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Protein Folding, Denaturation and Stability: A Brief Introduction

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Abstract

Protein folding refers to the process and path by which a nascent polypeptide obtains its 3-D or native state. Over the years the field of protein folding has evolved, as are the questions pertaining with this perplexing field. Levinthal, was first to propose that the folding code for a polypeptide to fold, is its primary structure or sequence of amino acids. But, still in present era, a major challenge is to predict the native structure of a protein solely from its sequence. This has become more challenging with ever increasing sequence data being accumulated with each day. Thus, protein folding problem continues to be a complex problem to be solved and has perplexed scientists over the decades. In this article, main focus will be to sum up the broad aspects of protein folding, protein denaturation and discuss in brief the methods to estimate protein stability.

Keywords: Protein Folding; Denaturation; Stability; Equilibrium Methods; Differential Scanning Calorimetry.

Introduction

It has long been established through the works of Anfinsen and his colleagues that the primary sequence holds the key for protein folding process, i.e., it has the code for folding a nascent polypeptide chain to the functional native state in a given milieu [1, 2]. Anfinsen's work on ribonuclease A (RNase A) clearly showed that the compact, three-dimensional structure (native state) could be reached from the primary amino acid sequence (denatured state) through purely physicochemical processes without any need of molecular chaperones [3, 4]. Folding into the native conformation is a very fast process with time scale ranging in milliseconds to microseconds. Thus, Cyrus Levinthal pointed out that it is impossible for a real unfolded protein to achieve native fold on the biological timescale if it goes randomly searching all the possible structural conformations [4, 5] . He suggested that this folding must occur according to specified pathways. Currently, it is believed that the folded native state of a protein is the main determinant of the folding process and ultimately provides the stability needed for the native protein to be functional [6, 7]. The complex process of protein folding has perplexed scientists for past several decades and different models have been suggested for the folding process.

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Folding Models

Various models of protein folding have been

proposed to describe the possible pathways through which a protein's primary sequence achieves the native fold. First, the "framework model" describes folding as a stepwise mechanism involving a hierarchical assembly of local elements of secondary structure from the primary sequence but independent from tertiary structure. This greatly reduces the conformational search and tertiary structure is attained by diffusion and collision of the local elements of secondary structure whereby favorable amalgamation occurs [8-10]. This model does not place emphasis on the formation of native tertiary contacts directly from the primary structure; thus, secondary structure formation is independent of how the final folded tertiary protein should look. Second, the "nucleation-condensation" model is a modified form of the "nucleation" model and describes folding from more of a helix-coil perspective. The model describes folding via the formation of a loosely packed (extended) nucleus, derived from initial helix or sheet "seeding" of native secondary structural elements, which becomes more compact in the transition state and is directly responsible for the formation (condensation) of higher order tertiary structure [11-14]. Third, the "hydrophobic collapse" model hypothesizes that the native protein fold is formed from a "molten globule" as a result of the polypeptide chain having a concentrated region of hydrophobic side chains [14-17]. The molten globule then quickly rearranges due to the narrowed conformational search leading up to the native fold.

Recent research in protein folding has lead to a more modern and general view of protein folding. The two main competing models have been proposed. The "predetermined pathway – optional error (PPOE)" model claims that all of a protein population folds by essentially the same stepwise pathway. This single pathway is defined by predetermined cooperative native-like foldon units (intermediates) and how those foldon units interconnect in the final native fold [18] . This model predicts the transition state as a single obligatory step having a few well-defined structures that all protein molecules need to pass through. The intermediates are all downhill from the transition state, thus are "hidden" and are only seen when there is an error in folding (misfold); thus, proteins behave as two-state or multi-state folders, depending on the spectroscopic probe being used [18-20]. This model is mainly derived from hydrogen exchange data. The main idea of this model is presented in **Figure 1**.

