K R K K A Y A D F Y R N Y D S M K D F E E M R K A G I F Q S A K
                      *                                   *
```

Subunit VIIa

```
                10                  20                  30                  40
F E N R V A E K Q K L F Q E D N G L P V H L K G G A T D N I L Y R V T M T L C L
                                                      *                 *
                50                59
G G T L Y S L Y C L G W A S F P H K K
```

Subunit VIIb

```
                10                  20                  30                  40
I H Q K R A P D F H D K Y G N A V L A S G A T F C V A V W V Y M A T Q I G I E W

                50          56
                *
N P S P V G R V T P K E W R E Q
                *
```

Subunit VIIc

```
                10                  20                  30                  40
S H Y E E G P G K N I P F S V E N K W R L L A M M T L F F G S G F A A P F F I V
                                                          *
           47
R H Q L L K K
```

Subunit VIII

```
                10                  20                  30                  40
             *     *
I T A K P A R T P T S P K E Q A I G L S V T F L S F L L P A G W V L Y H L D N Y

          46
K K S S A A
    * *
```

Fig. 23. Possible phosphorylation sites in the subunits of bovine heart cytochrome c oxidase for cAMP-dependent and cGMP-dependent kinases, and calcium activated protein kinases.

The consensus sequences for cAMP-dependent protein kinase are boxed, and for cGMP-dependent kinases and calcium activated protein kinases are underlined and overlined. The asterisks denote the residues which could be phosphorylated. The consensus sequences for the protein kinases are taken from Pearson and Kemp [1991].

4.4. Regulation of cytochrome c oxidase activity by cAMP-dependent phosphorylation

4.4.1. The allosteric ATP-inhibition of cytochrome c oxidase is protected by KF and EGTA in the isolation media

The allosteric ATP-inhibition of isolated cytochrome c oxidase [Arnold and Kadenbach, 1997] was found to vary in different enzyme preparations, depending on the degree of dephosphorylation during isolation [Bender and Kadenbach, 2000]. Therefore the effect of including the nonspecific protein phosphatase inhibitor KF and the calcium chelating agent EGTA in the isolation media on the allosteric ATP-inhibition was investigated. Cytochrome c oxidases from bovine heart, isolated in the presence of 10 mM KF and 2 mM EGTA by the Triton X-100 method, and in the absence of KF and EGTA by the cholate method, were solubilized in Tween 20 (see in 3.3.3.) and the activities were measured at increasing concentrations of cytochrome c (Fig. 24). Interestingly, the enzyme isolated in the presence of KF and EGTA shows a sigmoidal activity/substrate (v/s) relationship in the presence of ATP, with full inhibition of activity up to 2.5 μM cytochrome c. In contrast, the enzyme which was purified without KF and EGTA shows in the presence of ATP a hyperbolic activity/substrate relationship (saturation kinetics). Saturation kinetics are obtained with both enzymes in the presence of ADP. Cytochrome c oxidase which was isolated in the absence of KF and EGTA by the Triton X-100 method, has also hyperbolic kinetics in the presence of ATP and ADP (data not shown).

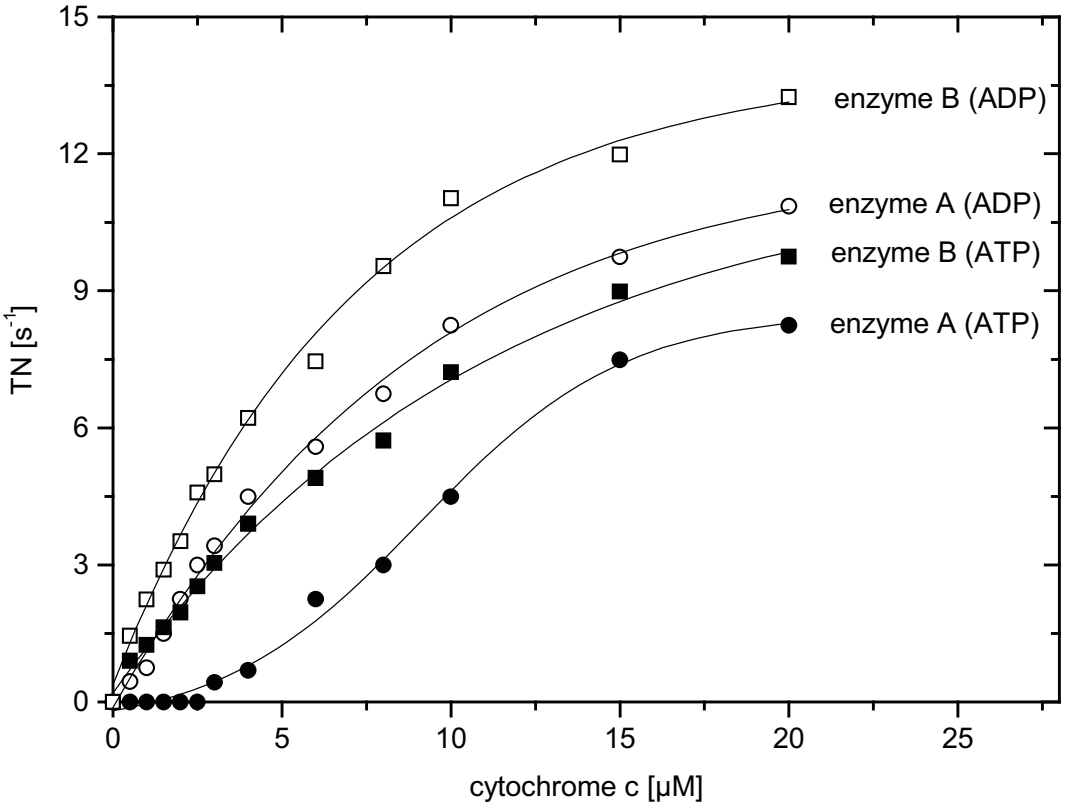


Fig. 24. Effect of including KF and EGTA in the enzyme isolation media on the kinetics of bovine heart cytochrome c oxidase.
Enzyme A (circles) was isolated by the Triton X-100 method in the presence of KF and EGTA and enzyme B (squares) was isolated by the cholate method in the absence of KF and EGTA. The enzymes were solubilized in Tween 20, 50 mM KPi (pH 7.4), 1 mM EDTA with 1 % cardiolipin (40:1 mol/mol, cardiolipin/enzyme) and dialysed against 0.5 mM ATP as described in 3.3.3. The ascorbate respiration was measured polarographically in the presence of 5 mM ATP (closed symbols) or 5 mM ADP (open symbols), as indicated in parentheses, with increasing concentrations of cytochrome c (0-20 μM).

4.4.2. Effect of cAMP-dependent phosphorylation on the allosteric ATP-inhibition of cytochrome c oxidase isolated with KF and EGTA

The allosteric ATP-inhibition of the bovine heart enzyme, purified with KF and EGTA, is only slightly enhanced by incubation with cAMP-dependent protein kinase (PKA), cAMP, ATP, and an ATP-regenerating system (see in 3.3.5.). Full inhibition of enzyme activity is extended only from 2.5 μM to 3 μM cytochrome c concentration (Fig. 25). This result suggests that the isolation conditions (presence of KF and EGTA) prevented the dephosphorylation of this enzyme at the sites for cAMP-dependent protein kinase(s) during purification.

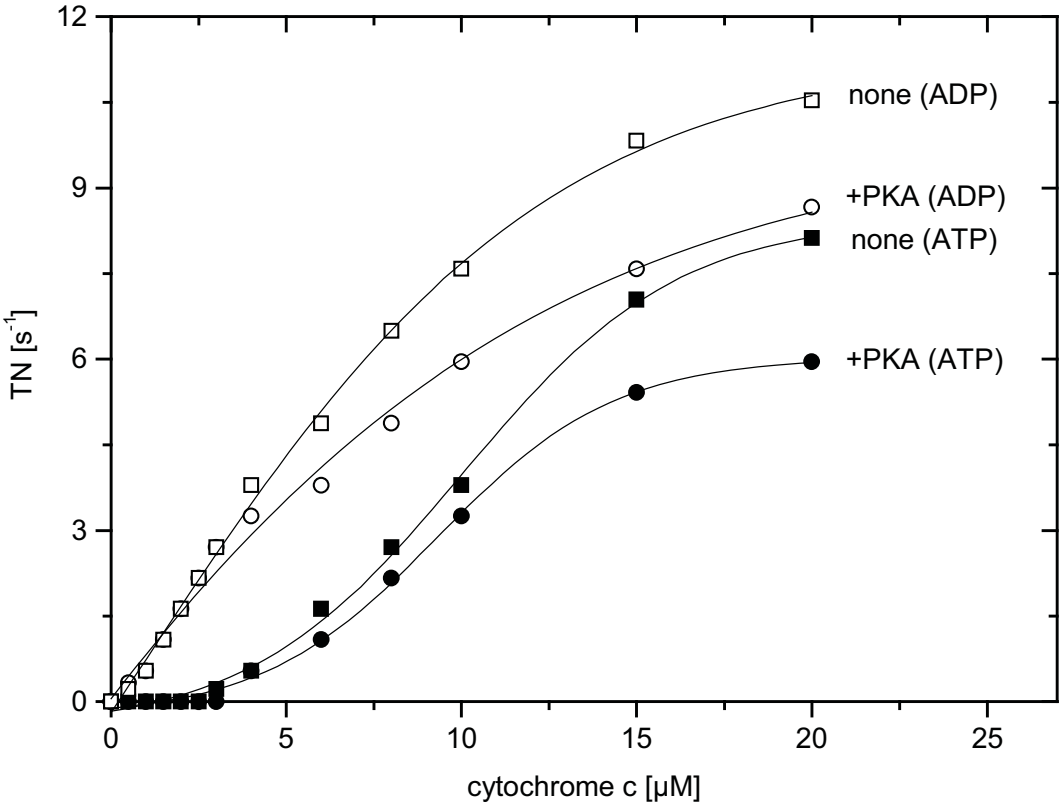


Fig. 25. Allosteric ATP-inhibition of bovine heart cytochrome c oxidase, isolated with KF and EGTA, is slightly intensified by incubation with protein kinase A, cAMP, and ATP.
Solubilized cytochrome c oxidase was incubated with 200 U/ml cAMP-dependent protein kinase (PKA), 50 μM cAMP, 5 mM ATP, and an ATP-regenerating system (10 mM PEP, 10 U/ml PK, and 5 mM $MgSO_4$) at 30 °C for 20 min (circles). As a control, cytochrome c oxidase was incubated with all substances except PKA under the same conditions (squares). The ascorbate respiration was measured in the presence of 5 mM ATP (closed symbols) or 5 mM ADP (open symbols).

4.4.3. TMPD abolishes the allosteric ATP-inhibition of cytochrome c oxidase

N,N,N',N'-Tetramethyl-p-phenylenediamine-hydrochloride (TMPD), an electron carrier between ascorbate and cytochrome c, was added to the solubilized enzyme during measurement. TMPD releases completely the allosteric ATP-inhibition of cytochrome c oxidase. The enzyme activity at 10 µM cytochrome c in the presence of ADP was increased to ca. 162 % by addition of TMPD and more than doubled in the presence of ATP (Fig. 26).

TMPD allows electron transfer from ascorbate via cytochrome c to the oxidase without dissociation of cytochrome c [Ferguson-Miller and Margoliash, 1978]. The release of allosteric ATP-inhibition indicates that TMPD eliminates the cooperativity of the two cytochrome c binding sites (in each monomer) of the dimeric enzyme.

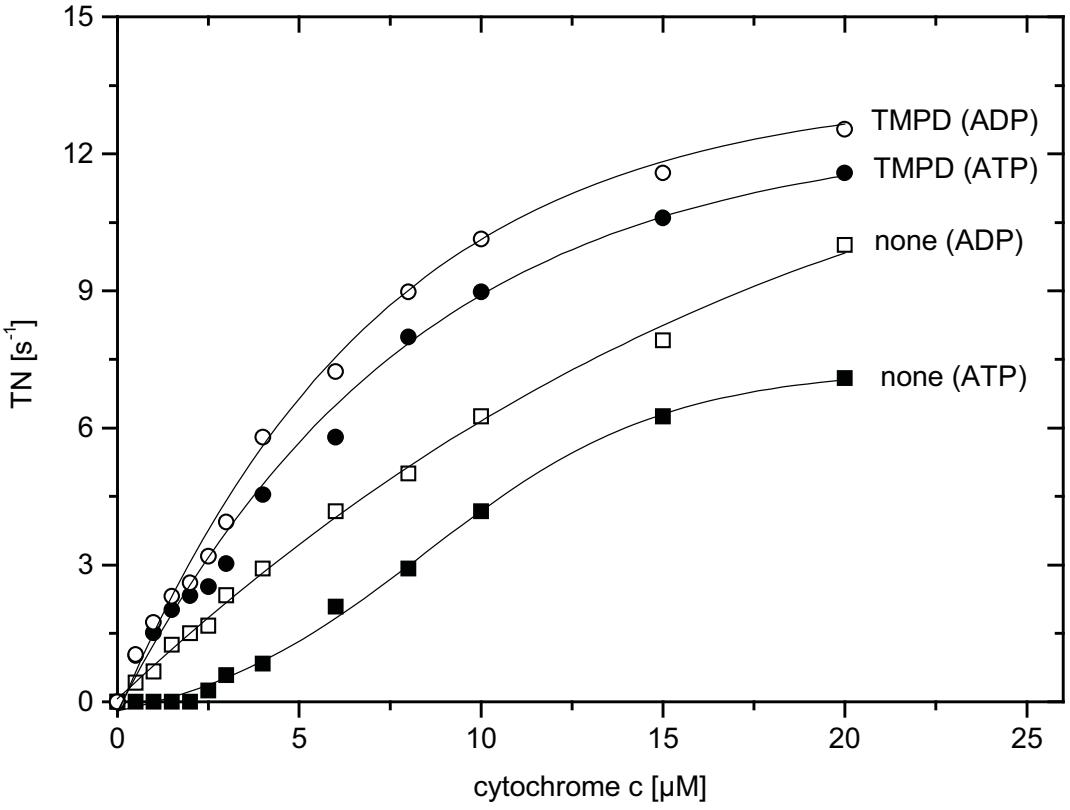


Fig. 26. TMPD releases the allosteric ATP-inhibition of isolated cytochrome c oxidase from bovine heart.
The respiration of solubilized cytochrome c oxidase, purified in the presence of KF and EGTA, was measured with (circles) and without (squares) 0.7 mM TMPD. 5 mM ATP (closed symbols) or 5 mM ADP (open symbols) was included in the measuring system.

4.4.4. Incubation with protein phosphatase 1 releases the allosteric ATP-inhibition of cytochrome c oxidase

Dephosphorylation of cytochrome c oxidase was performed to confirm the significance of phosphorylation for the allosteric ATP-inhibition of the enzyme. Solubilized bovine heart cytochrome c oxidase, isolated with KF and EGTA, was dephosphorylated by incubation with the catalytic subunit of protein phosphatase 1 (PP1) (see in 3.3.5.). After incubation with PP1 the allosteric ATP-inhibition of the enzyme is completely abolished. In the presence of ATP or ADP nearly the same activity of the enzyme is measured after treatment with PP1, as presented in Fig. 27.

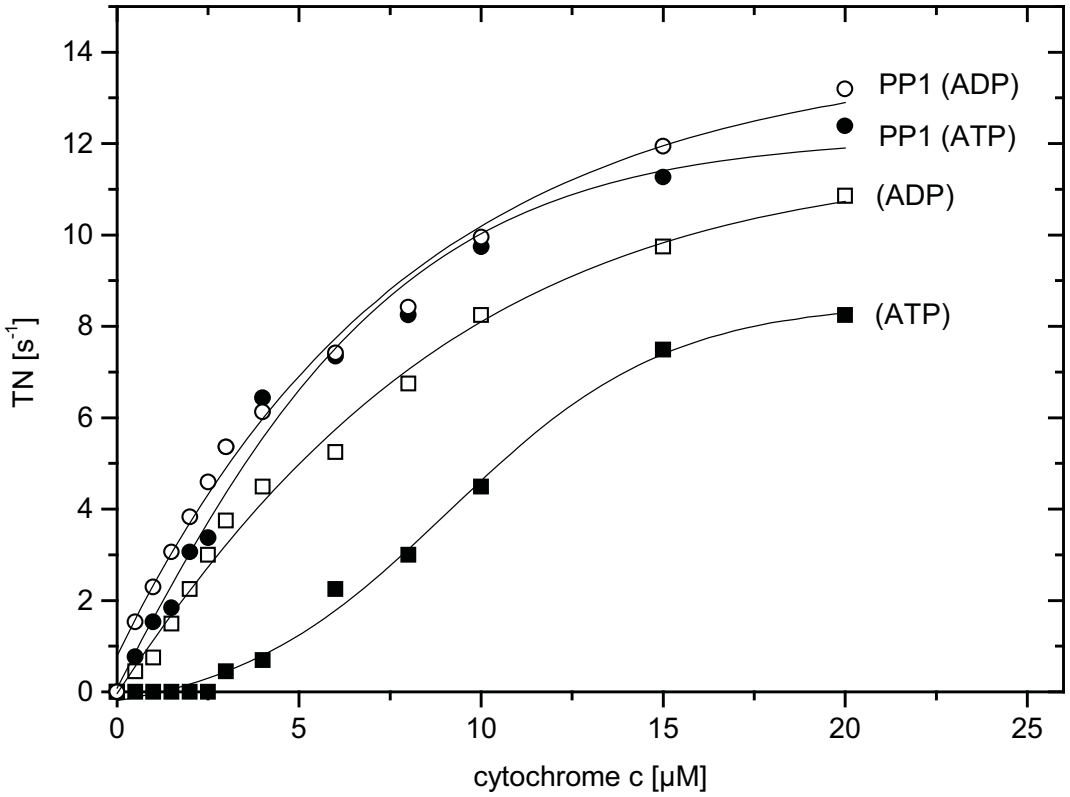


Fig. 27. Allosteric ATP-inhibition of bovine heart cytochrome c oxidase isolated in the presence of KF and EGTA, is released by incubation with protein phosphatase 1 (PP1). Solubilized enzyme was incubated at 30 °C for 40 min with 300 U/ml PP1 (catalytic subunit) and 100 μM $MnCl_2$ (circles). As a control the enzyme was incubated only with $MnCl_2$ under the same conditions (squares). The activity was measured in the presence of 5 mM ATP (closed symbols) or 5 mM ADP (open symbols).

