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Immobilised Macromolecules: Application Potentials

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Application Potentials

Edited by U. B Sleytr, P. Messner, D. Pum and M. Sára

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Cover Illustration: Electron micrograph showing the regular pattern of polycationised ferritin molecules attached to the S-layer of an archaebacterium

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Immobilised Macromolecules: Application Potentials

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Foreword from Series Editor

The Institute for Applied Biology was established by the Department of Biology at the University of York to consolidate and expand its existing activities in the field of applied biology. The Department of Biology at York contains a number of individual centres and groups specialising in particular areas of applied research which are associated with the Institute in providing a comprehensive facility for applied biology. Springer-Verlag has a long and successful history of publishing in the biological sciences. The combination of these two forces leads to the "Springer Series in Applied Biology". The choice of subjects for seminars is made by our own editorial board and external sources who have identified the need for a particular topic to be addressed.

The first volume, *"Foams: Physics, Chemistry and Structure"*, has been quickly followed by *"The 4-Quinolones: Antibacterial Agents in Vitro"*, *"Food Freezing: Today and Tomorrow"* and *"Biodegradation: Natural and Synthetic Materials"*. The aim is to keep abreast of topics that have a special applied, and contemporary, interest. The current volume describes how biomolecules have far reaching uses relating to the development of biosensors, optrodes, conjugated vaccines, biocompatible surfaces and membranes with novel properties.

Up to three volumes are published each year through the editorial office of *IFAB Communications* in York. Using modern methods of manuscript assembly, this streamlines the publication process without losing quality and, crucially, allows the books to take their place in the shops within four to five months of the actual seminar. In this way authors are able to publish their most up-to-date work without fear that it will, as so often happens, become outdated during an overlong period between submission and publication.

The applications of Biology are fundamental to the continuing welfare of all people, whether by protecting their environment or by ensuring the health of their bodies. The objective of this series is to become an important means of disseminating the most up-to-date information in this field.

York, July 1992 A. W. Robards

Editor's Preface

The last few years have seen astonishing progress in the fields of biotechnology, bioengineering and biomedical engineering. In particular, there have been many and far-reaching applications relating to the development of biosensors, optrodes, conjugated vaccines, biocompatible surfaces and membranes with novel properties. Common to all these interdisciplinary areas of research is a requirement for the immobilisation of functional macromolecules which, in turn, highlights the importance of knowing much more about how biomolecules interact and how they can be engineered to produce functionally useful surfaces and membranes. The contents of this book point to the way that scientists from many different disciplines are now coming together to bring their expertise to bear on the development of novel technology that can only arise from collaboration between protein- and physical chemists, enzymologists, biotechnologists, microbiologists, optical physicists and many others. The potential developments from such synergistic relationships are truly awe-inspiring whether they involve "clever" membranes that can be used to filter and/or adsorb molecules with a very high degree of specificity, the production of a wider range of biosensors, or opening the way for new vaccines. All such work needs to be underpinned by research on the molecular building blocks of the new devices and many of the relevant studies are reported in this book. The different chapters range across topics including: protein folding, stability and assembly; orientated immobilisation of antibodies; avidin/biotin interactions; 2D-protein crystals; and enzyme hapten or antigen immobilisation. From these fundamental studies it has been possible to lead on to discuss practical applications including; affinity and ultrafiltration membranes; biosensors; and diagnostics. In consequence, this book provides a unique and up-to-the-minute account of the state-of-the-art in the different ways that immobilised macromolecules can be used in practical applications.

U. B. Sleytr

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Chapter 1

Protein Stability, Folding and Association

R. Jaenicke

Introduction

Significant advances have been made in recent years in the technology of protein reconstitution. Making use of the stunning fact that even giant protein complexes such as ribosomes or multienzyme systems, or kringle structures like tissue plasminogen activator can be subjected to denaturation/renaturation, recombinant proteins are now "renativated" on large scale. The basis of this kind of industrial application is still alchemy, for two reasons: first, neither the mode nor the code of protein folding has been solved yet; and, second, protein stability is still not well understood in its thermodynamic principles.

