1. Introduction

1.1. Formulation Challenges with Biologicals

1.1.1. General Considerations

Unlike small chemical drugs (or New Chemical Entities (NCEs)), biologicals and especially protein pharmaceuticals form higher ordered structures. For the maximum efficacy and safety of the drug product it is mandatory to preserve this fragile system of interactions and arrangements until the drug is applied in the patient's body and arrives at the site of action (Porter 2001; Rosenberg 2006). In general, proteins are forming small specific and less hydrophobic surfaces. Usually the more hydrophobic amino acids are buried in the protein's core to reduce the contact area to water to a minimum. The more polar amino acids are predominant on the surface of the protein. This leads to strong hydrophobic interactions in the inner parts of the molecule and to an increased hydration at the surface. Only the target sites of e.g. antibodies contain a higher hydrophobic part at the surface (Ptitsyn 1987).

Proteins usually have only one specific target, e.g. receptors, antigens. This high specificity is their big advantage compared to NCEs, which often show side effects due to targeting multiple sites. NCEs act only partially specific. Opioids for example bind to several types of receptors. For some antipsychotic drugs their unspecifity makes them even more potent. But with more targets, the odds of a higher incidence of severe side effects are increasing.

Only proteins that are in the native state are active against their target (Weir et al. 2002). Furthermore, unfolded or aggregated protein molecules can in some cases lead to severe side effects. Different stresses can lead to chemical and physical denaturation of the protein. The next few examples should demonstrate the problems with handling pharmaceutical proteins as bulk substance or in the final dosage form. In the downstream process human Growth Hormone (hGH) aggregates can occur

within the filter during sterile filtration (Maa and Hsu 1998). During lyophilization various proteins form aggregates during the freezing process itself. E.g. Interleukin-1 receptor antagonist or tumor necrosis factor binding protein aggregate at the ice-liquid interface unless stabilized with surfactant (Chang et al. 1996b).
The oxidation of recombinant human monoclonal antibody HER2 is strongly enhanced in the presence iron ions derived from corrosive material during fill & finish processes (Lam et al. 1997).
Furthermore the storage and shipping temperature is an important factor for protein stability. Myofibrillar ATPase becomes more denatured at -5°C than at -20°C (Martino and Zaritzky 1989). This phenomenon is explained with recrystallization of the protein at higher temperatures in the frozen state (Williamson et al. 1999; Pham 2006; Fernandez et al. 2008).

Protein stability is not only an issue for content loss and hence a less efficient manufacturing process but may also lead to immunogenic side effects (Porter 2001; Patten and Schellekens 2003; Rosenberg 2006). Immunogenicity is a major concern especially when proteins are administered as multiple doses over prolonged periods (Patten and Schellekens 2003). The most prominent example for the breakdown of immune tolerance against a naturally occurring protein is probably the “erythropoietin (Epo) case”. The formation of non native like structures is related to the breakdown of immune tolerance against both the synthetic substance and Epo derived from the human body. This can be explained by the fact that the aggregated protein molecules form haptenes for the immune system. The human body in turn is not able later on to differentiate the origin of the haptene that also lies somewhere on the protein surface. When the body tries to eliminate all Epo the consequence for the patient is a so called pure red cell aplasia (PRCA). The only therapeutic option for this adverse effect is a complete blood transfusion to remove antibodies from the patient (Casadevall et al. 2002; Rossert et al. 2005; Schellekens 2005; Schellekens and Jiskoot 2006). Thus, the formulator in pharmaceutical industry has to try to prevent the protein from all types of chemical and physical degradation, which are explained in this chapter.

1.1.2. Physical Instability

Physical denaturation describes the unfolding of the native protein structure. One can differentiate between partial and complete denaturation (Figure 1). A frequent consequence of denaturation is unfolding. But also native proteins can form associates (Brange 2000).