4.4.5. Time dependent induction of allosteric ATP-inhibition of cytochrome c oxidase by cAMP-dependent phosphorylation

Bovine heart cytochrome c oxidase, isolated without KF and EGTA by the cholate method, was incubated for different times with PKA, cAMP, ATP, and an ATP-regenerating system and the enzyme activity was measured. The allosteric ATP-inhibition is not seen before incubation with PKA but is induced by incubation and intensified with increasing time of incubation. After incubation for 40 min, full inhibition of activity is obtained up to 3 µM cytochrome c concentration (Fig. 28). Extension of the incubation time for 60 min does not further intensify the allosteric ATP-inhibition. The activity of the enzyme in the presence of ADP is slightly decreased by phosphorylation but the hyperbolic activity/substrate relationship is not changed.

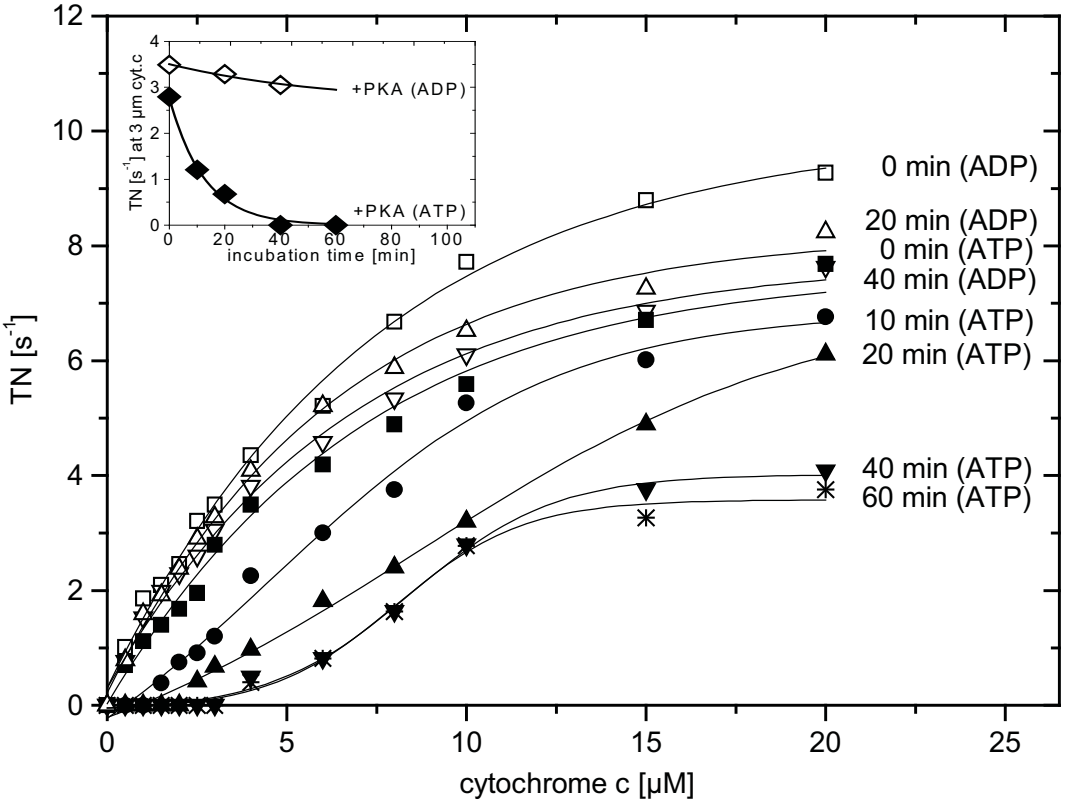


Fig. 28 The allosteric ATP-inhibition of bovine heart cytochrome c oxidase is dependent on the incubation time with PKA, cAMP, and ATP.
Solubilized heart enzyme, isolated under standard conditions (in the absence of KF and EGTA) by the cholate method, was incubated with 200 U/ml PKA, 50 µM cAMP, 5 mM ATP, and the ATP-regenerating system at 30 °C for 0, 10, 20, 40 and 60 min, respectively. The ascorbate respiration of the enzyme was measured in the presence of 5 mM ATP (closed symbols) or 5 mM ADP (open symbols). The enzyme activity at 3 µM cytochrome c (diamonds) is presented with increasing incubation time in the insert. Squares: incubation with PKA for 0 min; circles: 10 min; up-triangles: 20 min; down-triangles: 40 min; stars: 60 min.

4.4.6. Allosteric ATP-inhibition of cytochrome c oxidase is reversibly turned on by cAMP-dependent phosphorylation and turned off by dephosphorylation

The reversibility of allosteric ATP-inhibition of cytochrome c oxidase by phosphorylation with protein kinase A in a cAMP-dependent way, and by dephosphorylation with protein phosphatase, is presented in Fig. 29. The enzyme, isolated without KF and EGTA, showed no sigmoidal inhibition kinetics in the presence of ATP (see insert). Incubation with PKA induces the allosteric ATP-inhibition by changing the hyperbolic into a sigmoidal titration curve, whereas subsequent incubation with PP1 converts back the sigmoidal into a hyperbolic titration curve, resulting in the same activity in the presence of ATP and ADP.

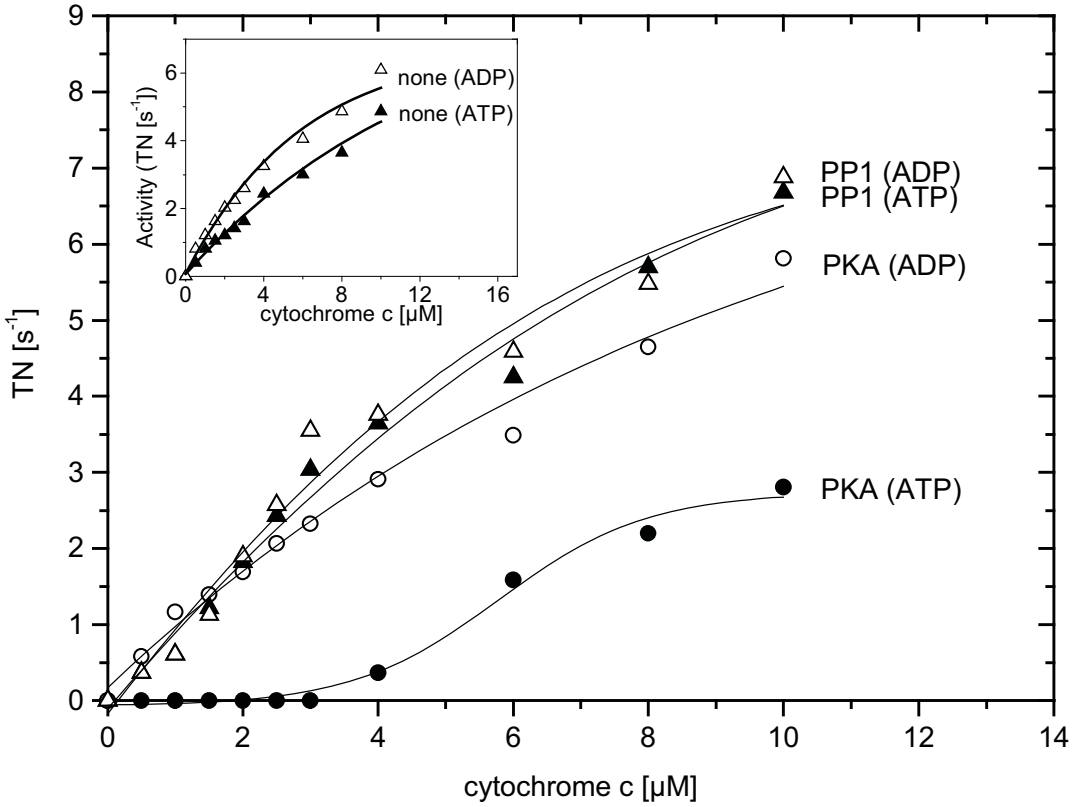


Fig. 29. Phosphorylation of bovine heart cytochrome c oxidase with PKA turns on, and subsequent dephosphorylation with PP1 turns off the allosteric ATP-inhibition.
Solubilized cytochrome c oxidase, isolated in the absence of KF and EGTA, was phosphorylated (circles) at 37 °C for 40 min with 200 U/ml PKA, 50 µM cAMP, 5 mM ATP, and an ATP-regenerating system. Subsequent dephosphorylation (up-triangles) was performed at 30 °C for 40 min with 500 U/ml PP1 and 100 µM $MnCl_2$. The insert presents the respiration of the enzyme before incubation with PKA (squares). The activity was measured in the presence of 5 mM ATP (closed symbols) or 5 mM ADP (open symbols).

4.4.7. The allosteric ATP-inhibition is obtained with cytochrome c oxidases from bovine heart, kidney, and testis

Cytochrome c oxidases from bovine heart, kidney, and testis were phosphorylated with PKA, cAMP, ATP, and an ATP-regenerating system, and the activity was measured at increasing concentrations of cytochrome c (Fig. 30). Before phosphorylation the kidney and testis enzymes present hyperbolic titration curves in the presence of ATP. The heart enzyme seems to be already phosphorylated to some extent, because its kinetic has a linear shape instead of a hyperbolic. After phosphorylation, all enzymes present allosteric ATP-inhibition. The hyperbolic curves in the presence of ADP, and the sigmoidal curves in the presence of ATP are very similar among each other.

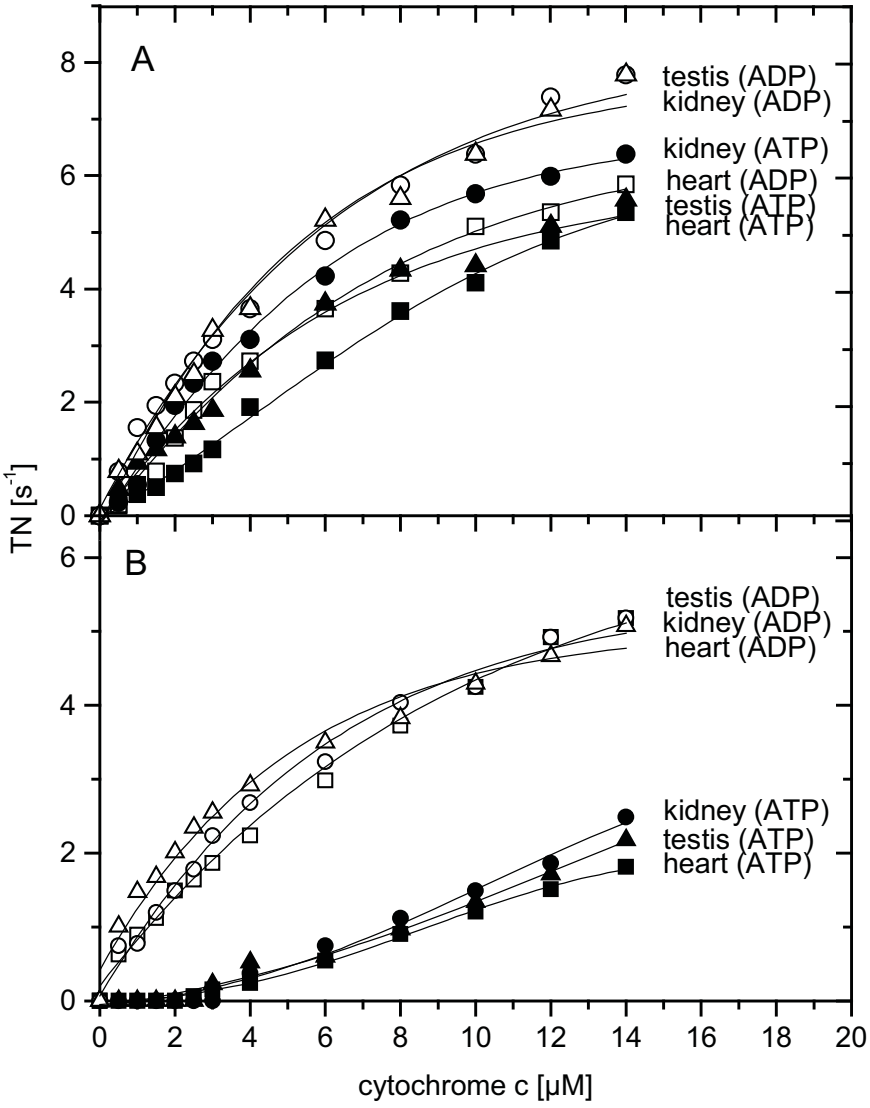


Fig. 30. Induction of allosteric ATP-inhibition of isolated cytochrome c oxidases from bovine heart, kidney, and testis by incubation with PKA, cAMP, and ATP.
The activity of the enzymes were measured before (A) and after (B) phosphorylation. Solubilized enzymes from heart (squares), kidney (circles), and testis (up-triangles) were phosphorylated at 37 °C for 40 min with 200 U/ml PKA, 50 µM cAMP, 5 mM ATP, and an ATP-regenerating system. The ascorbate respiration was measured in the presence of 10 mM PEP, 10 U/ml PK, 5 mM $MgSO_4$, and 5 mM ATP (closed symbols) or 10 mM PEP, 5 mM $MgSO_4$, and 5 mM ADP (open symbols).

4.4.8. Allosteric ATP-inhibition of cytochrome c oxidase is induced by phosphorylation from the cytosolic side

Most subunits of cytochrome c oxidase are transmembraneous, except subunits Va, Vb and VIb. Therefore it was of interest, whether the allosteric ATP-inhibition is due to phosphorylation from the cytosolic or matrix side. Bovine heart cytochrome c oxidase was reconstituted in the presence of ATP or ADP as described in 3.3.4. Then the proteoliposomes were phosphorylated only from the cytosolic side (outside). As shown in Fig. 31, allosteric ATP-inhibition is obtained only with phosphorylated proteoliposomes, reconstituted in the presence of ATP. This result demonstrates the sidedness (cytosolic side) of the phosphorylation site(s) in cytochrome c oxidase, which induces allosteric ATP-inhibition.

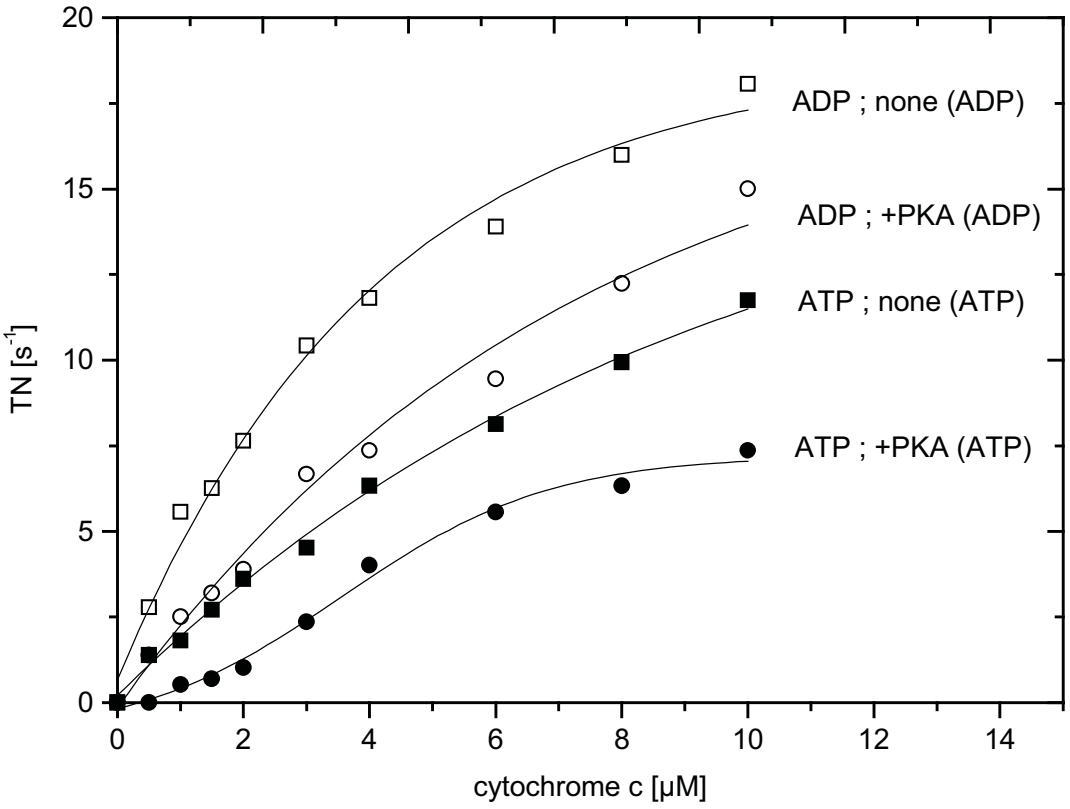


Fig. 31. Allosteric ATP-inhibition of reconstituted bovine heart cytochrome c oxidase is induced by phosphorylation from the outside.
Cytochrome c oxidase was reconstituted in the presence of 5 mM ATP (closed symbols) or 5 mM ADP (open symbols). The reconstituted enzyme (squares) was phosphorylated (circles) at 37° C for 40 min with 200 U/ml PKA, 50 µM cAMP, and an ATP-regenerating system. The descriptions before and after the semicolon indicate the conditions during reconstitution and during measurement of activity, respectively.

4.4.9. Proposed phosphorylation site for allosteric ATP-inhibition

A search for consensus sequences for cAMP-dependent phosphorylation in the bovine heart enzyme (Fig. 23) revealed only three sites: $RRYS^{441}$ in subunit I (cytosolic side), RES^{65} in subunit III (matrix side) and $RCPS^{84}$ in subunit Vb (matrix side). The consensus sequence in subunit Vb ($RCPS^{84}$) is not conserved, neither in mammals nor in other eukaryotes as shown in Fig. 32. Also the consensus sequence in subunit III (RES^{65}) is not conserved in animals (Fig. 33). In contrast the consensus sequence $RRYS^{441}$ in subunit I is completely conserved in all animals which are controlled by hormones, i.e. from mammals, over birds, reptiles, fishes, insects, molluscs, down to echinacea (sea urchin). This consensus sequence is absent in all other organisms, e.g. in nematodes *(C. elegans*), neodermata, plants, fungi (yeast), and bacteria (Fig. 34).