Second model, the more popular "folding funnel" model, is derived from statistical mechanics and concepts of polymer physics, rather than those of classical chemical dynamics – hence, is called the "new view" [21, 22]. This model represents the energy landscape of the protein folding pathway as an energy funnel as depicted in **Figure 2** [23]. At the top (rim) of the funnel is a heterogeneous mixture of rapidly-exchanging, high enthalpy, high entropy, polypeptide conformations in the unfolded denatured state ensemble (DSE). Polypeptides can explore funnel shaped potential energy surfaces downhill towards native state along several paths. [3]. Since the funnel is not smooth, the rugged nature of the funnel may lead to transiently populated various intermediate states [24]. Partially folded or misfolded states have tendency to aggregate because of exposed hydrophobic residues which are otherwise buried in native state [25]. The formation of these aggregates is prevented by the chaperone machinery in the cells but these highly ordered and thermodynamically stable aggregates accumulate under stress or when protein quality control fails [26]. Narrow bottom of the folding funnel indicates that there are few low-energy native like conformations and many more open unfolded structures [27,28]. This model has been widely agreed since it accommodates many of the ideas from previously mentioned models and also provides reasonable explanations for protein behavior both in vitro and in vivo.

Protein Denaturation

The conformation of a protein in which it is functional or active is called the "native state" of the protein. After a protein is synthesized on ribosome, a protein achieves its "native" conformation (out of millions & trillions possible conformation) on biological time-scale of few milliseconds. This is quite amazing and is difficult to explain how a protein achieves its native conformation at such a fast rate. Since starting from the primary structure of a protein is quite impractical given the number of possible conformations, protein folding is usually studied by how a protein unfolds or denatures from its native state. A protein has a certain pH, temperature, pressure and ionic concentration range where it remains in its native conformation. Any alteration in any of these parameters leads to loss of this native conformation. The first theory of protein denaturation was proposed by Hsien Wu in 1931 [29]. According to this, denatured state is viewed, as alteration of a highly compact and ordered structure into more or less open structure. The denaturation involves no change in the primary structure i.e. no effect on the covalent bonds linking the amino acids . The unfolding or denaturation is characterized by a

polypeptide chain becoming less compact, highly solvated and more flexible leading to increased intrinsic viscosity and partial or complete loss of secondary structural elements [30].

Protein denaturation can be brought about in different ways and the products of denaturation have been characterized by various techniques. Main modes of denaturation are (1) heat denaturation (3) organic solvents and solutes (4) inorganic salts, e.g., lithium chloride, lithium perchlorate, lithium bromide, calcium chloride, potassium thiocyanide and sodium bromide (5) detergents and (6) guanidinium chloride and urea. Though the denatured state of many proteins has been characterized using various biophysical techniques, the mechanism and the details of the chemistry underlying the mode of denaturation are still not clearly understood. Some methods employed for protein denaturation are discussed as under;

- ***Thermal Denaturation***

Temperature occupies a central and unique role as a perturbant of the equilibrium between different conformational species in macromolecules. Thermal denaturation or heat-induced denaturation is brought by heating a protein solution and following the change in observable physiochemical property as a function of temperature. Thermal denaturation may be reversible or irreversible. Reversibility is checked by regaining the native state if initial conditions are restored. Irreversibility is mainly due to aggregation or precipitation of protein. In some proteins, high temperature results in disulfide rupture or disulfide interchange (particularly at alkaline pH) [30], but usually only non-covalent interactions are affected by heat .

- ***Guanidinium Chloride (GdmCl) and Urea Denaturation***

Guanidinium chloride (GdmCl) and urea are the widely used protein denaturants and give the extensively unfolded state [30-32]. It is remarkable that a mechanistic understanding of how they affect protein structure is still elusive. The main difficulty arises from the fact that GdmCl and urea are weakly interacting molecules, and concentrations in the molar range are usually required to destabilize proteins [33,34]. It is still not clear whether the denaturant molecules modulate solution properties or interact with the protein directly by binding at specific sites which are otherwise occupied by H2O molecules [35]. If the latter is correct, it is still not clear what type of interaction (be it polar, hydrophobic, or van der Waals) is the driving force for denaturation, and whether the effect of the cosolvent on the polypeptide backbone or on the amino acid side chains is more important [36]. The molar concentrations at which chemical denaturants tend to operate make it certain that many cosolvent molecules will find themselves in close proximity to the protein chain. Thus, in the context in which chemical denaturation generally takes place, interaction between cosolvent and protein is a fact that must be accounted in the discussion of the process of denaturation [37]. It is also possible that some of these questions ultimately will have different explanations for urea than for GdmCl. With the advent of computer technology, molecular dynamics (MD) simulations have been carried out for both urea and GdmCl solutions. O'Brien et al. simulated both urea and GdmCl solutions with a model peptide and focused on the relative frequency of direct contacts between cosolvent molecules and polar atoms on the protein. They observed strong, direct associations between the guanidinium cation and charged or polar groups in both protein side chains and backbone, but did not observe similar associations for urea, and thus argued that direct electrostatic interaction is a driving force for denaturation mediated by GdmCl more strongly than for urea [38].