Starting points for the present discussion are native mesophilic globular proteins of average molecular mass. From the available data base with ca. 500 crystal structures it has become clear that beyond a critical length of the polypeptide chain of ≤ 150 amino acid residues, proteins consist of domains. They represent cooperative units with respect to both intrinsic stability and folding. The general principle underlying the three-dimensional structure of proteins in aqueous solution is the minimisation of hydrophobic surface area which leads to the well-known distribution of non-polar residues in the interior of the molecule, and polar residues on its surface. A given amino acid composition does not necessarily allow this "micellar structure" to be perfect so that polar residues may be buried in the interior and non-polar residues may be exposed to the solvent. Evidently, the distribution of residues and the packing of the core has important implications for protein dynamics and stability. Both are determined by the subtle balance of attractive and repulsive interactions between the residues on one hand and the solvent on the other (Jaenicke 1987a).

Protein Stability

Interactions Involved in Protein Stability

The contributions of the various weak interactions that determine the three-dimensional structure of proteins are more controversial than they used to be since Kauzmann wrote his classical review (Kauzmann 1959; Privalov and Gill 1989; Dill 1990; Creighton 1991; Jaenicke 1991a; Jaenicke 1991b). In the present context, it is sufficient to note that globular proteins in solution exhibit only marginal free energies of stabilisation. The subtle balance of hydrogen bonds, hydrophobic interactions, ion pairs and van der Waals forces yields values for the free energy of stabilisation, ΔG_{stab}, which are generally found to be below 60 kJ/mol, independent of the mode of denaturation (Pace 1990; Pfeil 1986). Expressed in ΔG_{stab} per residue, this means that the free energy is one order of magnitude below the thermal energy (kT). Thus, on balance, the stability of globular proteins in solution is based on the equivalent of a few weak interactions. This holds even in the case of highly stable proteins, e.g. from hyperthermophilic microorganisms (Jaenicke 1991b,1992).

Ribonuclease T1 (RNaseT1) may serve as an example: Its structure is stabilised by two disulfide bridges, apart from 87 intramolecular hydrogen bonds and prominent hydrophobic interactions involving 85% of the non-polar residues; however, the free energy of stabilisation, ΔG_{stab}, does not exceed 24 kJ/mol (pH 7.0, 25°C). The high-resolution X-ray analysis of the protein has shown that the hydrophobic core of the molecule is sandwiched between a long α-helix and an extended antiparallel ß-sheet. A number of mutants with single amino-acid exchanges have been investigated, indicating that the stability of the molecule can be increased or decreased when residues in the helix or in the ß-sheet gain or lose a charge. In addition, substitution of side chains involved in intramolecular hydrogen bonds has proven that there is a net loss in stability per H-bond of the order of 5 kJ/mol, settling the old controversy whether there is a difference in H-bond strength between water-water and water-protein hydrogen bonds. Making up the balance of the various intra- and intermolecular interactions involved in the total free energy of stabilisation, it becomes clear that H-bonding, at least in small proteins, contributes equally, or even to a higher extent, to the configurational stability than hydrophobic interactions. The following numbers illustrate this conclusion: 494 kJ/mol for the conformational entropy, as compared to 268 kJ/mol for hydrophobic interactions and 448 kJ/mol for hydrogen bonds (Pace *et al. 1991;* Shirley *et al.* in press).

Coulomb interactions are well understood in model systems, but they become highly complex in non-homogeneous environments such as folded proteins, mainly because of the ill-defined dielectric constant in the immediate surrounding of the charges (Dill 1990; Sharp and Honig 1990; Creighton 1991). Whether or not ion-pairs contribute significantly to protein stability has been questioned since it became clear that most charged groups in globular proteins are exposed to the aqueous solvent (Kauzmann 1959). On the average, only one ion-pair per 150 amino acid residues of a globular protein is buried within the interior core (Barlow and Thornton 1983). Thus, only surface ion-pairs are expected to be involved in stabilisation. As indicated by site-directed mutagenesis and X-ray analysis, their contribution may be significant to the extent that thermophilic adaptation has been attributed to charge interactions (Perutz and Raidt 1975; Anderson *et al.* 1990). However, even for these groups, effects of varying salt concentration suggest that a significant fraction of the electrostatic free

energy arises from the entropy of proton and water release rather than charge energetics (Stigter and Dill 1990). On the other hand, the destabilisation of proteins at extremes of pH has always been explained in terms of increased repulsive interaction at high net charge. Whether this is correct, or whether the ionisation of buried polar residues is involved is an open question (Privalov and Gill 1989; Pace *et al.* 1990).