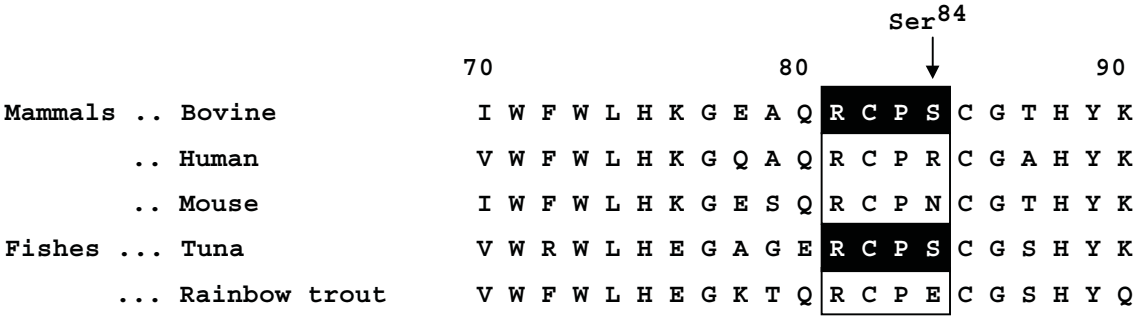


Fig. 32. The consensus sequence for cAMP-dependent phosphorylation in cytochrome c oxidase subunit Vb is not conserved.
The consensus sequence for cAMP-dependent phosphorylation is boxed in black.

In previous studies, cAMP-dependent phosphorylation of isolated bovine heart cytochrome c oxidase with [γ-^{32}P] ATP, cAMP, and PKA resulted in labelling of subunits I, II (and/or III), and Vb [Bender and Kadenbach, 2000]. This corresponds to the three cAMP-dependent phosphorylation sites in subunits I, III, and Vb from the search in Fig. 23. From the facts that (1) the consensus sequence $RRYS^{441}$ for cAMP-dependent phosphorylation in subunit I is oriented to the cytosolic side and completely conserved among animals, and (2) that phosphorylation from the cytosolic side is essential for turning on the allosteric ATP-inhibition in the bovine heart enzyme (Fig. 31), it is suggested that $RRYS^{441}$ in subunit I represents the phosphorylation site which induces the allosteric ATP-inhibition of animal cytochrome c oxidases.

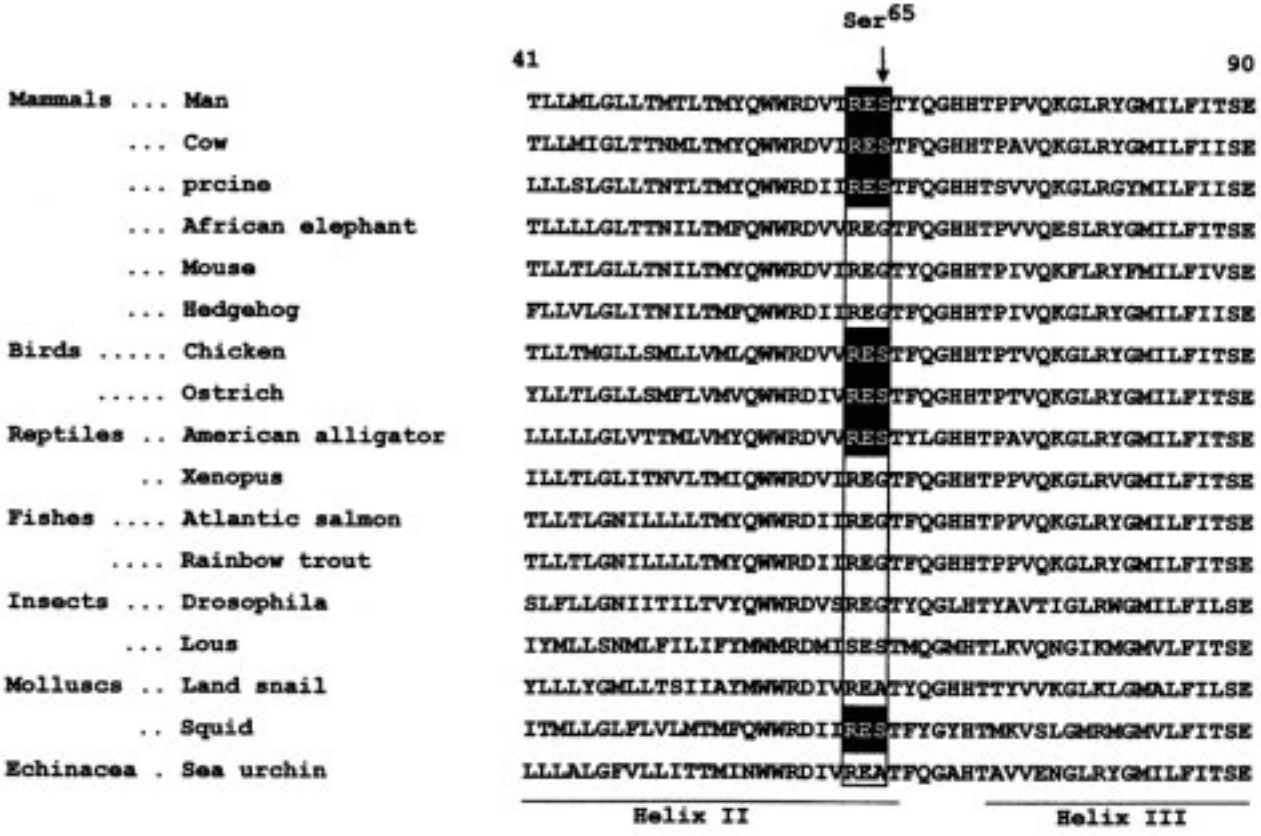


Fig. 33. The consensus sequence for cAMP-dependent phosphorylation in cytochrome c oxidase subunit III is not conserved.
The consensus sequence for cAMP-dependent phosphorylation is boxed in black. Helix II and III indicate parts of transmembraneous helices in subunit III, as derived from the crystal structure [Tsukihara et al., 1996].

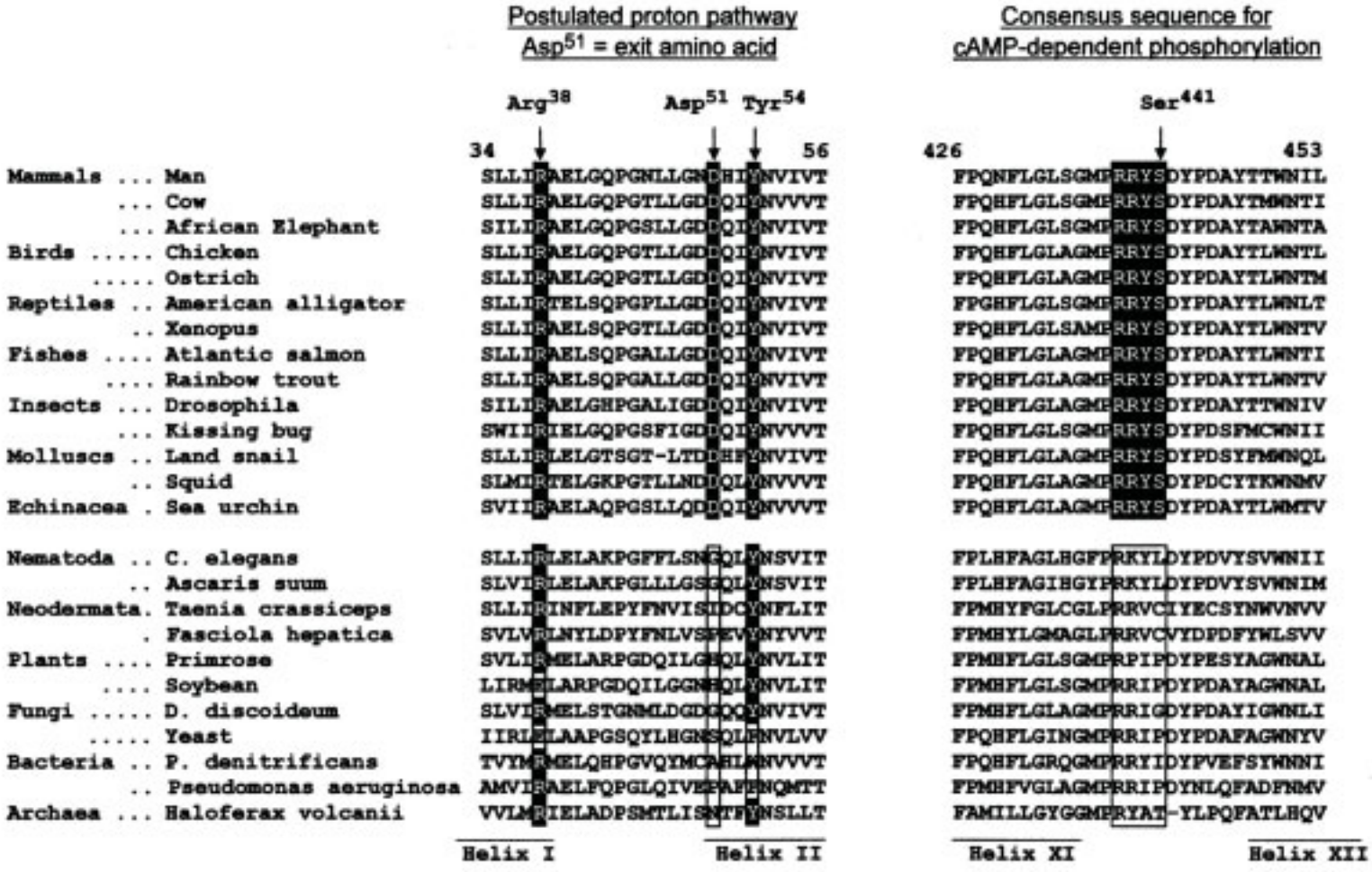


Fig. 34. Parallel conservation of a consensus sequence for cAMP-dependent phosphorylation and of the postulated proton pathway in subunit I of bovine heart cytochrome c oxidase, including Arg^{38}, Asp^{51} (exit amino acid), and Tyr^{54} [Yoshikawa et al., 1998].
The consensus amino acids of the proton pathway and the conserved consensus sequence for cAMP-dependent phosphorylation are presented white on black. Modified amino acids and modified consensus sequences are presented in boxes black on white. Helix I, II, XI, and XII indicate parts of transmembraneous helices in subunit I, as derived from the crystal structure [Tsukihara et al., 1996].

4.5. Proton translocation in eukaryotic cytochrome c oxidase has a variable H^+/e^- stoichiometry

A new regulation of the H^+/e^- stoichiometry in eukaryotic cytochrome c oxidase by the intramitochondrial ATP/ADP-ratio was found by Kadenbach and coworkers [Frank and Kadenbach, 1996; Hüttemann et al., 1999 and 2000]. At high ATP/ADP-ratios (half-maximal at ATP/ADP = 100), the H^+/e^- stoichiometry decreased from 1.0 to 0.5 in the enzyme from heart or skeletal muscle, containing subunit VIaH (heart type). It was suggested that the decreased proton pumping stoichiometry participates in thermogenesis in skeletal muscle at rest or during sleep [Rohdich and Kadenbach, 1993; Kadenbach et al., 1995; Hüttemann et al., 1999 and 2000].

4.5.1. Cardiolipin increases the H^+/e^- stoichiometry of reconstituted cytochrome c oxidase from bovine kidney but not from bovine heart.

In contrast to the decrease of H^+/e^- stoichiometry of the bovine heart enzyme from 1.0 to 0.5 at high intraliposomal ATP/ADP-ratios [Frank and Kadenbach, 1996], the H^+/e^- stoichiometry of reconstituted cytochrome c oxidases from bovine liver and kidney [Hüttemann et al., 1999] and from turkey liver and heart [Hüttemann et al., 2000] was constantly 0.5 under the same measuring conditions and was not influenced by the ATP/ADP-ratio. All these enzymes contain subunit VIaL (liver type of subunit VIa).

For the measurements of proton translocation of cytochrome c oxidase, generally cardiolipin had not been added during reconstitution. When 1% cardiolipin is included during reconstitution of the bovine kidney enzyme, the H^+/e^- stoichiometry increases to about 1.0, as presented in Fig. 35. After addition of 10.6 nmol of ferrocytochrome c, acidifications corresponding to $H^+/e^- = 0.47$ in the absence and 0.87 in the presence of cardiolipin are obtained, which are verified by H^+/e^- stoichiometries of -0.99 and -0.98 in the presence of CCCP, respectively (the theoretical alkalinization is 1.0). The addition of cardiolipin to asolectin before reconstitution increases the H^+/e^- stoichiometry of the kidney enzyme to the same value, as measured with the heart enzyme without addition of cardiolipin.

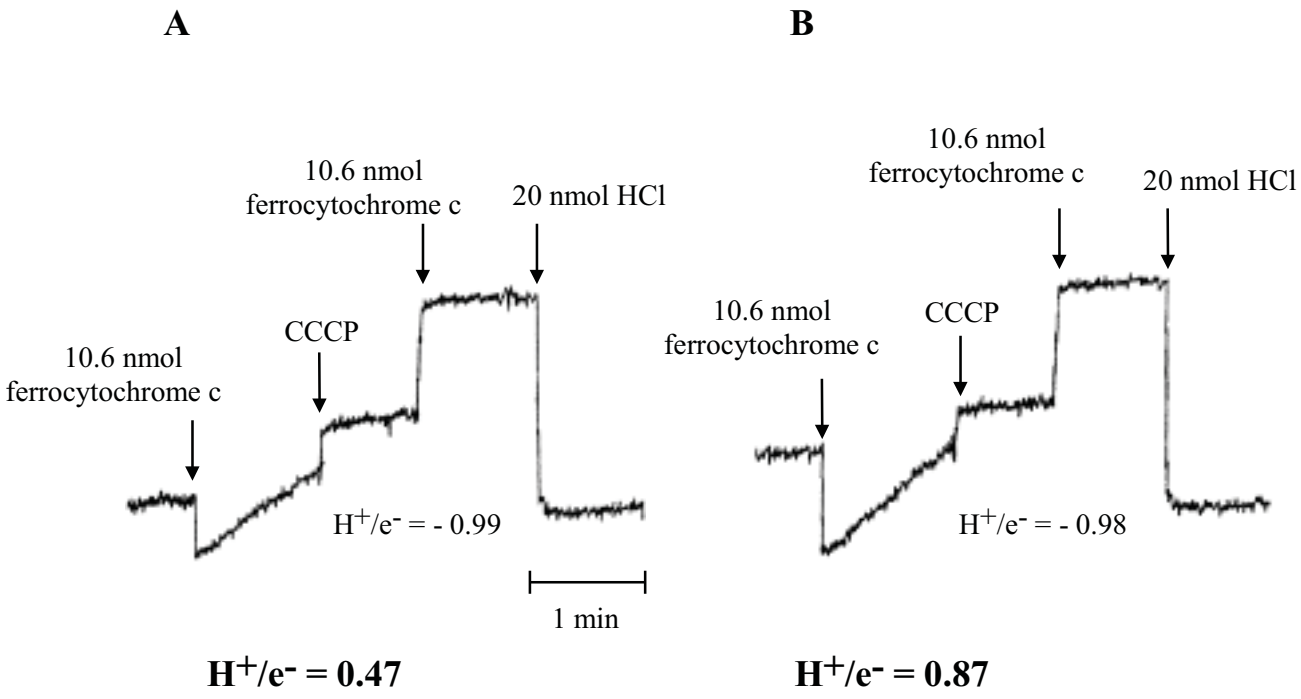


Fig. 35. H^+/e^- stoichiometry of cytochrome c oxidase from bovine kidney is increased by addition of cardiolipin.
Cytochrome c oxidase was reconstituted with (B) or without (A) 1 % cardiolipin in the presence of 5 mM ADP and dialysed as described in 3.4.1. The H^+/e^- stoichiometry of the enzyme was measured by the reductant-pulse method (see in 3.4.2.). The pH changes are recorded after addition of 10.6 nmol ferrocytochrome c (corresponding to 8 enzyme turnover) to 80 µl proteoliposomes (0.2 µM heme aa_3) and 1 µg/ml valinomycin in the presence and absence of 3 µM CCCP. The measuring system was calibrated with 20 nmol HCl.

Similar results are obtained with a stopped-flow spectrophotometric assay (Fig. 36). H^+/e^- stoichiometries of proteoliposomes were measured in collaboration with Maarten Ruitenberg in the group of Prof. K. Fendler, MPI of Biophysics, Department of Biophysical Chemistry, Frankfurt/Main, using the pH sensitive dye, phenol red, as described in 3.4.3. The absorbance before addition of cytochrome c to the cytochrome c oxidase vesicles was taken as zero value, and absorbance changes after addition of cytochrome c are presented by +/- numbers.

