Minireview

Proteins under extreme physical conditions*

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Life on earth is ubiquitous within the limits from −5 to 110°C for temperature, 0.1 to 120 MPa for hydrostatic pressure, 1.0 to 0.6 for water activity and pH 1 to 12. In general, mutative adaptation of proteins to changing environmental conditions tends to maintain 'corresponding states' regarding overall topology, flexibility and hydration. Due to the minute changes in the free energy of stabilization responsible for enhanced stability, nature provides a wide variety of different adaptive strategies. In the case of thermophilic proteins, improved packing densities are crucial. In halophilic proteins, decreased hydrophobicity and clustered surface charges serve to increase water and salt binding required for solubilization at high salt concentration. In the case of barophiles, high-pressure adaptation is expected to be less important than adaptation to low temperatures governing the deep sea. Nothing is known with respect to the mechanisms underlying psychrophilic and acidophilic:alkalophilic adaptation.

Barophile; Halophile; Stability; Thermophile

1. INTRODUCTION

The conformational stability of globular proteins can be defined by the free-energy difference between the folded and unfolded states, $\Delta G_{N\rightarrow D}$, under physiological conditions. This value is of the order of 45 ± 15 $kJ \cdot mol^{-1}$, reflecting only marginal stability of native proteins [1]. Adaptation to extreme environmental conditions can be accomplished by shifting the optimum curve such that similar $\Delta G_{N\rightarrow D}$ values are obtained at the respective optimum conditions. In asking biologically relevant questions with regard to the structure-function relationship of proteins, it has been most useful to investigate the stability of proteins in a wide range of environmental parameters.

Life on earth is ubiquitous, implying that organisms have evolved to cope with the wide ranges of temperature, pressure, pH and water activity observed in the biosphere. Defining 'extreme' biotopes by their low species diversity, the limits of viability are:

for temperature: −5 to 110°C.
for hydrostatic pressure: 0.1 to 120 MPa,
for water activity: 0 to 0.6 (corresponding to <6 M salt),
for pH: pH 1–12.

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* Dedicated to Professor Erwin Chargaff on occasion of his 85th birthday

Avoidance, tolerance and adaptation are the 3 ways of adjustment of life to environmental stress [2]. Considering pH and water activity, neutralization or compensation by 'compatible solutes' is frequently observed. In the case of temperature and pressure, the isothermal and isobaric conditions in a given biotope *require* adaptation for survival. This means that at extremes the intrinsic stability of the macromolecular cell constituents is found to be increased. In addition, extrinsic factors such as biogenic amines or cyclic polyphosphates may serve as protecting agents [3]. Since the building blocks and the covalent bonds of all proteins, including the extremophilic ones, are the same, in the case of extreme environmental conditions the chemical constituents and the covalent structure of the polypeptide chain set the ultimate limit.

2. CONFORMATIONAL STABILITY

The 3D structure of proteins is directed and stabilized by two classes of non-covalent interactions, electrostatic and hydrophobic. The electrostatic interactions include ion pairs, hydrogen bonds, weakly polar interactions and Van der Waals interactions, and have been reviewed in detail by Burley and Petsko [4]. The hydrophobic effect does not really imply a force; it includes Van der Waals interactions and hydration effects of non-polar groups [5–7]. Recent analysis of experimental data on protein denaturation and hydrocarbon solubility showed that the stabilization of the folded structure of proteins by the hydrophobic effect

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originates from Van der Waals interactions of tightly packed non-polar side-chains while the solvation effect of these groups causes destabilization [8,9]. As pointed out by Finney [10], ideally, in order to quantify solvent effects in biomolecules, we need to understand the *detailed structures and interactions* of both polar and non-polar groups in solution. Presently, by the very nature of the disorder involved, experimental information is only rudimentary, and its interpretation to a high degree model-dependent. Using isotopic substitution neutron scattering difference methods, the partial pair correlations from a given atom to its surroundings can be determined. In the case of aqueous solutions of simple model compounds such as urea of tetramethylammonium ions, attempts were made to describe hydrogen bonding and hydrophobic hydration of groups exposed to the solvent. At low concentrations, the amphiphilic ion appears to behave as a non-polarly hydrated molecule with a defective and disordered cage hydration shell; urea seems to break down part of the cage hydration structure [11].