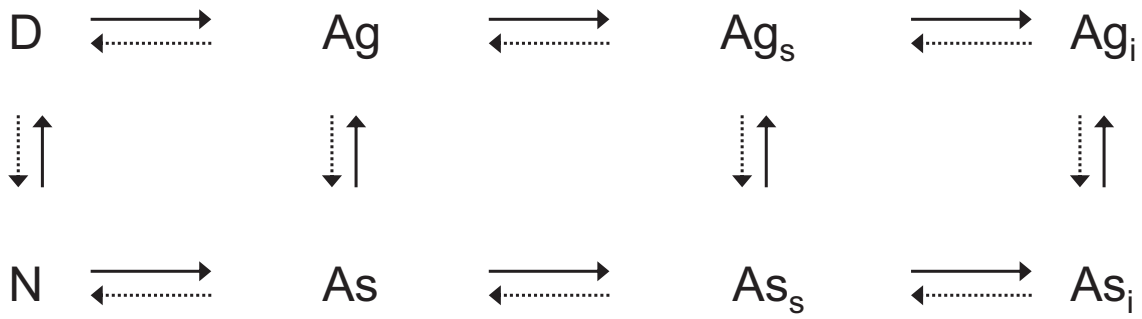

Figure 1: Simplified model of proposed protein aggregation and association mechanism (Mahler et al. 2005)

In Figure 1 N is the native state protein that can unfold to the denatured state D or form small associates As. As are oligomers and multimers whose monomer subunits have a preserved native structure. D or As can form aggregates Ag based on unfolded monomers. As can grow and build up large soluble As_s or when solubility limits are exceeded can form insoluble associates As_i. From smaller aggregates larger soluble Ag_s and insoluble aggregates Ag_I can be generated (Mahler et al. 2005).

As indicated by the dotted lines, the reaction back to a more native like state typically only occurs under special conditions like high pressure for the refolding of human Growth Hormone (St. John et al. 2001).

An important aspect of physical instability is the size of the associates or aggregates. Proteins can arrange in small units like dimers, trimers or tetramers, but also in larger oligomers or aggregate to large multimers. Although some protein pharmaceuticals have a native state that comprises monomers, dimers and hexamers, e.g. insulin (Manallack et al. 1985; Bhattacharyya and Das 1999) or glycogen phosphorylase (Paladini et al. 1994) that show enhanced stability or activity in an oligomeric state, for most pharmaceutically applied proteins the monomer is the only acceptable, stable, and active state.

The problem with bigger oligomers is that they can act as epitopes for the patient's immune system. This recognition can lead to acute immunogenicity or breakdown of the immune tolerance (see above) to exogenous substances (Schellekens 2005; Rosenberg 2006).

Often the first transition from N to D is going through a slightly unfolded state, the so-called molten globule state (Figure 2) (Ptitsyn 1987; Kumar et al. 1995; Bam et al. 1996). These intermediates are less compact than the native state, but show similar Stokes radii. The molten globule state is usually thermodynamically unstable and transforms into completely unfolded protein. But in some cases this partially unfolded protein can be preserved and can fold back to the native state by high pressure as applied for different proteins (Zhang et al. 1995; Bam et al. 1996).