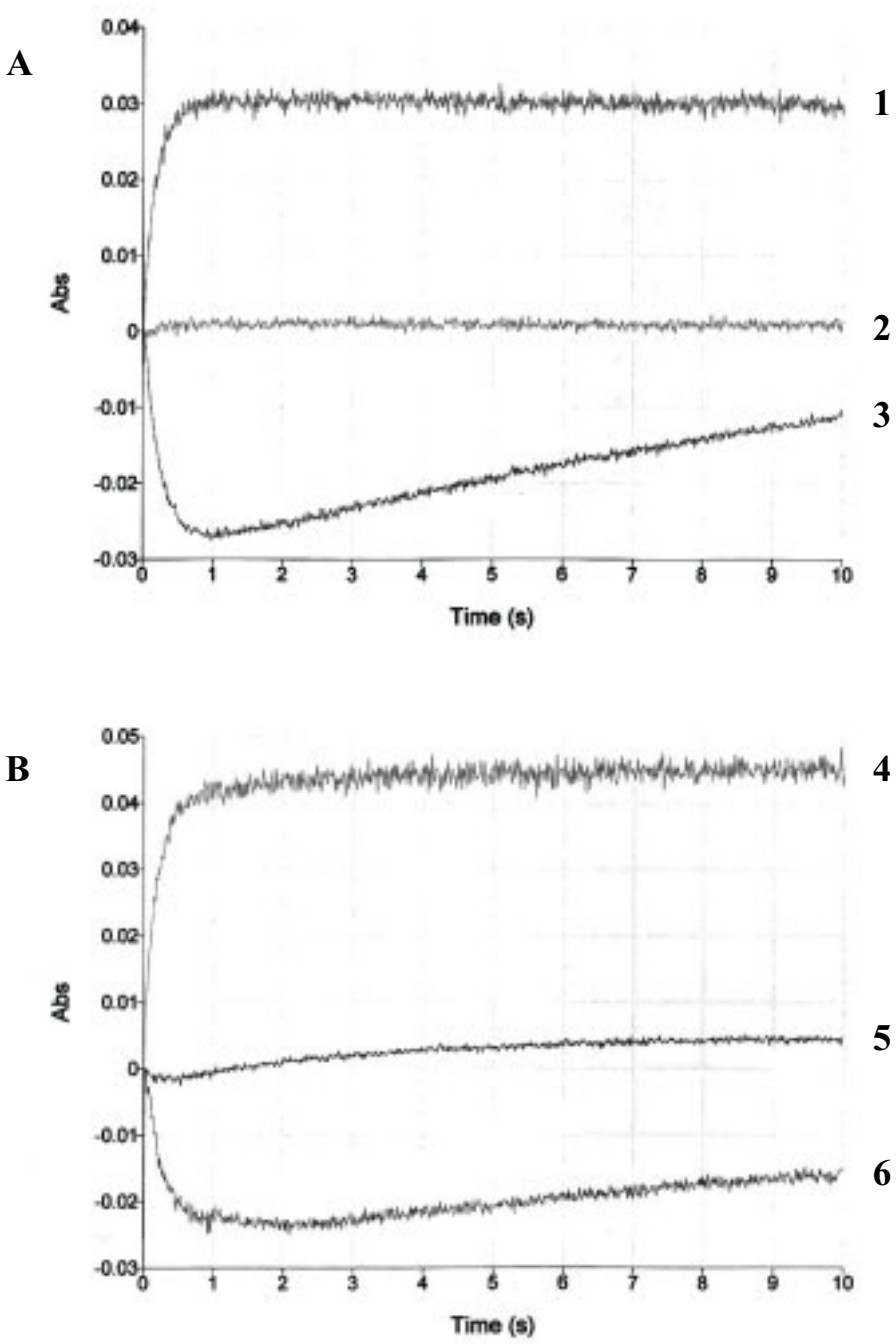


Fig. 36. Measurement of proton translocation of bovine kidney cytochrome c oxidase reconstituted with (A) and without (B) cardiolipin by a stopped-flow spectrophotometer. The proteoliposomes were prepared with or without 5 % cardiolipin in the presence of 5 mM ADP as described in 3.4.1. Absorbance changes of phenol red were recorded after addition of ferrocytochrome c to the proteoliposomes in the absence and the presence of CCCP at 555.6 nm. The absorbance before addition of reduced cytochrome c was taken as zero value. Positive changes represent alkalinization, negative changes acidification. Trace 1 and 4: addition of reduced cytochrome c in the presence of CCCP; trace 2 and 5: addition of oxidized cytochrome c in the absence of CCCP; trace 3 and 6: addition of reduced cytochrome c in the absence of CCCP.

After addition of cytochrome c to the reconstituted bovine kidney enzyme, the absorbance is decreased in the absence of CCCP, due to acidification of the measuring system by pumped protons from cytochrome c oxidase. Increase of absorbance is shown after addition of cytochrome c in the presence of CCCP, due to intravesicular alkalinization as a consequence of oxygen reduction and immediate pH equilibration between intra- and extraliposomal space. The extents of acidification and alkalinization with the kidney enzyme vesicles containing cardiolipin are almost the same in the presence and absence of CCCP, corresponding to a H^+/e^- stoichiometry of 1.0. In contrast, the extent of acidification is half of the alkalinization with the reconstituted kidney enzyme without cardiolipin, corresponding to a H^+/e^- stoichiometry of 0.5. Control experiments with oxidized cytochrome c showed that there was no contribution from cytochrome c to the phenol red absorbance changes. Calibration was performed by the released protons after tryptic cleavage of TAME in the incubation mixture (data not shown).

In Fig. 37 the stimulatory effect of increasing cardiolipin concentrations on the H^+/e^- stoichiometry of kidney cytochrome c oxidase is presented. Maximal increase of H^+/e^- stoichiometry is obtained at a concentration of 1 % in the lipid mixture. On the other hand, the H^+/e^- stoichiometry of the bovine heart enzyme is close to 1.0, independent of the addition of cardiolipin.

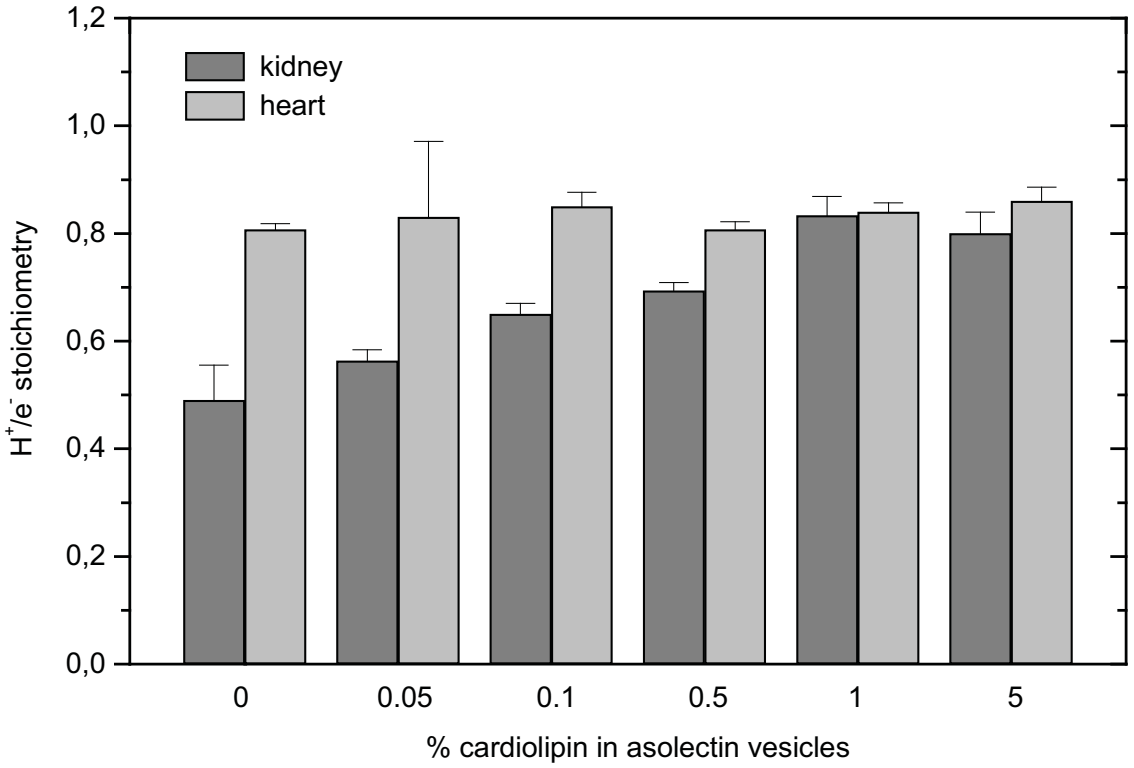


Fig. 37. Effect of different concentrations of cardiolipin on the H^+/e^- stoichiometry of reconstituted cytochrome c oxidases from kidney and heart.
Cytochrome c oxidase from bovine kidney and heart were reconstituted with 0-5 % of cardiolipin (percentage of the total lipid) and proton translocation was measured. The standard deviations were calculated from 3 independent determinations.

In order to exclude the possibility that the higher efficiency of proton translocation of the kidney enzyme in the presence of cardiolipin was due to a better proton tightness of the proteoliposomes, the proton permeability of the vesicles was measured by applying a potassium diffusion gradient, as shown in Fig. 38. The addition of 1 µM valinomycin stimulates the proton influx into the proteoliposomes at the same rate with or without 1 % cardiolipin. Therefore the increased H^+/e^- stoichiometry of the bovine kidney enzyme, reconstituted with cardiolipin, is assumed to be due to a change of the proton pumping property of the enzyme.

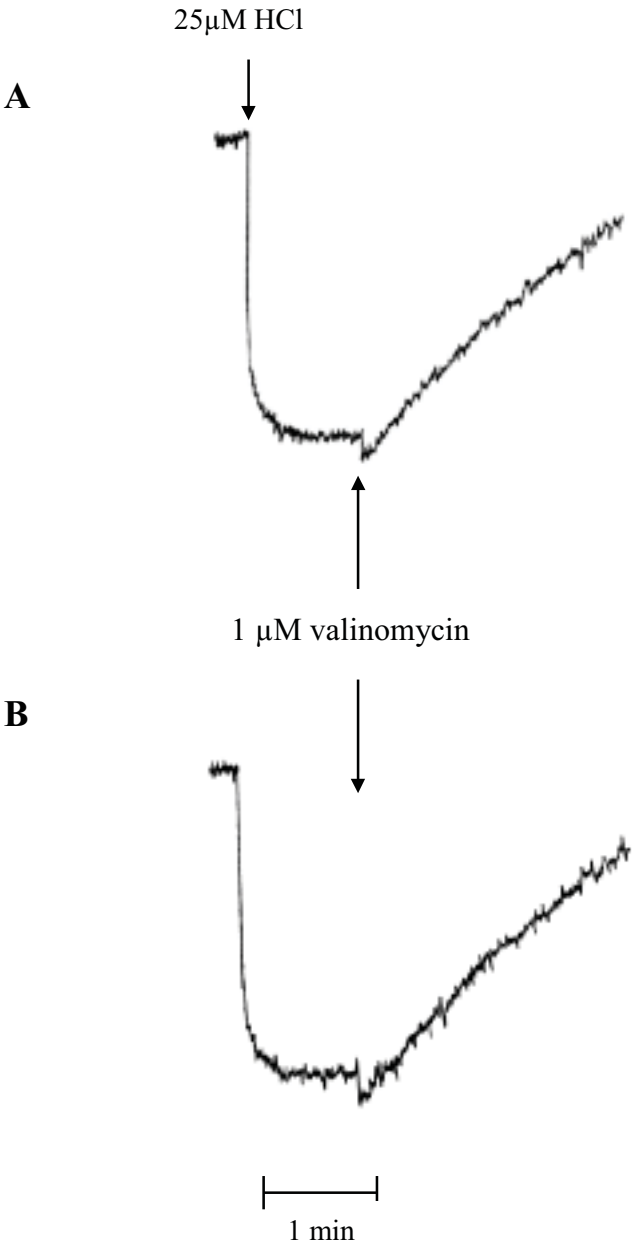


Fig. 38. The proton conductivities of proteoliposomes with bovine kidney cytochrome c oxidase reconstituted in the absence (A) or presence (B) of 1 % cardiolipin are the same. The proteoliposomes contained 100 mM K-Hepes inside the vesicles, and were suspended in 1 mM K-Hepes, pH 7.2, 100 mM choline chloride, 5 mM KCl. The proton conductivity is measured by recording the pH change of the suspension after addition of 1 μM valinomycin as described in 3.4.4. The H^+/e^- stoichiometry of the proteoliposomes without cardiolipin (A) was 0.47, that with cardiolipin (B) was 0.87. The ordinate was calibrated with 25 μM HCl, as indicated.

In order to exclude the possibility that cardiolipin improves incorporation of cytochrome c oxidase into the liposomes, the respiratory control ratios (RCRs) of the vesicles containing increasing amounts of cardiolipin was determined (better incorporation results in higher values of RCR). No change of RCR is obtained with proteoliposomes containing increasing cardiolipin concentrations up to 5 % of total lipids (Table 5).

Table 5. Effect of cardiolipin on respiratory control ratios (RCRs) of reconstituted cytochrome c oxidase from bovine kidney.
Cytochrome c oxidase was reconstituted with 0-5 % cardiolipin. RCRs were measured polarographically as described in 3.3.6. The standard deviations, sd (ΔyEr) were calculated from 3 independent determinations.

Concentration of cardiolipin (%)	RCR	sd (ΔyEr)	n
0	4.65	0.0212	3
0.05	4.86	0.0141	3
0.1	4.63	0.0565	3
0.5	4.82	0.0134	3
1	4.82	0.0287	3
5	4.59	0.0254	3

To characterize the chemical group in cardiolipin which is responsible for the stimulation of H^+/e^- stoichiometry in the kidney enzyme, the influence of 3-palmitoyl-sn-glycerol on the H^+/e^- stoichiometry was investigated. Palmitoyl glycerol has a similar structure to half of the cardiolipin molecule. Cytochrome c oxidase was reconstituted with palmitoyl glycerol in the presence and absence of cardiolipin. As shown in Table 6, no effect of palmitoyl glycerol on the H^+/e^- stoichiometry is detected, neither in the absence nor in the presence of cardiolipin.

Table 6. Effect of 3-palmitoyl-sn-glycerol on the H^+/e^- stoichiometry of reconstituted bovine kidney cytochrome c oxidase.
Cytochrome c oxidase was reconstituted with 0-1.5 % palmitoyl glycerol in the absence and presence of 1 % cardiolipin. The standard deviations (sd (ΔyEr)) were calculated from 3 independent determinations.

Cardiolipin (%)	palmitoyl glycerol (%)	H^+/e^- stoichiometry	sd(ΔyEr)	n
0	1	0.42	0.0400	3
1	0	0.86	0.0057	3
1	0.25	0.92	0.0095	3
1	0.5	0.87	0.0635	3
1	1.5	0.89	0.0057	3

4.5.2. Specific decrease of H^+/e^- stoichiometry by palmitate of cytochrome c oxidase from kidney but not from heart

Crystallized cytochrome c oxidase from bovine heart contains 5 binding sites for cardiolipin [Yoshikawa, personal communication]. The stimulatory effect of cardiolipin on proton pumping of the kidney enzyme but not of the heart enzyme, suggested a specific binding site for cardiolipin in the kidney enzyme with less tight binding affinity. Since binding of cardiolipin is assumed to involve binding sites for fatty acids, the effect of free fatty acids on the H^+/e^- stoichiometry of the kidney enzyme was investigated. Reconstituted kidney enzyme with 1 % cardiolipin was incubated for 0 to 75 min with 5 μM palmitate, as shown in Fig. 39. The H^+/e^- stoichiometry is decreased by incubation with palmitate and already after 30 min of incubation the maximal effect is obtained. On the other hand, no decrease of H^+/e^- stoichiometry with time is obtained with proteoliposomes in the absence of palmitate.

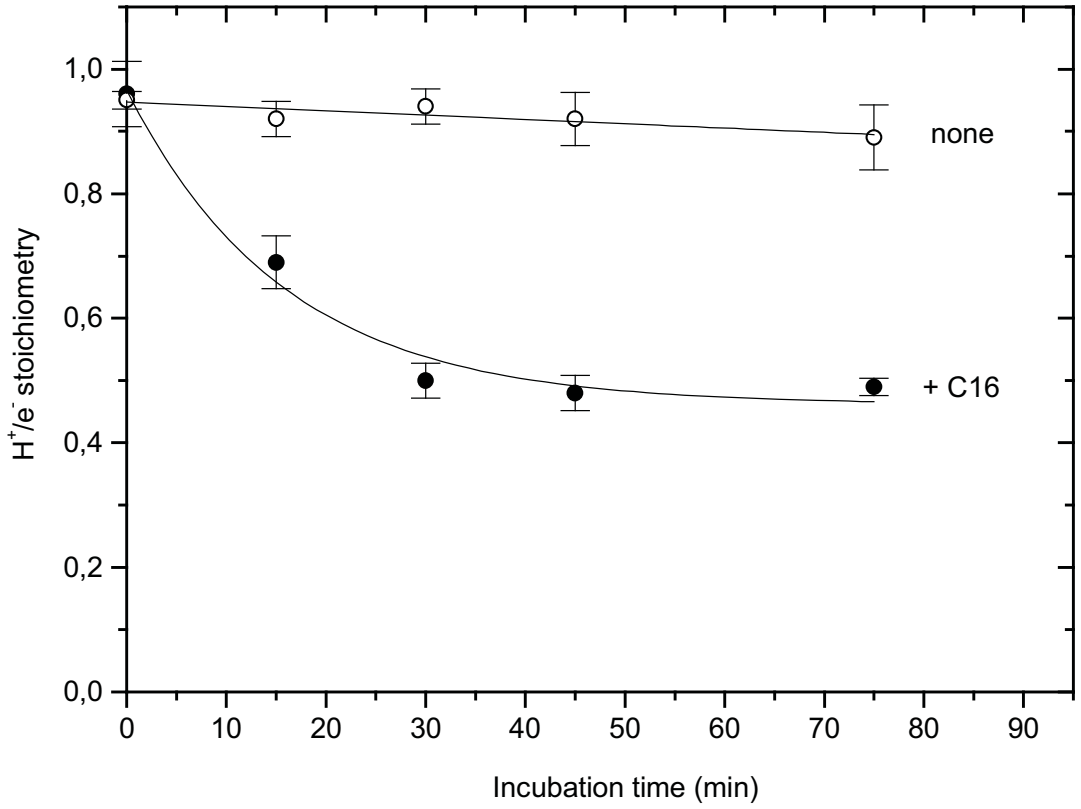


Fig. 39. Time dependent decrease of H^+/e^- stoichiometry of bovine kidney cytochrome c oxidase by palmitate (C16).
Bovine kidney cytochrome c oxidase was reconstituted with 1 % cardiolipin. The proteoliposomes were preincubated from 0 to 75 min at 4 °C with (closed symbols) or without (open symbols) 5 µM palmitate. Each individual point represents the average of 2-3 independent determinations.

In further studies the effect of other fatty acids and related compounds on the H^+/e^- stoichiometry of bovine kidney cytochrome c oxidase was investigated. Fig. 40 summarizes the concentration dependent effects of laurate (C12), myristate (C14), palmitate (C16), stearate (C18), oleate (C18:1 Δ^9), cetylalcohol (C16 alcohol, C16-OH), and palmitoyl CoA (C16-CoA). With concentrations up to 10 µM, a specific decrease of H^+/e^- stoichiometry was obtained only with palmitate. Half-maximal decrease was measured at 0.5 µM palmitate. This suggests a physiological effect, because *in vivo* the concentration of free fatty acids normally does not increase above 1 µM, due to the presence of large amounts of fatty acid binding proteins with dissociation constants below 1 µM [Börchers and Spener, 1994; Banaszak et al., 1994; Veerkamp and Maatman, 1995; Schaap et al., 1998]. By addition of either 1.5 µM palmitate or stearate the proton conductivity of proteoliposomes was not changed (data not shown).