As pointed out by Privalov and Tsalkova [12], protein stability can be expressed in terms of the free energy difference associated with the macro- and micro-unfolding of the conformation. The macrostability is characterized by $\Delta G_{N\rightarrow D}$ (calculated per mol of the cooperative unit), i.e. by the work required to transfer the protein from the folded to the unfolded macroscopic state. On the other hand, the microstability is characteristic of the rigidity of the structure, with ΔG^{mic} as the Gibbs free energy associated with the local non-cooperative unfolding reactions within the folded state.

The thermodynamic (macro-)stability of soluble globular proteins is the sum of exceedingly large contributions of diverse stabilizing and destabilizing interactions involved in the formation of the folded compact state [10,13]. As mentioned, $\Delta G_{N\rightarrow D}$ does not exceed the energy loss on breaking just one ion pair, or 2–5 hydrogen bonds, i.e. only a few percent of the total number of weak interactions involved in an average protein structure. The conformational stability of proteins depends on environmental factors in a complex way. Therefore, it is difficult to estimate the small differences in free energy between the limited ensemble of folded and the exceedingly large ensemble of unfolded states [14]. Despite the complexity of the problem the effect of environmental factors and amino-acid replacements on the individual groups of stabilizing forces can be estimated quantitatively [9].

3. THERMAL ADAPTATION

3.1. *Stability, folding and solvent interactions at high temperature*

As suggested by the upper limit of thermal adaptation around 110°C [15], and the reversible deactivation of enzymes below the normal degree of hydration [16], the ultimate requirement for life seems to be the presence of water. In testing this hypothesis, stabilization of the liquid state of water at high hydrostatic pressure shows clearly that the observed maximum temperature cannot be shifted any further. It is dictated by the susceptibility of the covalent structure of the polypeptide chain toward hydrolysis, and by the hydrothermal degradation of essential small molecules such as amino acids and metabolites [17,18]. The high reaction rates ($t_{1/2}$ for glutamine at 120°C $\approx$ 10 min, for ATP and NADH $<$ 1 s) do not allow the decay to be compensated by enhanced synthesis [19]. Synthetic proteins with thermal stabilities exceeding 110°C have been reported [20]; thus, the above limit is not absolute. In this context, it is interesting to note that above 110°C the hydrophobic effect changes from being entropy driven to being enthalpy driven, since the contribution of water solvation (which is always destabilizing) approaches zero [9].

A large body of experimental data on proteins isolated from thermophilic microorganisms has been accumulated in recent years. Some general features of thermophilic adaptation may be concluded from these results. Increased thermal stability of proteins is commonly based on mutative alterations of the amino-acid sequence; for extrinsic factors cf. [3,21,22]. Cloning and expression of genes from thermophiles in mesophilic hosts (resulting in the production of native thermostable protein) clearly demonstrate the intrinsic character of thermal stability [23–26]. At the same time, these experiments show that the physiological thermophilic conditions are not required for the folding of heat-stable proteins.

Most proteins isolated from thermophilic sources are characterized by increased conformational stability; however, this stability becomes marginal at the relevant physiological temperature [22,27–30]. This observation supports the view that molecular flexibility is critical for function [13,22,31,32]. Available data provide convincing evidence that thermophilic enzymes in terms of their basic topology, activity and mechanism are closely similar to their mesophilic counterparts. The same activity and catalytic mechanism suggest similar conformational mobility at the corresponding physiological temperature. As shown by amide proton and hydrogen-deuterium exchange rates, as well as resistance to proteolysis [33–35], thermophilic proteins at room temperature are less flexible than their mesophilic counterparts. Temperature-dependent NMR and X-ray studies and calculations based on normalized B-values also show that flexibility indices decrease with increasing thermal stability [36–38].

Based on the wide spectrum of experiments, a qualitative picture of the structural background of thermal stability of proteins emerges. The delicate balance between conformational entropy and hydration of the

unfolded macroscopic state on one hand, and the stabilizing interactions in the folded state, on the other, results in a marginally stable state of the protein. At physiological temperature, $\Delta G_{N\rightarrow D}$ represents a compromise providing optimum stability *and* flexibility. Since this compromise is the superposition of a vast number of stabilizing and destabilizing local interactions, there is an exceedingly large number of ways to end up with $\Delta G_{N\rightarrow D} \approx 50\text{–}100\ kJ \cdot mol^{-1}$.