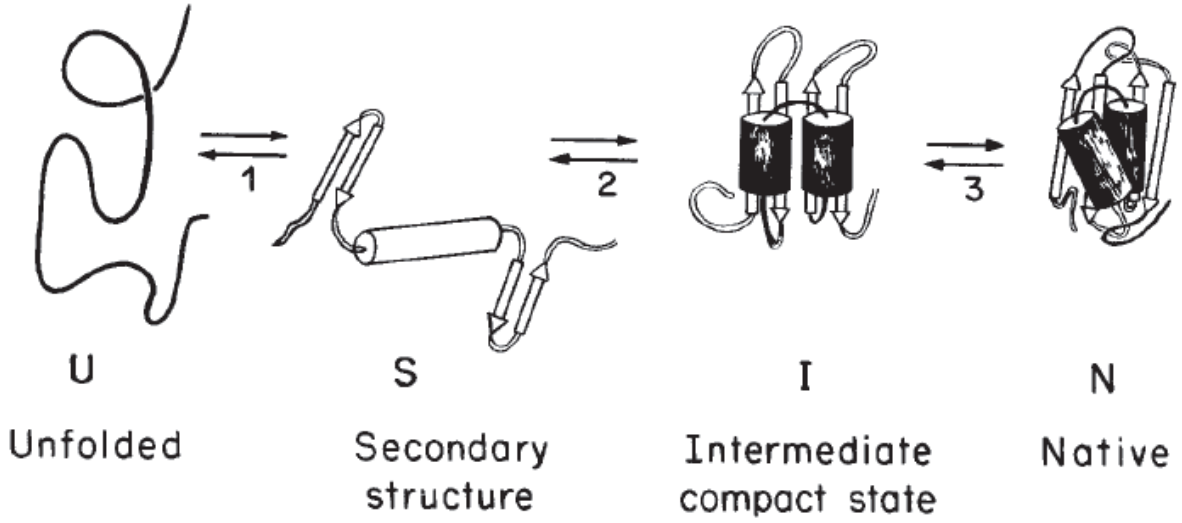

Figure 2: Proteins are folded via an Intermediate State (I), the molten globule state (N), into the native form. I is usually slightly bigger than N (from (Ptitsyn 1987)).

1.1.3. Chemical Instability:

Chemical degradation of protein comprises a number of different processes.

Deamidation is the most prominent degradation pathway for proteins (Robinson and Robinson 2001) for example insulin is deamidated at Asn21 under acid and at AsnB3 under neutral pH conditions (Brange et al. 1992). For deamidation one of the two amino acids with amid functions, asparagine (Asn) or glutamine (Gln), must be present in the protein (Brange et al. 1992; Sasaoki et al. 1992; Shire 1996). Asn is

much more susceptible to deamidation than Gln. For both amino acids the mechanism leads via cyclic imide products to the oxidized acid, aparagic acid or glutamic acid or their respective iso-form (Robinson and Robinson 2001; Robinson and Robinson 2004).

The amino acids most likely to undergo **oxidation** are methionine, cysteine and histidine (Gu et al. 1991; Stadtman 1993; Nabuchi et al. 1995; Fransson et al. 1996; Li et al. 1996a; Zhao et al. 1997). Methionine can react to its sulfoxide or under stronger conditions to its sulfone (Manning et al. 1989). Oxidized cystein is forming inter- and intramolecular disulfide bridges, under stronger conditions reactions to its sulfenic, sulfinic and finally sulfonic acids are possible, too (Florence 1980; Stadtman 1993). In this work Interleukin-11 and hGH are applied. Both proteins are susceptible to methionine oxidation (Pikal et al. 1991; Yokota et al. 2000).

When aspartic acid (Asp) is present in the protein, peptide bonds are eager to **hydrolize** at the N-terminal and C-terminal adjacent to an Asp residue. This behaviour is favoured when the following amino acid is proline and glycine. In some cases hydrolysis of Asn is following the deamidation of Asp to Asn (Manning et al. 1989; Brange et al. 1992; Li et al. 1995; Reubsaet et al. 1998). RhIL11 is also cleaved by hydrolytic mechanisms between Asp133 and Pro134 under acidic conditions (Kenley and Warne 1994).

The potential for **racemization** is present in all amino acids but Gly. Asp and Glu racemize via cyclic imide intermediate formation. The rate of racemization is strongly structure dependent (Stephenson and Clarke 1989; Kimber and Hare 1992; Ritz and Schutz 1993; Luthra et al. 1994; Shahrokh et al. 1994). Shifts in pH and high temperatures are the cause of this degradation mechanism.

Cys, Ser, Phe, Thr and Lys are prone to **β-elimination**. This is a special pathway of racemisation, where the intermediate product is cleaved after conversion. The products, originating from elimination mechanisms will contribute to physical instability such as aggregation, adsorption or precipitation. For example recombinant human Macrophage colony stimulating factor is supposed to be β-eliminated under alkaline conditions (Nashef et al. 1977; Schrier et al. 1993). Insulin shows β-elimination after thiol-induced interchange (Costantino et al. 1994).

A consequence of β-elimination can be **disulfide scrambling**. Free thiol groups can be oxidized forming disulfide bridges. Insulin shows disulfide scrambling in the dried state. This is even more pronounced when the lyophilizatates showed higher residual moisture (Costantino et al. 1994; Kuwata et al. 1994; Shahrokh et al. 1994).