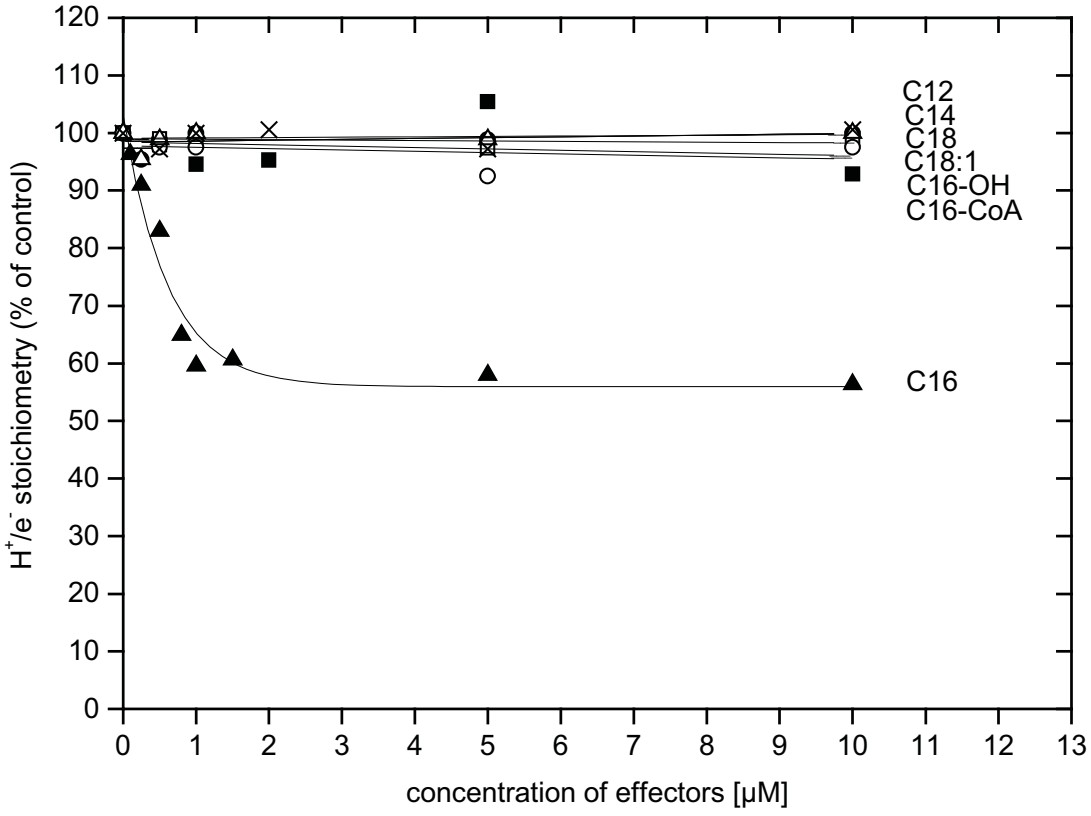


Fig. 40. Specific decrease by palmitate of H^+/e^- stoichiometry of bovine kidney cytochrome c oxidase.

Proteoliposomes reconstituted in the presence of 1 % cardiolipin, were preincubated at 4 °C for 30 min with increasing concentrations of the indicated fatty acids, cetylalcohol, or palmitoyl-CoA. The H^+/e^- stoichiometry of different proteoliposomal preparations in the absence of additions varied between 0.7-1.0. These values were taken as 100 % H^+/e^- stoichiometry, and changes are presented as % of control. Each individual point represents the average of 2-3, that of the palmitate titration of 12 independent determinations. Closed squares: laurate (C12); closed circles: myristate (C14); closed triangles: palmitate (C16); open squares: stearate (C18); open circles: oleate (C18:1); open triangles: cetylalcohol (C16-OH); crosses: palmitoyl-CoA (C16-CoA).

The effect of palmitate on the H^+/e^- stoichiometry of cytochrome c oxidase from bovine kidney was independent of the type of nucleotides (5 mM ATP or 5 mM ADP) within the proteoliposomes, as presented in Fig. 41. Furthermore, palmitate had no effect on the H^+/e^- stoichiometry of the kidney enzyme which was reconstituted in the absence of cardiolipin (Fig. 42).

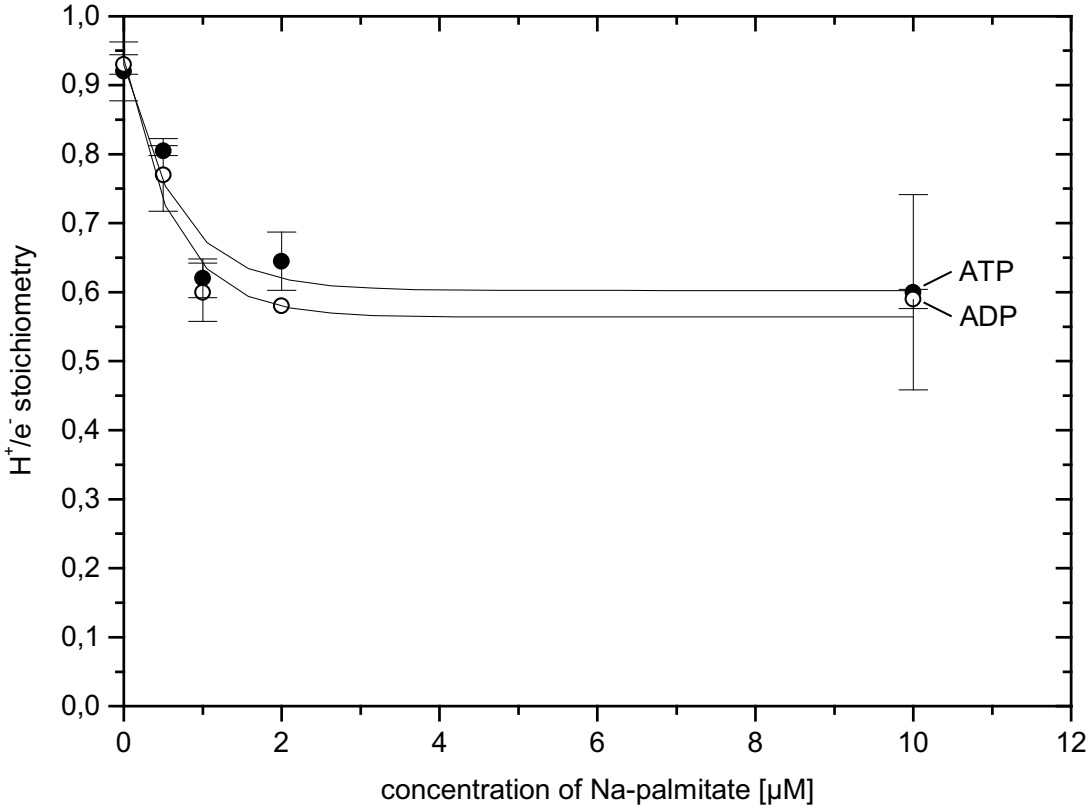


Fig. 41. The decrease of H^+/e^- stoichiometry by palmitate of bovine kidney cytochrome c oxidase is independent of the intraliposomal nucleotides.
Cytochrome c oxidase from bovine kidney was reconstituted in the presence of 1 % cardiolipin and of either 5 mM ADP (open symbols) or 5 mM ATP (closed symbols). The proteoliposomes were preincubated at 4 °C for 30 min with increasing concentrations of palmitate. The standard deviations were calculated from 3 independent determinations.

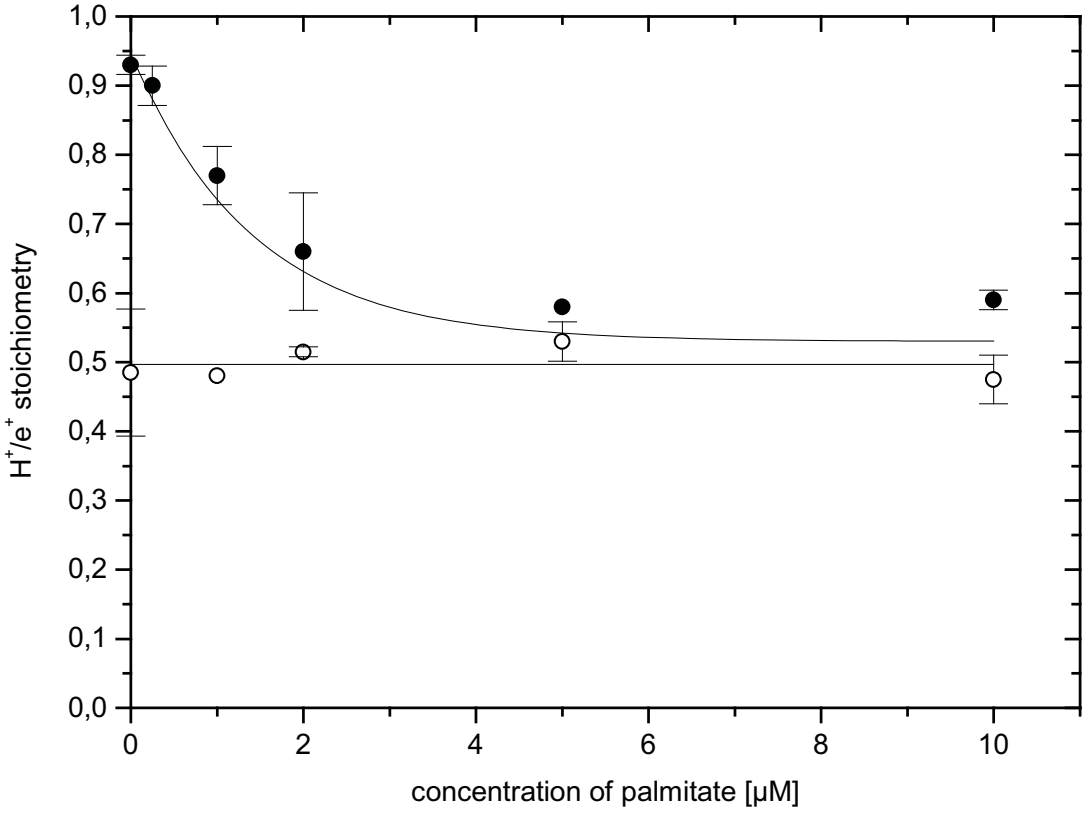


Fig. 42. Palmitate decreases only the cardiolipin-stimulated H^+/e^- stoichiometry of bovine kidney cytochrome c oxidase.
Bovine kidney enzyme was reconstituted in the presence (closed symbols) and absence (open symbols) of 1 % cardiolipin. Proteoliposomes were preincubated at 4 °C for 30 min with increasing concentrations of palmitate. Each individual point represents the average of 3 independent determinations.

The effect of palmitate on the H^+/e^- stoichiometry of cytochrome c oxidase is specific for the liver type isozyme containing subunit VIaL. No effect of palmitate on proton pumping of the bovine heart enzyme was found, containing subunit VIaH, independent of the type of nucleotides (5 mM ADP or 5 mM ATP) within the vesicles, as indicated in Fig. 43. The H^+/e^- stoichiometries of 1.0 were measured with intraliposomal ADP, and of 0.5 with intraliposomal ATP, as described in previous publications [Frank and Kadenbach, 1996; Hüttemann et al., 1999; Kadenbach et al., 1999].

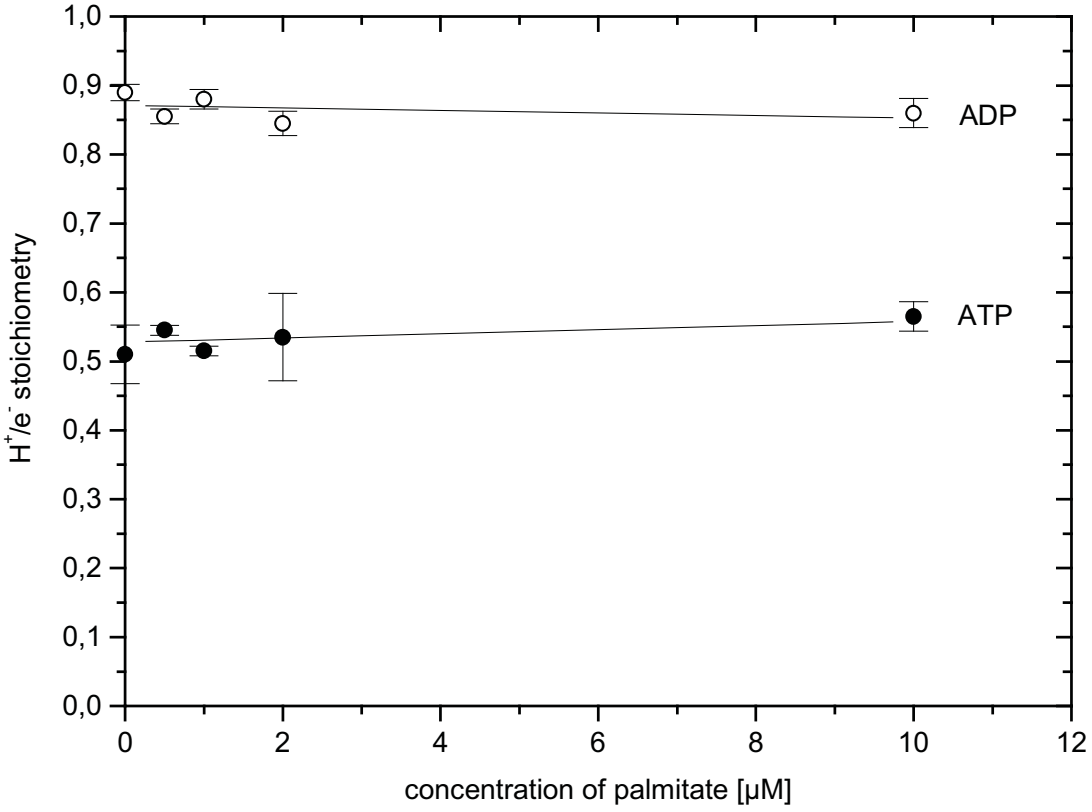


Fig. 43. Palmitate has no effect on the H^+/e^- stoichiometry of cytochrome c oxidase from bovine heart.
Bovine heart cytochrome c oxidase was reconstituted in the presence of 5 mM ADP (open symbols) or 5 mM ATP (closed symbols). The proteoliposomes were preincubated at 4 °C for 30 min with increasing concentrations of palmitate. The standard deviations were calculated from 3 independent determinations.

4.5.3. Palmitate increases the controlled respiration of kidney cytochrome c oxidase

From the decrease of proton pumping efficiency by palmitate, one would expect that also the respiratory control ratio (RCR = ratio of respiratory rate in the presence/absence of uncoupler of oxidative phosphorylation) of cytochrome c oxidase from kidney will be decreased by palmitate. This is indeed the case as presented in Fig. 44.

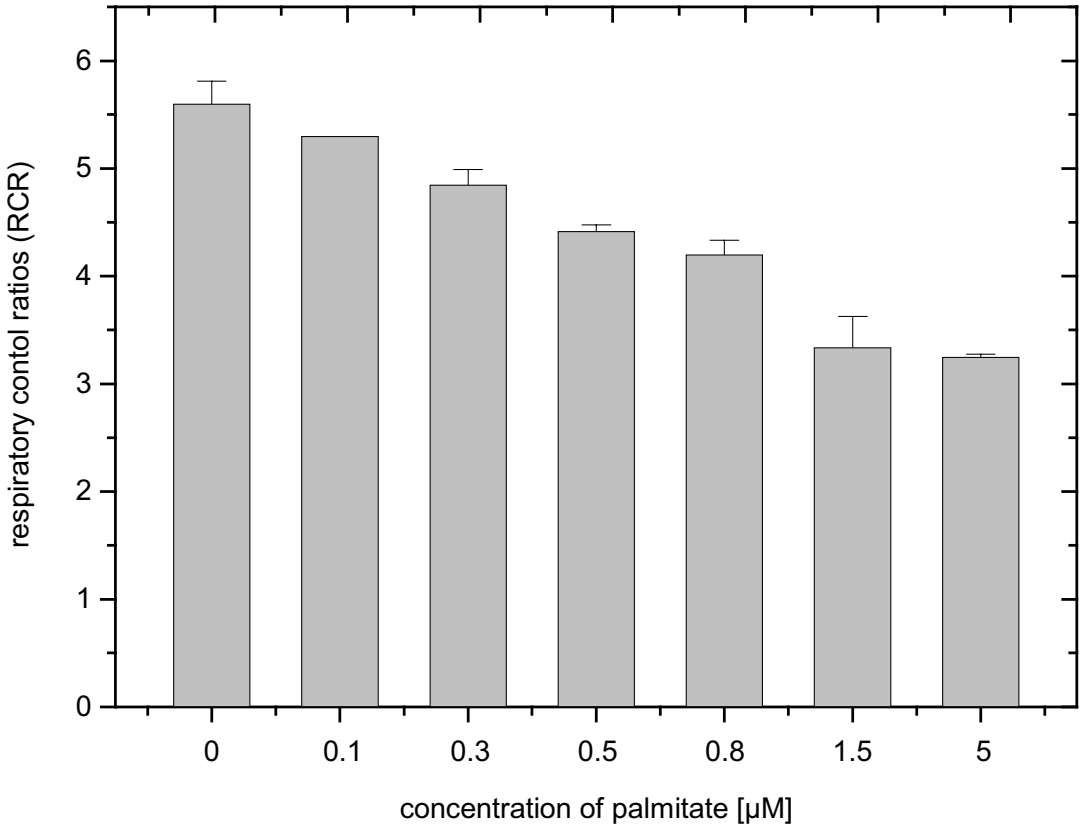


Fig. 44. Palmitate decreases the respiratory control ratio (RCR) of bovine kidney cytochrome c oxidase.
Proteoliposomes reconstituted with 1 % cardiolipin, were preincubated at 4 °C for 30 min with increasing concentrations of palmitate. The ascorbate respiration was measured in the presence and absence of 3 µM CCCP + 1 µM valinomycin, as described in 3.3.6. Each individual point represents the average of 3 independent determinations.