As demonstrated by site-directed mutagenesis, the structural background of altered stability is individual in each case [14]. Apart from numerous examples where single amino-acid exchanges result in active enzymes with altered thermal stability, double and triple mutants have been reported to exhibit cumulative stabilization.

X-ray analysis of ts mutants of lysozyme from bacteriophage T4 showed that sites with low mobility and low solvent accessibility in the native molecule are the most vulnerable positions [39]. In the case of point mutants of α-amylase from *Bacillus stearothermophilus*, exchanges of conserved residues were found to produce enzymes with widely differing thermal stability [40]. In λ-repressor, random alteration of hydrophobic core residues shows that certain mutant polypeptide chains can fold into a globular, enzymatically inactive protein with altered thermal stability [41]. Obviously, only a small set of topologies yields a compact structure with both minimum hydrophobic surface area and maximum exposure of hydrophilic residues in the surface of the protein. The tight packing of proteins strictly limits alterations in hydrophobic core positions. There is a higher chance to replace surface residues without affecting the 3D structure, but at the same time altering the stability. The observed Lys → Arg replacements in the surface of thermophilic proteins may illustrate this [2].

In evolution, the delicate balance between stabilizing and destabilizing forces has been optimized to maintain stability and, at the same time, optimal flexibility at physiological temperature. To mimic evolution in protein design related sequences have to be analyzed in the context of their known 3D structure. Ab initio structure predictions based solely on the amino-acid sequence are presently not feasible [13].

3.2. *Stability, folding and solvent interactions at low temperature*

Available evidence from temperature-dependent studies on proteins, surfactants and synthetic polymers suggests that for any system which can undergo (two-state) order/disorder transitions, the temperature profile of the free energy of stabilization takes the form of a skewed parabola with two characteristic transition temperatures [2,7,42,43]. Starting from the temperature of maximum stability, both heating and cooling lead to an equilibrium transition where $\Delta G_{N\rightarrow D}$ becomes zero and disruption of the folded structure occurs. Cold denaturation is accompanied by a release of heat and a decrease in entropy, in accordance with the concept of hydrophobic effects [5–7]. For details with respect to the correlation of structure, function and energetics cf. [44]. Recent work refers to thermodynamic and kinetic studies on T4 lysozyme and lactate dehydrogenase [43,46–48].

Stability and kinetic data for T4 lysozyme mutants at temperatures down to $-10°C$ (in the presence of 3 M guanidine hydrochloride) can be interpreted within the framework of the two-state assumption. Two classes of mutants have been studied: in the first, single amino-acid exchanges only 'perturb' the wild type structure at specific sites altering the local hydrophobicity or flexibility. In the second class, sets of substitutions of cysteines, and introduction of one or more disulfide bridges were investigated. Neither the transition temperature nor the enthalpy of unfolding show drastic changes, although some of the mutated proteins depart rather far from the native enzyme. In the case of the cystine-bridged variants, the parabolic stability profile is shifted to higher temperatures so that there are good prospects to compare thermodynamic data with data obtained from X-ray crystallography. This holds especially for a triple-bridged construct with considerable thermal stability ($\Delta T_m = 25°C$) the X-ray analysis of which is underway.

Franks and Hatley [43] in a recent review presented a thorough analysis of protein stability at low temperature, re-evaluating all experimental results presently available. Three general conclusions are of importance: (i) Undercooling methods (using microemulsions of aqueous solutions in an organic carrier) may be applied to extend the accessible temperature range down to the homogeneous-nucleation temperature of ice ($\approx -40°C$). Applying this technique, artificial destabilization by pH or chaotropic agents can be avoided; this is important because denaturants as well as cryosolvents may affect the thermodynamics significantly. (ii) In comparing the unfolding of proteins, the two-state assumption has to be tested; for multidomain proteins and oligomers, $\Delta H_{cal}/\Delta H_{van't\ Hoff}$ may be as high as 4, thus indicating highly populated folding intermediates persisting for significant periods of time. All thermodynamic quantities strongly depend on temperature. In the case of the change in heat capacity, ΔC, the non-linear temperature dependence resides mainly in the heat capacity of the denatured state(s) whereas the corresponding values for the native state are considered to increase linearly with T. Thus, ΔC is expected to change sign at low temperature. (iii) Contributions to ΔC come from hydrophobic as well as electrostatic interactions. Both are of a mainly entropic origin and become weaker at lower temperatures; on the other hand, direct polar group hydration is increased, again favoring denaturation.