An important observation in this context is the fact that at very low pH, charge effects seem to be inverted such that the acid denatured protein undergoes a second structural transition into its "A-state" or other "alternative states" (Goto *et al.* 1990; Buchner *et al.* 1991). These show structures distinct from both the native and denatured states, and are frequently identified as "molten globules" (see below); in certain cases they obviously differ from this rather ill-defined intermediate "collapsed state" by showing well-defined, but non-native structure (Buchner *et al.* 1991). The simplest explanation for the regain of spatial organisation of the polypeptide chain after preceding acid denaturation would be the fact that at pH $\leq$ 1 the high activity of the acid leads to a significant increase in ionic strength. This will screen part of the charge effects, thus modulating coulombic interactions. On the other hand, effects on water structure as well as preferential salt binding might be involved. These phenomena are expected to be relevant especially for peripheral proteins from acidophilic microorganisms.

The mechanisms underlying the stabilisation of proteins that undergo no or relatively slow turnover, such as basic pancreatic trypsin inhibitor (BPTI) or γ-crystallin, may be totally different from those discussed so far. The fact that reduction of the three cystine bridges in BPTI leads to complete unfolding, even in the absence of denaturants, indicates that in this case evidently folding and stability are coupled to disulfide bond formation (Creighton 1978, 1988a; Wetzel *et al.* 1990). Calorimetric studies on BPTI analogs, selectively modified with respect to the number of disulfide bonds, clearly show the gradual stabilisation of the protein with increasing crosslinking. In some cases, the stabilising effect is much larger than one would expect for the restricted increase in entropy of the denatured state. Since γ-crystallin does not contain disulfide bridges, in this case the low turnover must be related with the all-ß structure; a clear-cut explanation for the anomalous stability of the protein (at pH 1-10 and temperatures up to 75°C, or in the presence of 7M urea) has yet to be given (Rudolph *et al.* 1990).

The oxidation of cysteine residues to form cystine bridges is a cotranslational process which is speeded up by its "vectorial" character (Bergman and Kuehl 1979); in addition, the reaction is catalysed by protein disulfide isomerase (PDI). The enzyme resides in the endoplasmic reticulum (ER), where folding of secretory disulfide-bonded proteins is known to occur (Freedman 1984). It is a dimer with two internally homologous domains in each monomer which are structurally and functionally related with thioredoxin. Mechanistically the two proteins are linked as a redox pair with PDI as the weaker reductant; this property may be significant in ensuring that PDI does not reduce correct disulfide bonds that are already stabilised in the nascent native-like protein (Freedman 1989; Schmid 1991).

Structural Elements, Fragments, Domains and Subunits

Up to this point, intact proteins have been considered, with the general conclusion that stability is accomplished by the cumulative effect of non-covalent interactions at many locations within the globular entity of the protein molecule. In considering the

stability of local structural elements, one may ask what happens if we shorten the protein, e.g. by limited proteolysis, and what is the minimum length of a polypeptide chain that is required to still form an intrinsically stable native-like structure (Jaenicke 1987a, 1991a; Baldwin 1990). NMR data have shown that oligopeptides down to six residues do form stable (non-random) conformations, supporting the idea that local structures may serve as "seeds" in the folding process (Wright *et al.* 1988). However, there is some indication that the short fragments have no substantial tendency to adopt the same conformation in unrelated protein structures; for example, reverse turn motifs observed in small peptides seem to be absent in the known three-dimensional structures of proteins with these sequences (Creighton 1988b). With regard to the stability of protein fragments, it has been known for a long time that protein domains show high intrinsic stablity, not far from the free energies observed for the uncleaved parent molecule (Jaenicke 1987a). Reducing the chainlength further, it becomes evident that proteins are cooperative structures showing mutual stabilisation of structural elements. In order to find out at which fragment size native-like structure can no longer be formed, thermolysin was used as a model (Table 1.1). Folding/unfolding experiments with a variety of BrCN fragments show that the N-terminal portion of the enzyme stabilises the all-helical C-terminal domain. This may be shortened drastically, down to the 62-residues three-helix structure, without aggregating or losing much of the stability of native thermolysin; only the C-terminal 20-residue helix is too short to maintain its native structure in aqueous solution (Vita *et al.* 1989). Whether the N- and the C-terminal ends of the polypeptide chain are important for protein stability depends on the protein. Taking RNase and lactate dehydrogenase (LDH) as examples, it has been shown that the N-terminal ends of both proteins can be cleaved without altering the overall topology; however, the stability is greatly affected. Cleaving off the C-terminus of RNase is sufficient to block the oxidative reshuffling reaction (Opitz *et al.* 1987; Teschner and Rudolph 1989).