The decrease of RCR by incubation with palmitate is expected to be based on the increase of the controlled respiration of kidney proteoliposomes by palmitate. This is shown in Fig. 45, where the ascorbate respiration of the reconstituted enzymes from bovine kidney and bovine heart at increasing concentrations of cytochrome c are presented under coupled as well as uncoupled conditions. The increase of coupled respiration by palmitate is only measured with the kidney enzyme, not with the heart enzyme. After addition of uncoupler of oxidative phosphorylation (valinomycin and CCCP) neither the respiration of the heart nor of the kidney enzyme was increased by palmitate. Stearate at a concentration of 5 µM had no effect on the coupled as well as on the uncoupled respiration of reconstituted kidney cytochrome c oxidase, as presented in Fig. 46.

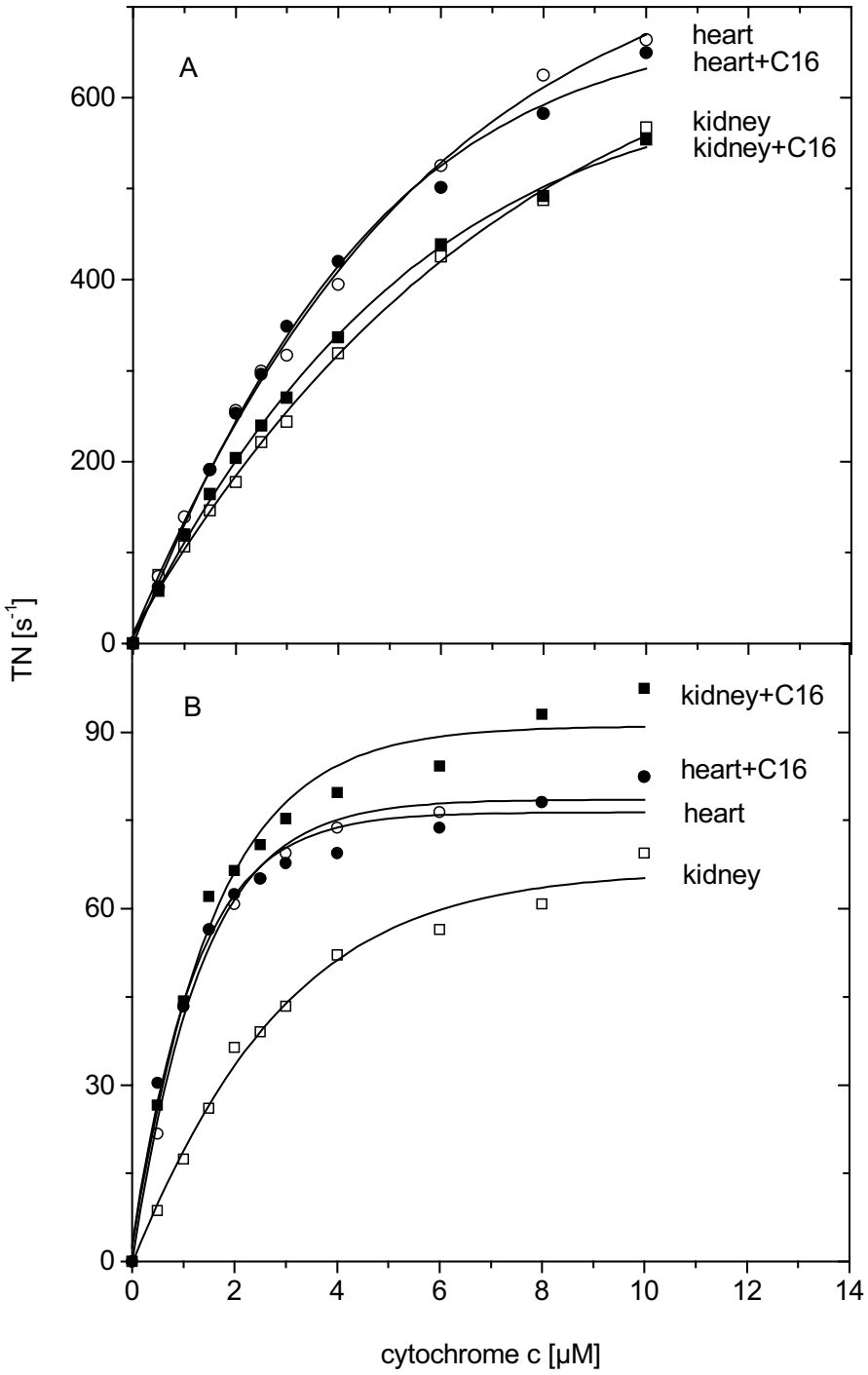


Fig. 45. Respiration of reconstituted cytochrome c oxidase from bovine kidney and heart in the absence and presence of palmitate under coupled and uncoupled condition. Cytochrome c oxidases from bovine kidney (squares) and heart (circles) were reconstituted with 1 % cardiolipin as described in 3.4.1. The proteoliposomes were preincubated with 1 µM palmitate (C16) at 4 °C for 30 min, when indicated (closed symbols). As controls, the proteoliposomes were incubated without palmitate (open symbols). The ascorbate respirations of reconstituted cytochrome c oxidases were measured at increasing cytochrome c concentrations in the presence (A) or absence (B) of 3 µM CCCP and 1 µM valinomycin.

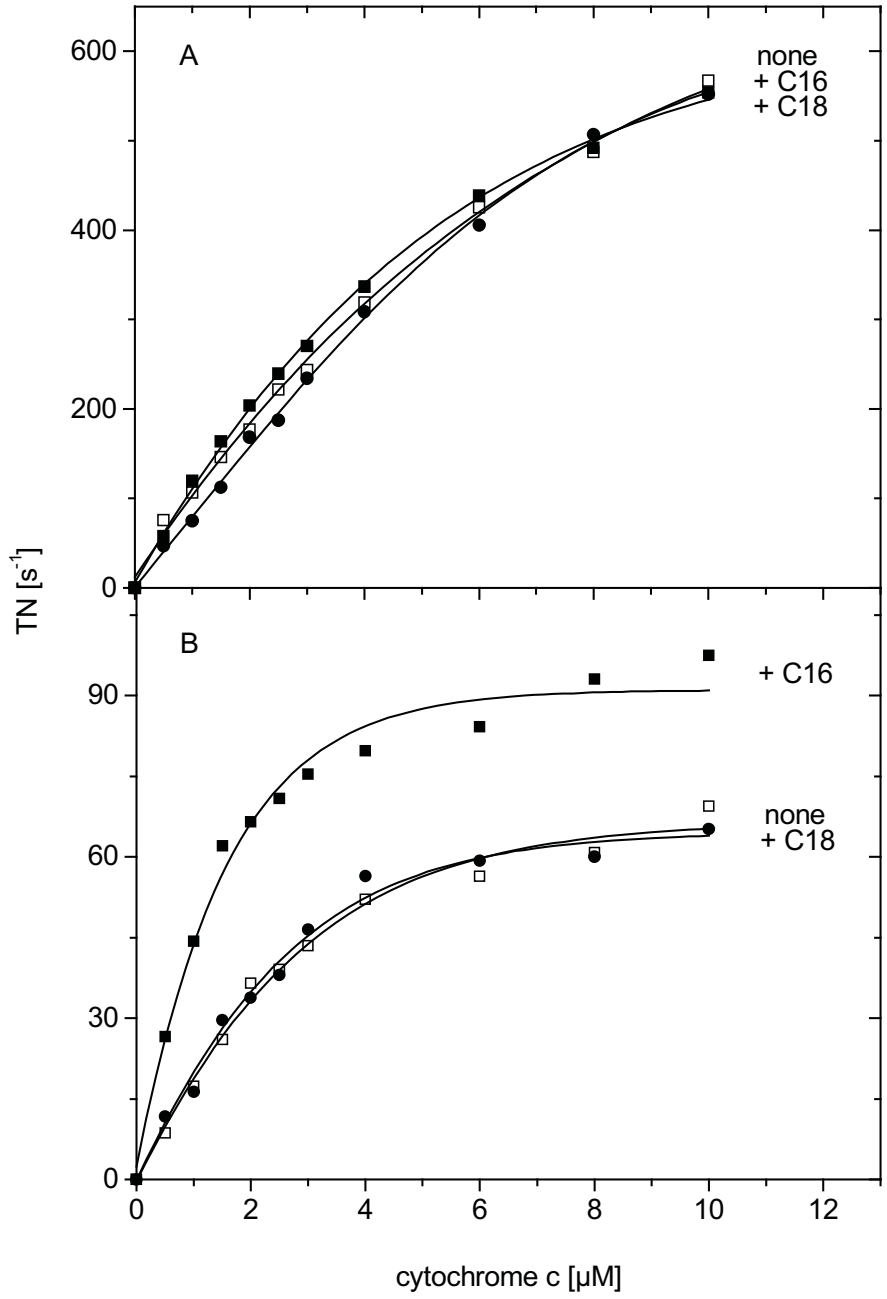


Fig. 46. Respiration of reconstituted cytochrome c oxidase from bovine kidney in the presence of palmitate or stearate under coupled and uncoupled conditions.
Cytochrome c oxidase from bovine kidney was reconstituted with 1 % cardiolipin as described in 3.4.1. The proteoliposomes were preincubated with 1 µM palmitate (C16, squares) or 5 µM stearate (C18, circles) at 4 °C for 30 min (closed symbols). As controls, enzyme vesicles were incubated without fatty acids (open symbols). The ascorbate respirations of proteoliposome were measured at increasing cytochrome c concentrations in the presence (A) or absence (B) of 3µM CCCP and 1 µM valinomycin.

5. Discussion

5.1. Search for cytochrome c oxidase subunit IV-2 isoform

One aspect of this dissertation was the identification of the cDNA for the proposed isoform of subunit IV (IV-2) of cytochrome c oxidase in mammals and birds by molecular biological methods. The two isoforms for subunit IV (IV-1 and IV-2) had just been described in tuna by Hüttemann [2000]. Experiments to isolate the cDNA for subunit IV-2 using degenerated PCR primers related to the cDNA of subunit IV-1 were unsuccessful. The reason was the low homology between the two isoforms (identities of amino acid sequences between subunit IV-1 and subunit IV-2 are 44.2 % in rat and 46.9 % in human). Subsequent continued search by Maik Hüttemann using numerous other primers was finally successful and the complete cDNAs for subunit IV-2 from rat, mouse, and human were identified [Hüttemann et al., 2001].

Screening for the mRNA expression of subunit IV-2 in rat by Northern Blot analysis had demonstrated its high expression only in lung tissue [Hüttemann et al., 2001]. Therefore, in this dissertation cytochrome c oxidase was isolated from bovine lung where the isozyme containing subunit IV-2, was expected to be expressed. By N-terminal and internal amino acid sequencing of subunit IV separated from the isolated enzyme by SDS-PAGE, only the known subunit IV-1 was found. According to the deduced amino acid sequence, subunit IV-2 from bovine contains two cysteines which do not occur in subunit IV-1 (Fig. 15). Hence, it was investigated whether the isolated enzyme contains cysteines in subunit IV. By incubation of reduced cytochrome c oxidase with the fluorescent reagent DACM, labelled polypeptides were detected on SDS-gels at the position of subunit IV of enzymes from different tissues. The lung enzyme had the strongest fluorescence intensity at the position of subunit IV compared to the heart and kidney enzyme, which corresponds to the highest expression of subunit IV-2 mRNA in lung. The less pure enzyme illuminated more strongly than the purer enzyme. The amount of isozyme containing subunit IV-2 seems too small to be detected by amino acid sequencing. It seems that subunit IV-2 was bound to other proteins by S-S bonds, and thus the isozyme could have been coeliminated during the enzyme isolation steps. An improvement of isolation conditions to obtain enough amount of subunit IV-2 should be considered to investigate the subunit IV-2 function.

5.2. Phosphorylation of Ser^{441} in subunit I of cytochrome c oxidase induces allosteric ATP-inhibition

In this dissertation, phosphorylation states of cytochrome c oxidases from bovine heart that had been isolated under different conditions, were studied by antibodies against phosphoamino acids (Fig. 22). With an antibody against phosphoserine, subunits II and/or III, and subunit VIc of the enzymes isolated with KF and EGTA, were stained stronger than those of the enzymes isolated without KF and EGTA. The most remarkable staining with an antibody against phosphothreonine was subunit IV of the enzyme which had been isolated with KF and EGTA (Fig. 22). Somehow the addition of KF and EGTA seems to prevent protein phosphatases to dephosphorylate the cytochrome c oxidase during enzyme purification. Fig. 23 shows many possible phosphorylation sites for serines and threonines in bovine heart cytochrome c oxidase, searched by consensus sequences for protein kinases [Pearson and Kemp, 1991]. Even though some of them are not accessible to protein kinases and protein phosphatases in the structure, because of structural hindrance by neighbouring amino acids, phosphorylated serine(s) and threonine(s) from most subunits (except subunits Va and Vb) were detected by Western Blot analysis (Fig. 22). The reason might be that the subunits had been phosphorylated before assembling when protein kinases could still approach each polypeptide. Permanent phosphorylation is supposed to have roles in regulation of enzyme activity or in stability of enzyme conformation. Interestingly, in the crystal structure of bovine heart cytochrome c oxidase, one phosphate was detected on threonine^{15} of subunit VIaH [Yoshikawa, personal communication]. Threonine^{15} is part of a possible phosphorylation site for cGMP-dependent protein kinase and protein kinase C. In the previous study, cAMP-independent phosphorylation of cytochrome c oxidase subunit IV from rat liver was described [Steenaart and Shore, 1997], but the effect of phosphorylation on enzyme activity is unknown.

Here, the induction of the allosteric ATP-inhibition by phosphorylation with protein kinase A, and its abolition by dephosphorylation of cytochrome c oxidase [Bender and Kadenbach, 2000] were reproduced, and further the phosphorylation site which responds to the allosteric ATP-inhibition was investigated. It was shown that variable kinetic properties of purified enzymes were caused by different isolation conditions (Fig. 24). The enzyme isolated in the presence of KF (unspecific inhibitor of protein phosphatases) and EGTA, showed allosteric ATP-inhibition without phosphorylation with protein kinase A. Since the inhibition was not further intensified by phosphorylation with protein kinase A but was abolished by dephosphorylation with protein phosphatase 1, this cytochrome c oxidase is assumed to have

been endogenously phosphorylated at the phosphorylation sites for cAMP-dependent protein kinase.

Furthermore, it could be shown that the phosphorylation site which turns on the allosteric ATP-inhibition, is located outside of the proteoliposomes at the cytosolic side of the reconstituted enzyme (see Fig. 31). The search for consensus sequences for protein kinases [Pearson and Kemp, 1996], in all 13 subunits of cytochrome c oxidase from bovine heart, revealed only three cAMP-dependent phosphorylation sites (Fig. 23). These correspond to the three subunits that had been previously labelled by [γ-^{32}P]ATP with protein kinase A and cAMP [Bender and Kadenbach, 2000]. Among the three cAMP-dependent phosphorylation sites, only the sequence of RRYS^{441} in subunit I is located on the cytosolic side (Fig. 23). The other two subunits III and Vb are located on the matrix side. While those in subunits III and Vb are not conserved, the consensus sequence in subunit I (RRYS^{441}) is completely conserved in animals which are regulated by hormones down to sea urchin, but is absent in all other organisms including plants, fungi, bacteria, and archaea (Fig. 34). From these results, it is suggested that the reversible phosphorylation of Ser^{441} in subunit I turns on the allosteric ATP-inhibition of cytochrome c oxidase. The phosphate on Ser^{441} of subunit I, however, remains to be identified chemically in the purified enzyme in the future.

The crystal structure of bovine heart cytochrome c oxidase indicates a close neighbourhood of Ser^{441}, Asp^{51}, and a sodium ion in subunit I. Ser^{441} forms hydrogen bonds to Asp^{51}, and is a ligand of the sodium ion (see Figures 47-49). Asp^{51} was suggested as the exit amino acid of a proposed proton pathway in mammalian cytochrome c oxidase, because only Asp^{51} has a different location in the oxidized and reduced crystal structure, i.e. the accessibility to the cytosolic side (= intermembrane space) is increased in the reduced state [Fig. 47, Yoshikawa et al., 1998 and 2000]. This proton pathway, however, could not be corroborated in the bacterial enzyme [Pfitzner et al., 1998; Lee et al., 2001]. The Na^+ is complexed in addition to Ser^{441} by Gly^{45} and Glu^{40} [Yoshikawa et al., 1998]. It has been shown by Konstantinov and coworker [Kirichenko et al., 1998] that the Na^+ ion can be exchanged by Ca^{2+} associated with a spectral changes of the enzyme. The sodium site, Ser^{441}, and Asp^{51} are all accessible from the cytosolic side (Fig. 48). The functional meaning of the close location of a sodium ion, a phosphorylation site, and a proton pathway on the cytosolic side of cytochrome c oxidase is not known. One could speculate, however, that phosphorylation of Ser^{441} (in addition to turning on the allosteric ATP-inhibition) and the exchange of Na^+ by Ca^{2+}, modify the H^+/e^- stoichiometry of mammalian cytochrome c oxidase. In fact, in the crystal structure of the oxidized and reduced bovine heart enzyme, the

hydroxyl group of Ser^{441} forms hydrogen bonds to the free carboxyl group of Asp^{51} [Yoshikawa et al., 1998]. After phosphorylation of Ser^{441}, this interaction is no longer possible due to electrostatic repulsion between the negatively charged phosphate group at Ser^{441} and the carboxyl group of Asp^{51} (see Fig. 49). Phosphorylation of Ser^{441} is thus expected to induce conformational changes. In addition, exchange of the monovalent Na^+ by the divalent Ca^{2+} would also induce strong conformational changes. Further experiments are required to solve the physiological role of this regulatory site.