A long debate is going on the nature of the denatured states obtained by heat and guanidinium chloride (GdmCl) or urea denaturation. Work by Tanford and coworkers suggested that thermally denatured proteins have more residual structure than GdmCl-denatured proteins [30,39]. Cooperative loss of structure from thermally-denatured lysozyme, chymotrypsin and RNase A upon addition of GdmCl provides strong evidence that thermally denatured states contain residual structure [40]. NMR studies of thermally denatured barnase showed that some fraction of denatured ensembles contain residual, non random structure [41]. Singh et al. also have shown that the heat-denatured state is less unfolded than the GdmCl (or urea)-denatured state in case of lysozyme, RNase-A, α-lactalbumin, and α-chymotrypsinogen A [42]. Very recently, heat denatured state and GdmCl-danatured were also shown to be different in case of yeast iso-1-cytochrome c [43].

- ***Other Salt Denaturations***

Salts are known to affect the protein solubility, stability and biological activity[44]. Salts mainly affect the electrostatic and hydrophobic interactions. The effect of a salt depends on the concentration: salting in at lower concentration and salting out at

higher concentration. Salts mainly affect the electrostatic and hydrophobic interactions [45]. Salting out phenomena i.e. decreased solubility and aggregation of the protein at high salt concentration is mainly due to the effect of salt on the hydrophobic interactions [46]. Based on their ability to salt in or salt out proteins Hofmeister arranged them in a series known as Hofmesiter series [47,48]. Anions: SO_4^{2-} > HPO_4^{2-} > acetate > Cl^- > NO_3^- > ClO_3^- > I^- > ClO_4^- > SCN^-; Cations: NH_4^+ > K^+ > Na^+ > Mg^{2+} > Ca^{2+} > Gdn+. The mechanism of the Hofmeister series is not entirely clear. It has been argued that denaturation does not seem to result from changes in general water structure, instead more specific interactions between ions and proteins and ions and the water molecules directly contacting the proteins are important [49]. Inorganic salts induce conformational changes in proteins at room temperature. Kugimiya and Bigelow found that LiCl and $LiClO_4$ denatured states of lysozyme do possess some residual structures [50]. LiCl-denaturation in case of horse L94G mutant cytochrome and yeast iso-1-cytochrome c induces pre-molten globule formation [51, 52].

- ***pH Denaturation***

The dependence of protein stability on pH has been the focus of researchers for a long time. Linderstrom- Lang [53] was the first to explain the lower stability of proteins at extremes of pH. Extremes of pH far away from the isoelectric point favor denaturation because sensitive areas of the protein molecule acquire more charge causing internal repulsion or perhaps lose charges, which were previously involved in attractive forces holding protein together. In the native state, a number of acidic and basic amino acids are placed in environments that modify the pKa's of their side chains. In the denatured state the pKa,s of some or all of these residues return to values typical free amino acids. As a result, additional protons bind to the acidic denatured state and additional protons are lost from the alkaline denatured state. These protons must be lost (or retrieved) in order for the protein to refold and the free energy cost of doing so exceeds the intrinsic free energy stabilizing the native state when the pH is sufficiently low (or high).

Both activity and stability are pH dependent and very often pH optimum of activity is correlated with the pH optimum of stability and changes in pH are not unexpected for cellular function [54]. This suggests that other factors in addition to overall charge are important in determining the contribution of ionizable groups to the overall folding energy of globular proteins. Lysozyme and RNase A have been found to be stable at even pH 2.0 without any conformational change [55]. Acid or alkaline denaturation of proteins is mainly due to instability of the buried groups [56]. pH denaturation can induce a minor conformational change to nearly random coil conformation (at low ionic concentration) [57].