Table 1.1. Physicochemical characteristics of thermolysin and its fragments: M, molecular mass; helicity from CD; ΔG_{stab}, free energy of stabilisation

Sequence	M(calc)	M(obs)	Helicity	T (denaturation)	ΔG_{stab}
1-316	34 227	34 800	100%	87°C	55 kJ/mol
121-316	20 904	23 000	96	74	47
206-316	11 829	11 900	95	67	31
225-316	9 560	9 000	92	65	26
255-316	6 630	≤12 000	100±10	64	20

As mentioned in connection with domain proteins, cooperative effects contribute significantly to the intrinsic stability of proteins. This feature is even more pronounced in protein assemblies.

To illustrate this, lactate dehydrogenase may again serve as an example. The *native tetramer* represents a dimer of dimers. Medium denaturant concentrations, as well as cleaving off the N-terminal decapeptide allow the *dimer* to be characterised: its stability is drastically reduced, it shows no enzymatic activity unless structure-making salts are added, and its folding is strongly impaired by irreversible side reactions. The *monomer* is only accessible as short-lived intermediate on the pathway of folding and association: limited proteolysis indicates a still higher degree of flexibility compared with the

dimer, and no residual enzymatic activity can be observed. The domains (after "nicking") are able to pair correctly: they recognise each other in a topologically correct manner, so that one may assume that under quasiphysiological conditions "structured monomers" possess a native-like tertiary structure, but dramatically reduced stability (Jaenicke 1987a).

Physiological Stress and Protein Stability

Since it is not trivial to design proteins with specific stability characteristics, it seems useful to try to elucidate the long-term experiment that has been going on in nature, where adaptation to extremes of physical conditions in the biosphere has led to proteins with highly specified stabilities toward temperature, pressure, pH and low water activity. As taken from more than one century of careful investigation, life on earth is ubiquitous, except for centers of volcanic activity. The biosphere (including zones where "cryptobiosis" prevails) refers to the oceans with pressures up to 120 MPa (1200 atmospheres), the soil to a depth of 10-20 meters, and the atmosphere and stratosphere. Thus, organisms had to adapt their cell inventory to the extremes summarised in Table 1.2.

Table 1.2. Limits of viability and extremophilic adaptation[a]

Temperature	-5 - 110°C	Psychro- and thermophily
Pressure	0.1 - 120 MPa	Barophily
pH	0 - 12	Acido- and alkalophily
Water activity	0.6 - 1.0	Halophily

[a] Short-term survival and cryptobiosis may exceed the given limits

In comparing mesophiles and extremophiles, it has been shown that the range of viability is commonly shifted rather than broadened (Jaenicke 1981). Focusing on homologous proteins from organisms taken from different extreme environments, it turns out that adaptation to extremes of physical conditions tends to maintain "corresponding states" regarding overall structure, flexibility and ligand affinity. Generally, evolution is geared to maintain optimum function in widely differing solvent environments. It does so with an amazingly high degree of conservatism with respect to the three-dimensional "topology" of proteins and their constituent amino acids. This is beautifully illustrated by the fact that hemoglobin has preserved its characteristic peptide fold in spite of only two out of ca. 150 residues along the polypeptide chain having been absolutely conserved over the whole time span of evolution; on the other hand, all proteins, including those from the most extremophilic organisms, do not contain other than the 20 natural amino acids as amide-linked polypeptides. The increase in overall stability is of the same order as mentioned before: $\Delta\Delta G_{stab}$ for a typical thermophilic protein is $\approx$ 50 kJ/mol, again only marginal compared with the inner energy which is of the order of 10^4 kJ/mol (Baldwin and Eisenberg 1987). Faced with this ratio, it is evident that there may be many ways to accumulate the $\Delta\Delta G_{stab}$ required to stabilise a protein; on the other hand, one would predict that it is highly improbable to uncover general strategies of molecular adaptation. In responding to extreme physical conditions, organisms may use strategies other than molecular adaptation: acidophiles or alkalophiles avoid extremes of pH in their cytoplasm by pumping protons; halotolerant organisms may compensate salt