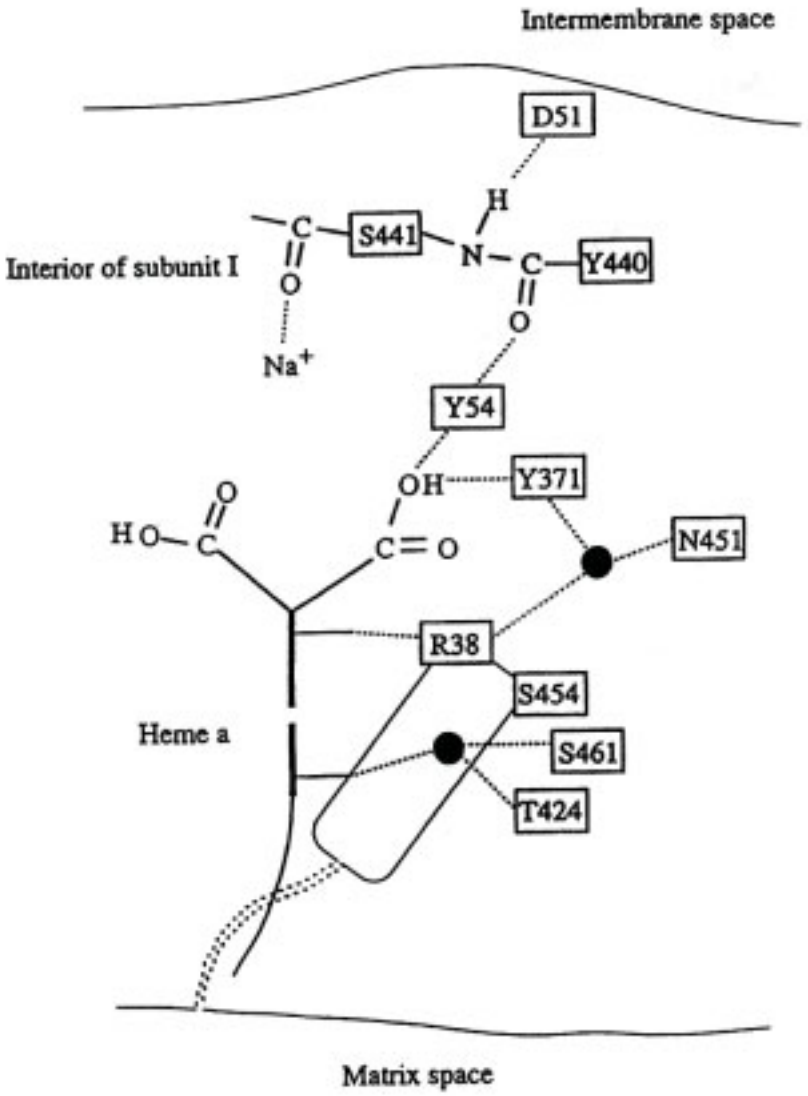


Fig. 47. Schematic representation of the possible proton pathway from the matrix space to Asp^{51} at the cytosolic side in the fully oxidized state (taken from Yoshikawa et al., 1998).

Dotted lines, dark circles, and a rectangle denote hydrogen bonds, fixed water molecules, and a cavity, respectively. The cavity is connected to the matrix space with a water path shown in a pair of dotted lines. Thick and thin sticks are a side view of the porphyrin plane of heme a and its side chains.

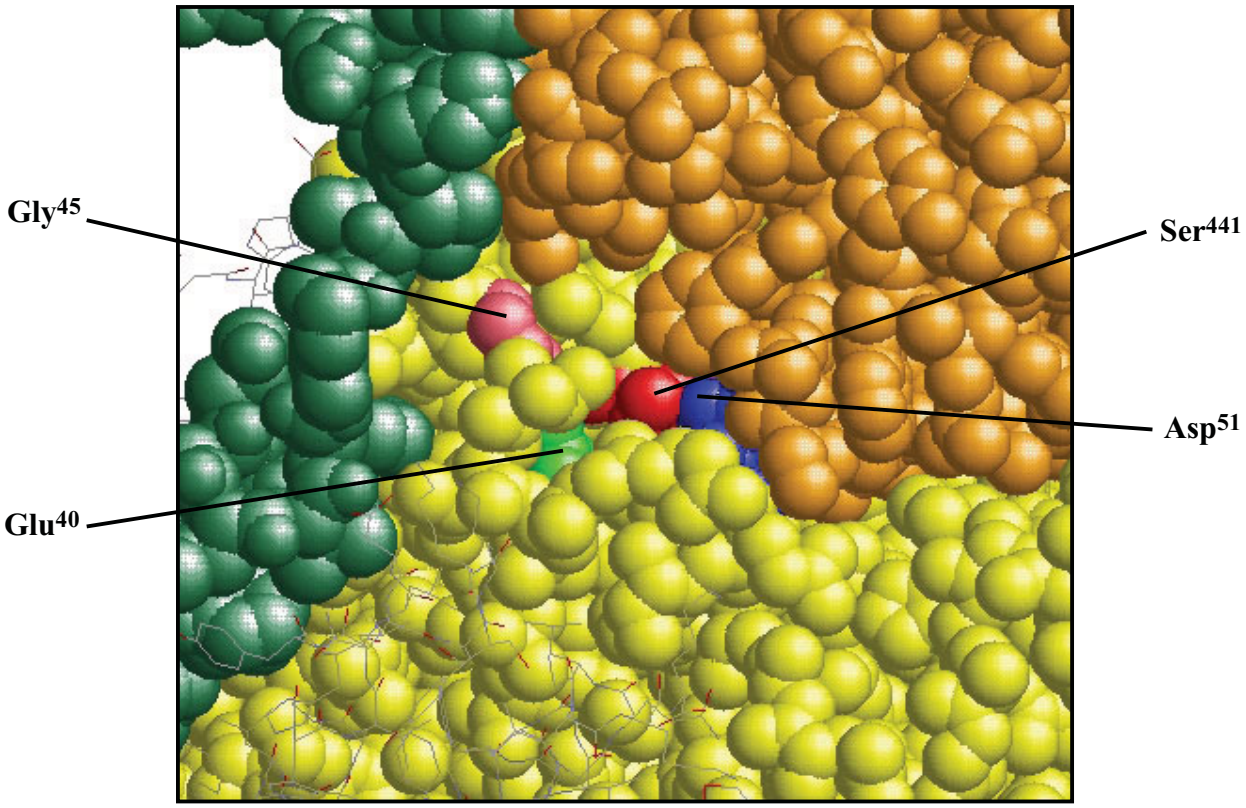


Fig. 48. The exit amino acid of a proton pathway (Asp^{51}, blue), the phosphorylatable Ser^{441} (red), and a Na^+ site complexed by Ser^{441}, Gly^{45} (pink), and Glu^{40} (green) all in subunit I, are accessible from the cytosolic side in bovine heart cytochrome c oxidase. Subunits I (yellow), II (brown), and IV (dark green) are presented in spacefill, the other subunits are in backbone. Coordinates of the crystal structure [Tsukihara et al., 1996] are from protein Data Brookhaven [COX.pdb; 1HRC].

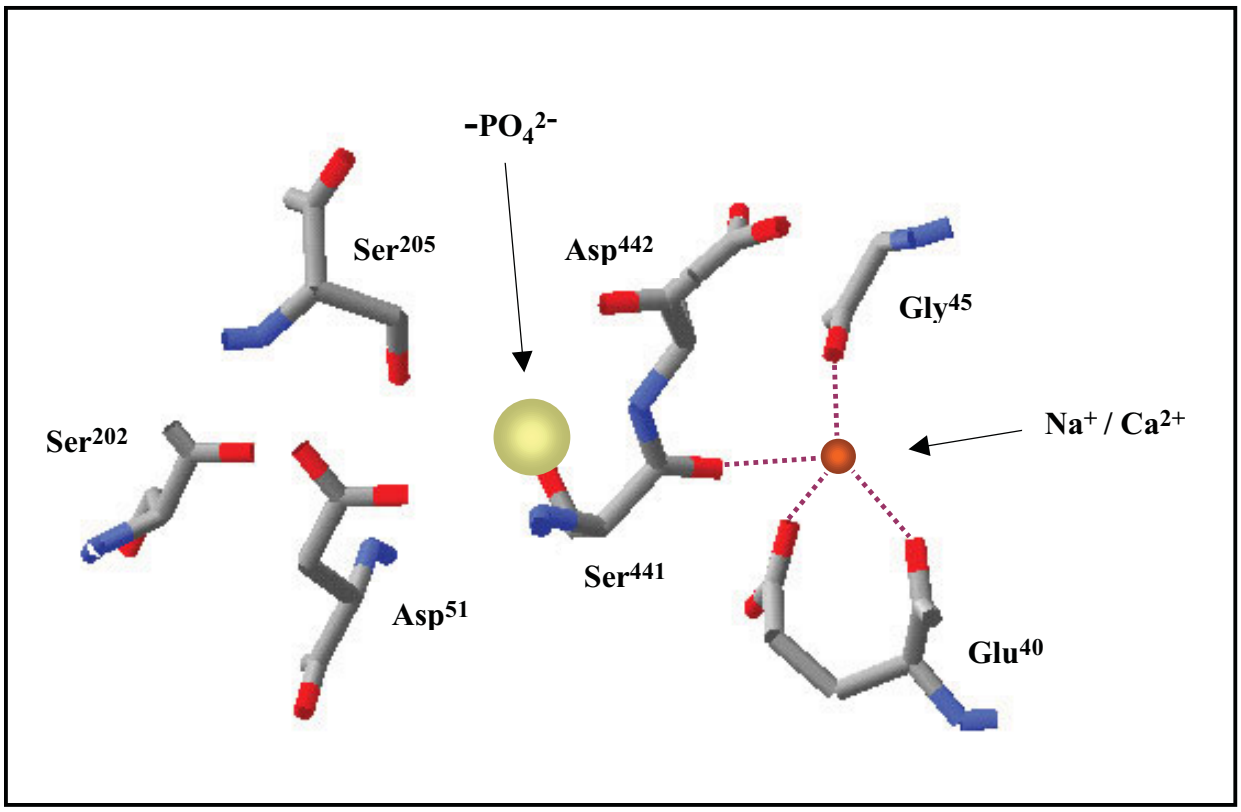


Fig. 49. The postulated regulatory triad in bovine heart cytochrome c oxidase subunit I: Asp^{51} (the postulated exit amino acid of a proton pathway), Ser^{441} (a cAMP-dependent phosphorylation site), and a Na^+/Ca^{2+} site liganded by Ser^{441}, Gly^{45}, and Glu^{40}.
Shown are the amino acids from the oxidized crystal structure [Tsukihara et al., 1996], including Ser^{202} and Ser^{205} from subunit II. A phosphate group at Ser^{441} and a metal ion are indicated schematically.

5.3. Regulation of oxidative phosphorylation by allosteric ATP-inhibition of cytochrome c oxidase

The allosteric ATP-inhibition of cytochrome c oxidase presents a "second mechanism of respiratory control" [Kadenbach and Arnold, 1999], and is based on the exchange of ADP by ATP at the matrix domain of subunit IV at high intramitochondrial ATP/ADP-ratios [Arnold and Kadenbach, 1997]. The term "respiratory control" is defined as the control of mitochondrial respiration by the availability of ADP [Lardy and Wellman, 1952; Chance and Williams, 1956]. According to the chemiosmotic hypothesis the "first mechanism of respiratory control" is based on the inhibition of mitochondrial proton pumps (complexes I, III, and IV) at high proton motive force Δp (140-180 mV) [Nicholls and Ferguson, 1992]. ADP increases mitochondrial respiration via activation of the ATP synthase with consequent decrease of Δp and activation of the proton pumps. By the second mechanism of respiratory control ADP directly activates mitochondrial respiration via decreasing the ATP/ADP-ratio and relieving the ATP-inhibition of cytochrome c oxidase. The control of mitochondrial respiration by the ATP/ADP-ratio will in consequence decrease the mitochondrial membrane potential $\Delta\psi_m$ (the main component of Δp), based on the potential dependence of the ATP synthase. Kaim and Dimroth [1999] have shown that the ATP synthase from E. coli is saturated and has maximal rates of ATP synthesis at $\Delta\psi_m = 120$ mV. Therefore at $\Delta\psi_m = 120$ mV also the ATP/ADP-ratio will be maximal and inhibition of cytochrome c oxidase activity will be optimal. The allosteric ATP-inhibition keeps thus $\Delta\psi_m$ at low values (100-140 mV) [Lee et al., 2001; Lee et al., 2002]. This assumption is in accordance with estimations of $\Delta\psi_m$ between 100-140 mV in a perfused rat heart, where the absolute values varied, depending on the substrate in the perfusion medium and decreased slightly after increasing the work load (rate of ATP consumption) by a factor of five [Wan et al., 1993]. In contrast, in isolated mitochondria membrane potentials of 150-180 mV have been frequently measured [Nicholls and Ferguson, 1992]. This could be explained by turning off the allosteric ATP-inhibition during isolation of mitochondria by calcium stimulated dephosphorylation of cytochrome c oxidase.

Above $\Delta\psi_m$ values of 140 mV an exponential increase of ROS (reactive oxygen species) formation has been measured in isolated mitochondria [Liu, 1997 and 1999; Korshunov and Skulachev, 1997]. The "molecular-physiological hypothesis" on regulation of oxidative phosphorylation *in vivo* [Kadenbach et al., 2000; Ludwig et al., 2001; Lee et al., 2001] proposes the control of mitochondrial membrane potential and ROS formation by

hormones via turning on and off the allosteric ATP-inhibition of cytochrome c oxidase by reversible phosphorylation (Fig. 50). It was proposed that stress hormones, which increase cellular [Ca^{2+}], result in dephosphorylation of cytochrome c oxidase, accompanied by an increase of mitochondrial membrane potential and ROS formation. Under relaxed conditions other hormones are expected to increase cellular [cAMP], resulting in phosphorylation of cytochrome c oxidase which reestablishes the "relaxed" state of oxidative phosphorylation with low $\Delta\psi_m$ and no ROS formation (Fig. 50).

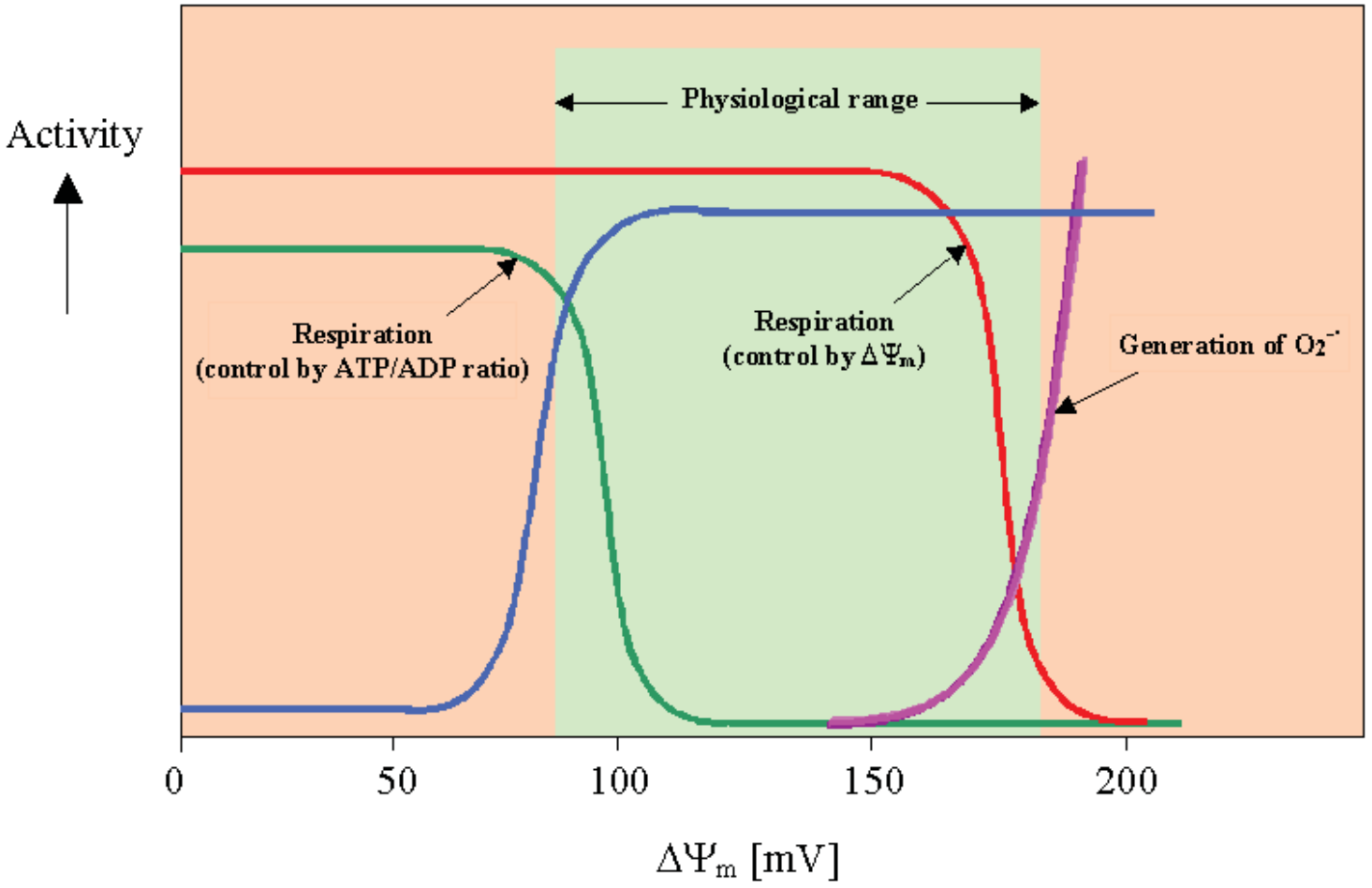


Fig. 50. Scheme of postulated control of mitochondrial $\Delta\psi_m$ and ROS formation.
The figure presents schematically the inhibition of respiration at high $\Delta\psi_m$ via the first mechanism of respiratory control (red line), the inhibition of respiration by high ATP/ADP-ratios via the allosteric ATP-inhibition (green line), based on the membrane potential dependence of the ATP synthase (blue line), and the $\Delta\psi_m$ dependence of ROS formation (violet line). The suggested physiological range of $\Delta\psi_m$ is presented in light green.