The formation of anhydrids from Asp and Glu is another possible degradation pathway where intramolecular bonds are formed. These reaction are strongly dependent on pH and are sometimes proposed to occur in the presence of formaldehyde, e.g. for vaccines (Schrier et al. 1993; Prestrelski et al. 1995; Schwendeman et al. 1995). **Non-disulfide cross-linking** (also called non-reducible cross-linking) is also a degradation pathway of Interleukin-2, that is increased by the presence of polysorbate 80 in the liquid protein formulation (Wang et al. 2008). This degradation mechanism, usually oxidative, can e.g. be thioether formation and is detectable via SDS-Page (Wang 1999).

A big number of therapeutic proteins, especially monoclonal antibodies show a specific level of glycosilation (Wang et al. 2005). **Deglycosilation** can impact thermal stability and function of the protein as shown for human Interferon-β and phytase. Furthermore, the isoelectric point can be shifted due to this degradation and impair physical stability of a formulation (Runkel et al. 1998; Bagger et al. 2007a). Deglycosilation can occur pH triggered and lead to thermally unstable proteins that are prone to intracellular degradation (Wang et al. 1996; Dobson 2003).

In the presence of reducing sugars, such as glucose and fructose, proteins can undergo a degradation pathway called the **Maillard reaction**. This type of reaction is well known from food browning during baking. Products are usually yellowish to brownish and heterogenous. A free amino group of an amino acid and a hemiacetal in the sugar are essential for this kind of reaction (Reyes et al. 1982). E.g. for human relaxin in the lyophilized state it is important to refuse reducing sugars from the formulation as they significantly react with the protein (Li et al. 1996b).

Physical and chemical instability are usually affecting each other. On the one hand side aggregation can occur due to covalent linkage of two unfolded monomers (Muhammad et al. 2009) and on the other hand chemical reactions may be enhanced in the unfolded state (Kendrick et al. 1997).

1.2. Stabilizing Additives in Pharmaceutical Protein Formulation

There are several types of excipients applied to prevent physical degradation of pharmaceutical proteins. Surfactants are applied to prevent surface induced

unfolding, as artificial chaperones to reverse unfolding and in some cases to prevent chemical denaturation. This group of excipients is discussed in detail in the paragraphs below. Other applied excipients are sugars and polyols, amino acids, buffer salts, polyethylene glycols (PEG), other polymers, metal ions.

1.2.1. Other Excipients than Surfactants

Sugars and polyols act through the preferential exclusion mechanism, introduced by Timasheff et al. into the pharmaceutical field (Timasheff 1993). Preferential exclusion of the excipient from the protein leads to a stronger hydration of the protein, which in turn leads to a denser packing of the protein molecules to minimize exposure of hydrophobic protein parts at the surface. Furthermore, reduced protein surface due to preferential exclusion reduces the chemical potential and is thus less prone to oxidative processes (Kendrick et al. 1997). Sugars are also able to remove metal salts from the solution and may prevent metal ion catalyzed chemical degradation or act in other ways as antioxidant (Lam et al. 1997). In freeze-dried formulation, sugars are acting as water replacement by providing hydroxyl functions to the protein (Crowe et al. 1993a; Crowe et al. 1993b; Schuele et al. 2008). The tendency of in particular sucrose and trehalose to form amorphous cakes makes them ideal bulking agents in dried protein formulations (Arakawa et al. 1993; Chang et al. 1996a). Furthermore these disaccharides lead in many cases to solid systems with a glass transition temperature and relaxation rates high enough for effective storage stability. In numerous cases trehalose stabilizes slightly better than sucrose (Tanaka et al. 1991; Hora et al. 1992; te Booy et al. 1992; Pikal and Rigsbee 1997; Cleland et al. 2001; Maury et al. 2005). Reducing sugars should not be applied, as they have the tendency to react to Maillard-products with terminal amino groups as shown for human relaxin (Li et al. 1996b). The use of the sugar alcohol mannitol usually yields crystalline solid formulations (Akers 2002). Shorter polyols like glycerol can be added to freeze-dried formulations for the suppression of local, nanosecond relaxations and frequency shifting of collective vibrations that occur upon addition of diluents. Therefore, they increase protein stability although the glass transition temperature is decreased (Cicerone et al. 2005).