4. HIGH HYDROSTATIC PRESSURE: BAROPHILISM

In considering effects of low temperature on biochemical processes, the combination of low temperature and high pressure is of considerable importance since deep-sea biotopes are generally characterized by $T \leq 5°C$. In contrast to our intuitive assessment, high hydrostatic pressure is widespread in nature: the average and maximum pressures in the biosphere are ≈ 380 and 1200 atm (≈ 38 and 120 MPa), respectively. Within this pressure range, non-pressure adapted microorganisms show anomalous growth, morphological modifications, and cell death [2].

How pressure adaptation of barophilic microorganisms works is still unresolved. The main reason for this information gap is the difficulty of obtaining viable samples which have to be transferred from their natural habitat at their respective physiological pressure, and have subsequently to be grown, harvested and analyzed under pressure. The corresponding technology has only recently become available; thus, unambiguous proof for the occurrence of true barophilism has been a recent finding [49,50].

Model studies on simple chemical reactions and complex biochemical processes allow predictions to be made on what kind of processes might be crucial in high-pressure adaptation. Using the size and sign of the reaction- and activation-volumes as criteria, changes in both hydrophobic solvation and Coulomb interactions are expected to have major effects. pH shifts (due to electrostriction upon ionization) are less important [51].

As taken from experiments on non-barophilic organisms and their molecular inventory at pressures up to 400 MPa, typical effects of high pressure on biomolecules are (1) deactivation of enzymes, (2) dissociation of protein assemblies, (3) dissociation of ionogenic complexes such as nucleoproteins (viruses), (4) gel-sol transition of polysaccharides, (5) phase transitions of phospholipids, etc. Nucleic acids are stable in the ecologically relevant pressure range [52].

How barophiles compensate for the pressure-induced ionization or hydrophobic solvation is unknown. The assumption that ion pairs and hydrophobic interactions are replaced by hydrogen bonds is putative and needs to be confirmed experimentally. What has been found in non-adapted microorganisms is that pressure stress is accompanied by the de novo synthesis of basic proteins not expressed at normal pressure. Whether they show any similarity to heat-shock proteins remains to be shown [54]*.

* Similar observations have been reported for the deep sea bacterium strain SS9, as well as a moderately halophilic bacterium, *Deleya halophila*; in the latter case changes in external salinity have been shown to cause alterations in the protein pattern [55].

Experiments devised to find out whether high pressure is able to extend the temperature range of viability of thermophilic microorganisms gave negative results. The growth rate of *Methanococcus thermolithotrophicus* is increased at pressures up to 50 MPa; at 30 MPa morphological anomalies and cell lysis become detectable. The temperature optimum remains unaltered ($65 \pm 1°C$) [56]. In trying to pin down growth inhibition at high pressure to specific cellular components, dissociation of assembly structures and altered kinetics within the metabolic network have been discussed. Since ligand binding commonly exhibits large volume effects, high pressure is expected to cause 'metabolic dislocation'. On the other hand, high pressure affects dissociation equilibria of oligomeric and multimeric proteins. In this context, the extreme pressure sensitivity of ribosomes may be assumed to be responsible for the inhibition of growth at elevated pressure [57,58].

In spite of the fact that exceedingly large reaction volumes are involved in a variety of biochemical processes, adaptation to deep sea conditions is expected to be dominated by low temperatures rather than high pressure. The reason is that, on changing from sea level to the ocean floor, 20 degrees temperature difference may decelerate the reaction rate by a factor of 4–10 (depending on the energy of activation), while effects of the increase in pressure hardly exceed 15%.

5. STABILITY OF PROTEINS AT LOW WATER ACTIVITY

Osmoregulatory adaptation of halophilic or xerophilic organisms to changing external water activity may be achieved by balancing the inside and outside electrolyte concentration, or by de novo synthesis of compatible constituents of the cytoplasm [2]. Proteins from halobacteria are halo*philic*, i.e. they show a specific requirement for high salt concentrations; at low salt they undergo denaturation [59–61]. In this case, molecular adaptation means that the polar and dissociated groups on the protein surface must compete for their hydration with excess electrolyte in the cytosol [59]. From the salting-out effect of unpolar molecules, one may predict that halophiles adapt their proteins by favoring strongly hydrated amino acids (such as glutamic acid and arginine), at the same time decreasing their ratio of non-polar/polar residues [59]. As in the case of thermophiles, the central issue in adaptation is the conservation of flexibility. According to available criteria, a decrease in overall hydrophobicity is expected to serve this purpose.