- ***Denaturation by Organic Solvents***

Organic solvents are known to perturb the protein structure. The effects of the water-miscible straight chain and branched alcohols and glycols on the native conformation of sperm whale myoglobin, cytochrome c, and a-chymotrypsinogen have been investigated by spectral, difference spectral, and circular dichroism methods [58-60]. Based on the midpoints of the denaturation transitions, it is concluded that the effectiveness of the alcohols as protein denaturants increases with increasing chain length. The glycols are found to be less effective than the corresponding alcohols [61]. The action of organic solvents on proteins is a function of their proton acceptor and proton donor tendency. Solvents such as dioxin, acetonitrile, dimethylformamide, pyridine and dimethylsulphoxide, which are good proton acceptors but weak proton donors, have a very weak tendency to disturb peptide hydrogen bonds [62]. The ability of alcohols to induce a-helical conformations in proteins was first noted in optical rotatory dispersion experiments by Tanford et al. on a-lactoglobulin [63]. Tamburro et al. [64] studied the effects of trifluoroethanol (TFE) on the conformations of the ribonuclease S-peptide; TFE was found to stabilize the small peptide in the same (a-helical) conformation that it adopts in native protein. Since then, alcohols have been used widely to examine the conformational (particularly helical) propensities of peptides [65-68] and to induce conformational changes in intact proteins [69-72]. TFE has also been shown to induce to induce β-turns, β hairpins and also β-strands[73]. It can also promote switching between different secondary structures, generally from a β-sheet to an a-helical structure [74].

Methods for Determination of Protein Stability

Protein stability is quantitatively described by the standard Gibbs energy change, ΔG_D^o, involved in unfolding the unique, three dimensional structure to randomly coiled polypeptide chains (N↔D).

This is also referred to as conformational stability of a protein. Studying of conformational stability can provide an insight into the understanding of the forces that determine the conformation of a protein and sometimes it may reveal additional features of a protein like existence of domains or the presence of stable folding intermediates [75]. Almost all the estimates of protein stability come from thermal denaturation and GdmCl or urea-induced denaturations and microcalorimetry.

- ***Differential Scanning Calorimetry (DSC)***

Differential scanning calorimetry is a direct, non-perturbing technique, developed in early 1960s to estimate protein stability. This technique measures the thermodynamic properties of a thermally induced transition and has been applied to variety of biological macromolecules such as lipids and proteins [76,77]. DSC endotherm gives direct estimate of constant-pressure heat capacity (ΔC_p), T_m (mid-point of thermal denaturation) and enthalpy change (ΔH_m) of a protein solution against a reference buffer. Thus DSC provides all the thermodynamic parameters of unfolding of a protein in a single experiment as shown in **Figure 3**.

DSC measures the excess heat capacity of a solution (C_p) of the molecule of interest as a function of temperature. The transition is recognized as a sharp endothermic peak centered at Tm and the maximum in C_p occurs directly at T_m. Integration of the C_p versus T curve yields the transition enthalpy (ΔH_m) and the shift in the baseline yields the ΔC_p (see **Figure 3**). DSC is the only method for the direct determination of ΔH_m. The value ΔH_m is calculated from the area under the transition [78]. DSC curve can be integrated incrementally to give a progress curve (C_p vs T), i.e. the proportion of the total heat absorbed as a function of temperature. Fitting of the progress curve will yield a van't Hoff enthalpy (ΔH_{vH}) which can be different from the calorimetric enthalpy, ΔH_m. The calorimetric enthalpy is the total enthalpy change including the contribution from all processes and determined independently of any model while the corresponding ΔH_{vH} assumes a simple two-state transition [79]. Comparison of ?Hm with ΔH_{vH} therefore allows one to assess whether the transition occurs as a two state or any intermediate is involved [80]. If $\Delta H_{vH} = \Delta Hm$ then the denaturation is considered to be well approximated by a two-state process. If $\Delta H_{vH} < \Delta H_m$ then most likely there is an unfolding intermediate (i.e. not a two-state process). If $\Delta H_{vH} > \Delta H_m$ then there is intermolecular association; the molecule may be a dimer or multimer or may be due to aggregation [78]. In DSC, ΔC_p is obtained by the difference between pre-transitional and post-transitional baselines of endotherm [81], though a good approach is to take several DSC scans in which Tm is perturbed as a result of change in pH. The slope plot of ΔH_m against corresponding T_m gives ΔC_p [54 82].