5.4. Palmitate decreases the H^+/e^- stoichiometry of bovine kidney cytochrome c oxidase

Cardiolipin is known to interact with a number of inner mitochondrial membrane proteins including several anionic carriers [Noel and Pande, 1986; Kadenbach et al., 1982] and some electron transport complexes [Fry and Green 1981; Goormaghtigh et al., 1986; Nicolay and de Kruijff, 1987] which require it for full activity [Hoch, 1992 and 1998]. Cytochrome c oxidase activity is essentially dependent on cardiolipin [Robinson, 1993] which occurs in the inner mitochondrial membrane at 10-20 % of the total phospholipids [Hoch, 1992]. The H^+/e^- stoichiometry of bovine heart cytochrome c oxidase, reconstituted with asolectin (purified soyabean phospholipids), is 1.0, as generally accepted for most cytochrome c oxidases [Babcock and Wikström, 1992]. In contrast, the reconstituted enzyme from bovine liver and kidney exhibited a H^+/e^- stoichiometry of 0.5 under the same conditions [Hüttemann et al., 1999].

In this dissertation it was shown that addition of cardiolipin during reconstitution increased the H^+/e^- stoichiometry of bovine kidney cytochrome c oxidase from 0.5 to 1.0. Cardiolipin had no influence on the H^+/e^- stoichiometry of the heart enzyme which exhibited a value of 1.0 with or without cardiolipin. These results can be explained by assuming a specific binding site for cardiolipin at the liver type isozyme which induces high proton pumping efficiency ($H^+/e^- = 1.0$). In the crystal structure of bovine heart cytochrome c oxidase, five tightly bound cardiolipin molecules were identified [Yoshikawa, personal communication]. In contrast, the kidney enzyme appears to contain a loosely bound cardiolipin which is lost during purification. The previous data concerning low H^+/e^- stoichiometry of 0.5 with cytochrome c oxidase from bovine liver and kidney [Frank and Kadenbach,1996; Hüttemann et al., 1999] turns out to be an isolation artifact. The stimulation of H^+/e^- stoichiometry by cardiolipin and its reversal by palmitate suggests competition between both for a specific binding site for a palmitoyl group. The lacking effect of palmitoyl glycerol on the H^+/e^- stoichiometry of the reconstituted kidney enzyme, neither in the presence nor in the absence of cardiolipin, however, does not support this assumption. Additional properties of cardiolipin, not present in the palmitoyl glycerol molecule, may be necessary to stimulate the H^+/e^- stoichiometry.

The H^+/e^- stoichiometry of 1.0 measured with the kidney enzyme after addition of cardiolipin, was decreased to 0.5 with low concentrations of palmitate (half-maximal effect at

0.5 µM) (Fig. 40). This decrease is highly specific for palmitate (C16), because no other fatty acid (C12, C14, C18, C18:1) as well as 1-hexadecanol, palmitoyl CoA, and palmitoyl glycerol decreased the proton pumping efficiency of the kidney enzyme. The H^+/e^- stoichiometry of the bovine heart enzyme, containing subunit VIaH (heart type) was not decreased by up to 10 µM palmitate. Also Labonia et al. [1988] found no effect of 30 µM palmitate on the H^+/e^- stoichiometry of the bovine heart enzyme. On the other hand, high intraliposomal ATP/ADP-ratios (half-maximal at ATP/ADP = 100) decreased the H^+/e^- stoichiometry of the heart enzyme from 1.0 to 0.5 [Frank and Kadenbach, 1996; Hüttemann et al., 1999]. The decrease of H^+/e^- stoichiometry by ATP of the heart enzyme was reproduced in this dissertation (Fig. 43). The tissue-specificity and the same decrease of H^+/e^- stoichiometry from 1.0 to 0.5, obtained by palmitate with the kidney enzyme, strongly suggest that the effects of ATP and palmitate are due to binding of the effectors to the corresponding subunit isoform. Each effector is ineffective to the other isozyme. Binding of both palmitate and ATP (instead of ADP) causes an increase of one negative charge at the binding site. This additional negative charge could induce a conformational change within the enzyme complex, associated with a specific decrease of H^+/e^- stoichiometry from 1.0 to 0.5 by ATP or palmitate. The amino acid sequences of subunits VIaH and VIaL reveal about 50 % identity [Linder et al., 1995]. It could be envisioned that the difference of amino acid sequence contributes in forming specific binding pockets for ADP or ATP at subunit VIaH and for palmitate at subunit VIaL. From these considerations it is postulated that palmitate binds to subunit VIaL of the kidney enzyme.

If the postulated binding site for the palmitoyl group of cardiolipin on subunit VIaL is the same site as that for palmitate binding (competition), at least 1 mol of palmitoyl diphosphatidyl glycerol per mol enzyme is required to saturate it. The minimal concentration of cardiolipin required to increase the H^+/e^- stoichiometry of the bovine kidney enzyme to 1.0 was determined to 1 % of asolectin, and this is about a 100 times excess of cardiolipin to cytochrome c oxidase (mol:mol). Interestingly, this means that the required amount of palmitoyl diphosphatidyl glycerol to increase the H^+/e^- stoichiometry of cytochrome c oxidase is about 1 mol per mol enzyme, because the palmitic acid content in the total fatty acids of the applied cardiolipin is ca. 1 % [data from Fluka catalogue, 1997/1998]. To prove the competition between cardiolipin and palmitate, experiments using cardiolipin from *E. coli* should be done, which has a palmitate content of ca. 44 % from total fatty acids [data from Fluka catalogue, 1997/1998]. This cardiolipin, however, was no more available.

The low half-maximal concentration of 0.5 µM palmitate suggests a specific binding site with high affinity for palmitate within the liver type of cytochrome c oxidase. Free palmitate is formed in animal cells by two pathways (1) from degradation of triacylglycerides by cAMP-regulated lipases, and (2) as the final product of the cytosolic fatty acid synthetase complex. *In vivo* free fatty acid concentrations are very low, due to (1) ATP-dependent esterification with coenzyme A by acyl-CoA synthetase on the outer mitochondrial membrane with subsequent transport via the carnitine shuttle and ß-oxidation in the mitochondria, and (2) the abundant presence of fatty acid binding proteins in mitochondria and in the cytosol with dissociation constants below 1.0 µM [Börchers and Spencer, 1994; Banaszak et al., 1994; Veerkamp and Maaman, 1995; Schaap et al., 1998]. Low amounts of free palmitate might diffuse through the inner mitochondrial membrane in the protonated form. The diffused palmitate is suggested to bind to a specific binding site on cytochrome c oxidase subunit VIaL. The described time dependency of the palmitate effect on the H^+/e^- stoichiometry of reconstituted cytochrome c oxidase from bovine kidney can be explained by the above interpretation. The palmitate signal in the cell can be abolished via acylation to form ineffective acyl-CoA. It was shown (Fig. 40) that esterified palmitate (palmitoyl-CoA) does not influence the H^+/e^- stoichiometry of kidney cytochrome c oxidase.

The physiological relevance of the decrease of H^+/e^- stoichiometry from 1.0 to 0.5 by palmitate in the liver type isozyme of cytochrome c oxidase, could be to increase oxidative metabolism associated with increased thermogenesis in warm-blooded animals. In brown adipose tissue, thermogenesis is generally known to be switched on by noradrenalin via cAMP, activation of a lipase, and release of free fatty acids which activate UCP1 [Nicholls and Locke, 1984; Cannon and Nedergaard, 1985; Klingenberg, 1990; Klingenberg and Huang, 1999; Winkler and Klingenberg, 1994]. Activation of UCP1 requires relatively high concentrations (10-100 µM) of different free fatty acids [Jezek et al., 1994], with laurate (C12) and myristate (C14) being most effective [Klingenberg and Huang, 1999]. Recently coenzyme Q was shown to be required for high rates of fatty acid stimulated H^+ transport by UCP1 [Echtay et al., 2000], as well as by UCP2 and UCP3 [Echtay et al., 2001]. But again, high concentrations of free fatty acids (10-100 µM) were required to stimulate H^+ transport which, however, may not occur *in vivo* due to the presence of large amounts of fatty acid binding proteins (see above). Therefore it is suggested that in analogy to its specific effect on liver type cytochrome c oxidase, palmitate represents also the specific signal transduction molecule which stimulates UCP1 and thus thermogenesis in brown adipocytes (see also Fig. 51). This conclusion is supported by two observations: (1) a specific stimulation of brown

adipocyte respiration by palmitate was described by Cunningham et al. [1986], and (2) Gonzalez-Barroso et al. [1998] observed stimulation of respiration of mitochondria from transformed yeast containing mammalian UCP1 by nanomolar concentrations of palmitate, whereas micromolar concentrations were required to stimulate respiration of wild type yeast mitochondria.

The above results suggest palmitate as a signal transduction molecule for thermogenesis in most (non-skeletal muscle) tissues of warm blooded animals (Fig. 51). In addition, the effect of palmitate on the respiratory rate of cytochrome c oxidase in non-skeletal muscle tissues could also prevent obesity after a big meal, by increasing the degradation rate of nutrient. Interestingly, palmitate is not only released by hydrolysis of fats (triacylglycerides) which is stimulated by noradrenalin, but is also released as the end product of fatty acid synthetase in animals, which contrasts with palmitoyl coenzyme A, the end product of the fatty acid synthetase in bacteria and plants. Therefore, also after a big meal with carbohydrates free palmitate increases via synthesis from acetyl coenzyme A and thus could result in accelerated oxidative metabolism, which could partially prevent obesity from excess food.

The data presented above present for the first time a physiological explanation for the tissue-specific expression of a nuclear coded subunit (VIa) of cytochrome c oxidase in warm blooded animals.

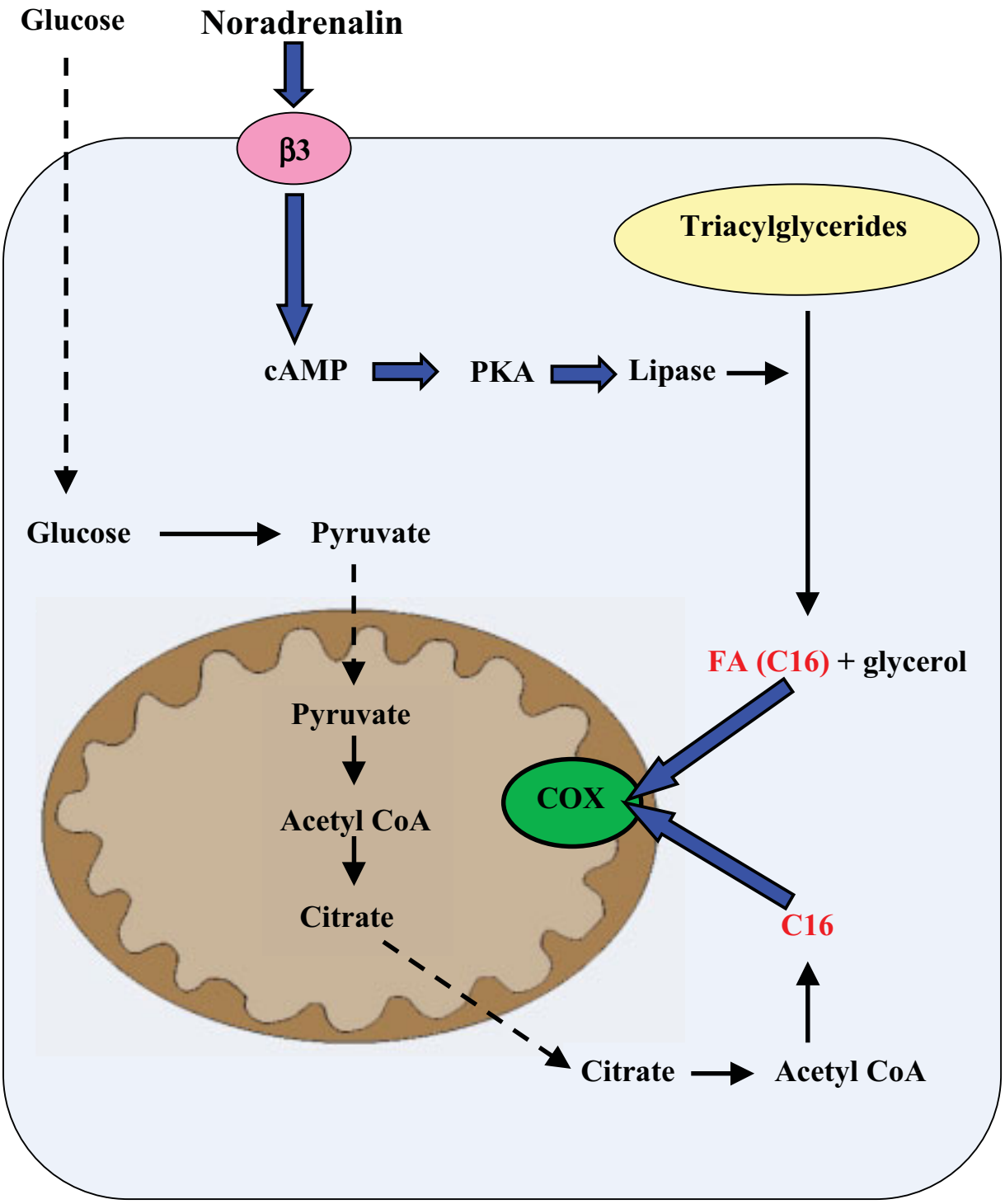


Fig. 51. Postulated signal cascade for thermogenesis including palmitate in non-skeletal cells of warm blooded animals.
FA: fatty acids; C16: palmitate; COX: cytochrome c oxidase

6. Summary

In this dissertation, the cDNA for the postulated new isoform of cytochrome c oxidase subunit IV (subunit IV-2) was screened using molecular biological methods. Using degenerated primers derived from subunit IV-1, several clones were amplified by RACE-PCR which all turned out to correspond to subunit IV-1. Later subunit IV-2 was identified by others, showing two cysteines not found in subunit IV-1, and its preferential expression in lung. Cytochrome c oxidase was isolated from bovine lung, separated by SDS-PAGE and cysteines were identified by DACM, a fluorescent thiol reagent.

Cytochrome c oxidase was isolated from turkey liver, heart, and skeletal muscle, and N-terminal and internal amino acid sequencing demonstrated the exclusive expression of the liver isoform of subunit VIa (VIaL) in all tissues.

The allosteric ATP-inhibition of cytochrome c oxidase (second mechanism of respiratory control) was investigated in more detail. Its reversible turning on by cAMP-dependent phosphorylation with protein kinase A and its dephosphorylation by protein phosphatase 1 was demonstrated. From screening for possible phosphorylation sites in bovine heart cytochrome c oxidase, and from the sidedness of the phosphorylation site, Ser^{441} on the cytosolic side of subunit I was concluded to represent that amino acid, which after phosphorylation turns on the allosteric ATP-inhibition.

The H^+/e^- stoichiometry of bovine heart cytochrome c oxidase was previously shown to be reduced from 1.0 to 0.5 in the presence of high intramitochondrial ATP/ADP-ratios. In contrast, the enzyme from bovine liver and heart revealed H^+/e^- ratios of 0.5. Here it could be demonstrated, that the presence of 1 % cardiolipin during reconstitution of the bovine kidney enzyme resulted in H^+/e^- ratios of 1.0. This high proton pumping efficiency could be reduced to H^+/e^- ratios of 0.5 by low concentrations of palmitate (half-maximal effect at 0.5 μM). Laurate (C12), myristate (C14), stearate (C18), oleate (C18:1), cetylalcohol (C16-OH), palmitoyl-CoA, and palmitoyl-glycerol were without effect. Palmitate had no effect on the heart enzyme. The controlled respiration of the reconstituted kidney enzyme, but not of the heart enzyme, was specifically increased by palmitate. It is concluded that the described decrease of proton pumping efficiency by palmitate in cytochrome c oxidase from non-skeletal muscle (containing subunit VIaL) participates in thermogenesis of warm blooded animals and in preventing obesity.

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8. Abbreviations

AP	alkaline phosphatase
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
bp	base pair
BSA	bovine serum albumin
CCCP	carbonlycyanide-3-chlorphenylhydrazone
cDNA	complemetary DNA
CL	cardiolipin
COX	cytochrome c oxidase
cyt. c	cytochrome c
DACM	N-(7-dimethylamino-4-methyl-3-cumarinyl)-maleinmide
DCCD	dicyclohexylcarbodiimide
DEAE	diethylaminoethyl
DEPC	diethylpyrocarbonate
ddNTP	dideoxynucleoside triphosphate
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNase	deoxyribonuclease
dsDNA	double strand DNA
DTE	dithioerythreitol
DTT	dithiothreitol
D.W.	deionized water
E_{280}	extrinction of light at 280 nm wavelength
ε_{280}	molar extinction coefficient at 280 nm wavelength
EDTA	ethylene diamine tetraacetic acid
EEDQ	N-ethoxycarbonyl-2-ethoxy-1,2-dihydrochinolin
EGTA	ethylene glycol-bis-(β-aminoethyl ether), N,N,N´,N´-tetraacetic acid
Hepes	N-(2-hydroxyethyl)-piperazine-N´-(2´-ethanesulfonic acid)
HPLC	high-performance liquid chromatography
IgG	immunoglobulin type G
ITPG	isopropyl-ß-D-thiogalactopyranoside
kb	kilobase(s)
K_D	Michaelis-Menten constant
LB	Luria-Bertani-medium
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
MCS	multiple cloning site
NBT	4-nitrotetrazolium chloride blue
nm	nanometer
O.D.	optical density
PBS	phosphate buffered saline
PEP	phosphoenolpyruvate
PCR	polymerase chain reaction
PEG	polyethylenglycol
Pi	inorganic phosphate
PK	pyruvate kinase
PKA	cAMP-dependent protein kinase
PKC	Ca^{2+} activated-phospholipid dependent serine-threonine kinase
PP1	protein phosphatase 1
RACE	rapid amplification of cDNA ends

RCF	relative centrifugal force
RCR	respiratory control ratio
RNase	ribonuclease
ROS	reactive oxygen species
rpm	rotation per minute
RT-reaction	reverse transcriptase reaction
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
ssDNA	single strand DNA
$T_{annealing}$	annealing temperature
TAME	N-α-tosyl-L-arginly-o-methyl ester
TBE	Tris-boric-EDTA buffer
TCA	tricholoacetic acid
TE	Tris-EDTA buffer
TEMED	N,N,N′,N′-Tetramethylethylenediamine
TMPD	N,N,N′,N′-Tetramethyl-p-phenylenediamine-hydrochloride
TN	turnover number
Tricin	N-Tris-(hydroxymethyl)-methylglycine
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
U	unit of enzyme activity
UCP	uncoupling protein
UV	ultraviolet light
Val	valinomycin
X-Gal	5-bromo-4-cholore-3-indoyl-ß-galactoside

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