Model systems illustrating the modulation of molecular flexibility by altering the hydrophobic properties of a protein have been reported for a number of enzymes: in the case of ferredoxin, the sequence of the protein from *Halobacterium halobium* is 22 residues

longer than the protein from blue-green algae; the extra piece contains 9 acidic residues and no basic one [62]. Tetrameric mammalian lactate dehydrogenase shows a drastic decrease in activity/flexibility with increasing salt concentration. The 'proteolytic dimer' of the enzyme (which lacks an N-terminal hydrophobic decapeptide, apart from hydrophobic interactions involved in quaternary structure formation) gains activity only in the presence of 'structure making salts' such as 1 M $(NH_4)_2SO_4$. At higher salt concentration, the further increase in rigidity causes deactivation of the proteolytic dimer, as in the case of the intact enzyme [63].

Increased hydration and salt binding as a consequence of halophilic adaptation have been determined directly by measuring mass density, electron density, neutron scattering length density, and classical small angle scattering in solution with contrast variation [59]. The subtle balance in the competition of protein and salt for water as their common solvent is stressed by the salt requirement of halophilic proteins; at low salt, deactivation, denaturation and (for oligomers) dissociation take place. Generalizations with respect to the salting-in and salting-out properties of the salts involved cannot be given. Taking malate dehydrogenase from *Halobacterium marismortui* as an example, it has been shown that the structure and stability of the enzyme are different in different salt solutions in which the enzyme shows unaltered catalytic activity. Thus, in phosphate, stabilization and hydration are similar to those of non-halophilic soluble proteins in which the hydrophobic effect dominates; in KCl, NaCl or $MgCl_2$, particles are formed in which the protein shows the anomalous water and salt binding mentioned above. Obviously, under these conditions, hydrophobicity of the protein core is insufficient to stabilize the folded state, and the main stabilization mechanism is the formation of 'cooperative hydrate bonds' between the protein and hydrated salt ions. Available data may be summarized in terms of a model in which the protein has a *core* similar to the non-halophilic protein, and *loops* extending into the solvent where the exceptional hydration interactions take place [59,64,65].

Presently, no high-resolution X-ray data are available to prove or disprove this model. It might be worth mentioning that in the case of dihydrofolate reductase the sequence of the halophilic enzyme may be fitted into the non-halophilic 3D structure. Thus, the overall topology of the homologous enzymes does not seem to be altered significantly (G. Böhm and J. Jaenicke, unpublished results).

As mentioned, instead of halophilic adaptation, de novo synthesis of 'compatible solvent components' such as glycerol, betaine, proline, or sugars may serve to cope with osmotic stress. A well-understood example illustrating this strategy is the halotolerant green alga *Dunaliella* [66]. Similar mechanisms have been described for the anhydrobiosis of sporulating bacteria, as well as for frost-hardened plants and 'hibernating' insects. It is obvious that in these cases nature has found ways to combine avoidance with intrinsic structure stabilization of cellular components by preferential solvation and/or specific protection mechanisms [67].

6. CONCLUSIONS

Adaptation of proteins to extreme conditions makes use of the common repertoire of the 20 natural amino acids, apart from 'extrinsic factors' such as ions or specific ligands. Homologous enzymes from extremophiles and mesophiles are closely similar in terms of their 3D structure and catalytic mechanism. Their activity tends to be comparable under their respective physiological conditions. Two levels of protein stability may be distinguished: The macro-stability maintains the integrity of the native folded conformation, while the micro-stability determines the dynamics of the protein responsible for optimum function.

There is a delicate balance of stabilizing and destabilizing interactions within the native 3D structure giving rise to a marginal free energy of stabilization. This balance can be easily adjusted to substantial environmental changes just by one or a few changes in the amino-acid sequence, keeping stability and flexibility at optimum levels. The general mechanism of thermal and salt adaptation or tolerance may be explained in qualitative terms making use of present knowledge about electrostatic and hydrophobic interactions in aqueous solution. Because of the complexity of the compensating forces maintaining the native protein structure, 'protein design' in terms of planned biotechnological modifications of a protein with the aim to adapt its stability properties to a given set of conditions is presently not feasible. It would require the correlation of structure, energetics and function of proteins to be known in an unambiguous way. Ab initio structure predictions are still far ahead.

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