A concern regarding DSC studies is the concentration of the protein needed. Considerably, higher concentration of protein is needed, more than 1mg ml^{-1} and volume inbetween. 5 ml to 1 ml. This high concentration of protein may lead to difficulties arising from aggregation or intermolecular association of the denatured state, or possibly self-association of the native state. Accurate DSC studies thus require an assessment of the concentration dependence of the thermodynamics. Thus accurate determination of protein concentration is needed. However, sensitive commercial instruments are now available with higher sensitivity and quality data can be obtained from samples at $1/10^{th}$ the concentration previously required.

- *Equilibrium Method*

Protein stability is mainly discussed in terms of thermodynamic stability of a protein that unfolds and refolds rapidly reversibly with a two-state mechanism: N (native state)↔D (denatured state); the equilibrium between N and D is given by equilibrium constant KD, as, KD=[D]/[N]. The

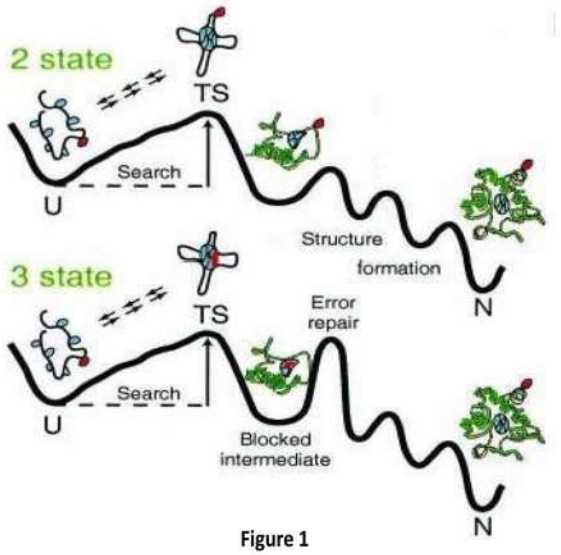

Fig. 1: A schematic energy profile representation of a two-state and three-state folder having hidden intermediates as described by the "Predetermined Pathway – Optional Error" method [20]. U represents the unfolded state, TS is the transition state and N is the native state. "Copyright (2001) National Academy of Sciences, U.S.A."

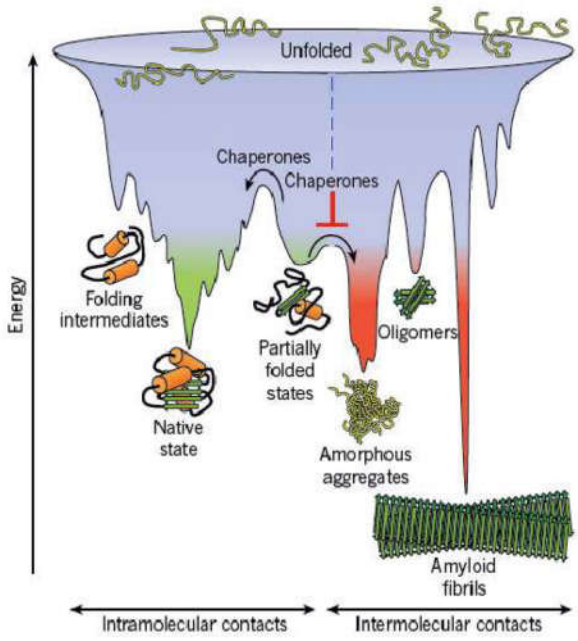

Fig. 2: Rugged "folding funnel" showing protein folding and aggregation. Reprinted by permission from Macmillan Publishers Ltd: [Nature] [23], copyright (2011).

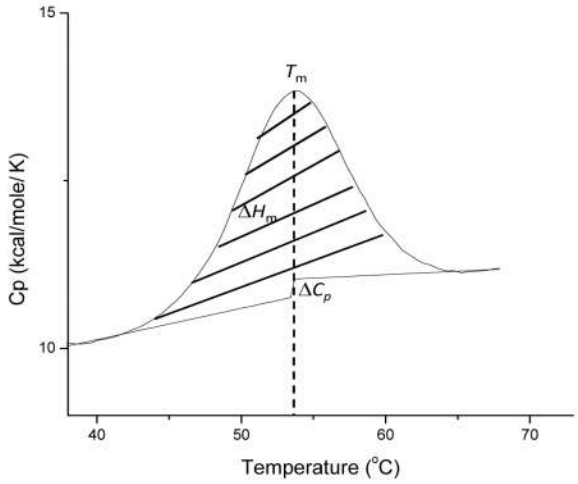

Fig. 3: DSC endotherm of yeast iso-1-cytochrome *c* at pH 6.0 in native buffer

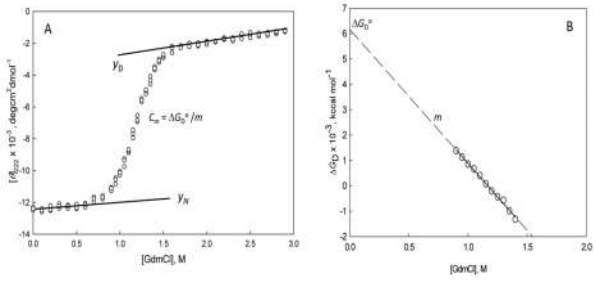

Fig. 4: A. GdmCl-induced denaturation curve of yeast iso-1-cytochrome *c* at pH 6.0 obtained by monitoring $[\varphi]_{222}$ Vs [GdmCl]. **B.** Plot of DG_D as a function of [GdmCl] and extended using least-squares analysis to zero concentration of denaturant to calculate DG_D^o. The slope of the line gives '*m*'.

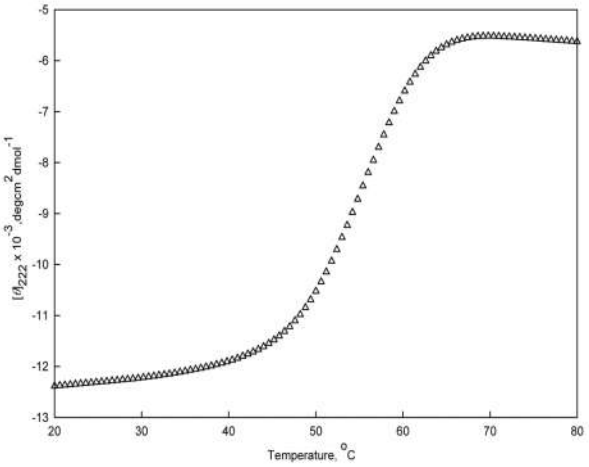

Fig. 5: Thermal denaturation curve of yeast iso-1-cytochrome *c* monitored by following change in $[\varphi]_{222}$ Vs temperature at pH 6.0.

difference in free energy between denatured and native state (stability) is give by $\Delta G_D^o = -RT \ln K_D$. More the ΔG_D^o, more stable is the protein to denaturation. Thus to estimate ΔG_D^o, we have to study the equilibrium between the N and the D state. The equilibrium can only be studied by perturbing this equilibrium using denaturants like GdmCl, urea or other solvents [83, 84] and heat. The method involves measuring of some optical property (at which N and D differ) as a function of denaturant concentration. This conformational transition is used to evaluate ΔG_D^o of a protein. Some of the methods used are;

Chemical Denaturant-Induced Denaturation

In these denaturation transition, a chemical denaturant is used to induce denaturation, mainly three procedures are used to evaluate ΔG_D^o from the analysis of conformational transitions; [85]. These are linear extrapolation method, denaturant binding model and transfer free energy model. Here, we will discuss the first method, i.e., linear extrapolation model since this is widely used model to evaluate ΔG_D^o of chemical-induced transition [39, 55, 83-88]. In this method, an observable spectral property is plotted as a function of denaturant concentration. The resulting plot, i.e., iso-thermal denaturation curve is sigmoidal (**Figure 4A**), signifying a denaturant concentration-induced cooperative transformation of N state to the D state. Assuming this represents only two states N and D and the denaturation process is reversible (to be checked), the equilibrium is put as;

N (Native Conformation)↔D (Denatured Conformation) (1)

For this process, one can calculate f_N (the fraction

of native protein molecules) and f_D (the fraction denatured protein molecules, where;

$$f_N + f_D = 1 \quad (2)$$

the value of any observed conformational property, y, is directly related to the fraction of the native and denatured protein

$$y = f_N y_N + f_D y_D \quad (3)$$

fD, the fraction of dentured protein can be calculated from

$$f_D = (y - y_N)/y_D - y_N) \quad (4)$$

where y is the observed optical property at the particular pH, temperature and denaturant concentration, while y_N and y_D are respectively the properties of the native and denatured states measured under the same experimental conditions in which *y* has been measured. The values of y_N and y_D for any point in the transition region are obtained by the intrapolation of the pre- and post-transition baselines, which is generally obtained by least-squares analysis [55] under the same experimental conditions in which y has been observed. Plots of f_D versus molar concentration of denaturant, at which they were calculated, gives the normalized transition curve. For each observed point within the transition region and equilibrium constant, KD can be calculated using the relation:

$$K_D = f_D/(1 - f_D) = (y - y_N) / (y_D - y) \quad (5)$$

The free energy change (ΔG_D) for folding unfolding reaction ($N \leftrightarrow D$) can be calculated using the relation;

$$\Delta GD = - RT \ln K_D \quad (6)$$

where R is the gas constant (1.987 calories/deg/mol) and T is the absolute temperature in Kelvin (K). The value of equilibrium constant, KD can be measured most accurately near the mid-point of denaturation curve, and the value of KD outside the range 0.1—1.0 ($0.1 \leq f_D \leq 0.9$) the error becomes substantial [30]. It has been observed that, the plot of -1.3 ≤ dGD (kcalmol-1) ≤ 1.3 versus [denaturant] is linear in the transition region. From this linear plot of ΔG_D values against the molar concentration of each denaturant, ΔGD^o can be estimated from the least-squares analysis according to the relation (**Figure 4B**) [86],

$$\Delta G_D = \Delta G_D^o - m\,[d] \quad (7)$$

where ΔG_D^o is the value of ΔG_D in the absence of denaturant and *m* is the slope of the line i.e., $(\partial \Delta G_D / \partial [d])_{T,P.}$ and [d] is molar denaturant concentration. The midpoint of transition curve, Cm is calculated from $C_m = \Delta G_D^o / m$.

Alternatively, the method which is commonly used nowadays, the entire equilibrium transition curve obtained by measuring any observable property *(y)* as a function of denaturant concentration *(d)* can be fitted to a two-state unfolding model with the help of Sigma Plot, v.10 or Origin softwares to analyze the values for ΔG_D^o, m_d and C_m using the relation [89]

$$y = y_N(d) + y_D(d) * \mathrm{Exp}[-(\Delta G_D^o + m_d[d])/RT]/(1 + \mathrm{Exp}[-(\Delta G_D^o + m_d[d])/RT]) \quad (8)$$

where *y* is the observed optical property at [d], the molar concentration of any denaturant, $y_N(d)$ and $y_D(d)$ are optical properties of the native and denatured protein molecules under the same experimental conditions in which *y* was measured, ΔG_D^o is the value of the Gibbs energy change in the absence of the denaturant, m_d is the slope $(\partial \Delta G_D / \partial [d])$, *R* is the universal gas constant, and *T* is the temperature in Kelvin. It should be noted that the analysis of each equilibrium transition curve was done assuming that unfolding is a two-state process and reversible and dependencies of $y_N(d)$ and $y_D(d)$ are linear *(i.e.,* $y_N(d) = a_N + b_N[d]$ and $y_D(d) = a_D + b_D[d]$, where a and b are [d]-independent parameters, and subscripts *N* and *D* represent these parameters for the native and denatured protein molecules, respectively. Equation (9), fits the native state $a_N + b_N[d]$ and denatured state $y_D(d) = a_D + b_D[d]$ baselines as well as the unfolding transition region simultaneously, assuming a linear dependence of free energy on denaturant concentration [d].

Heat-Induced Denaturation

Protein stability in terms of Gibbs free energy change (ΔG_D^o) is determined from the measurements of reversible heat-induced denaturation of proteins using calorimetric or equilibrium methods [90]. DSC is a direct method which gives the thermodynamic parameters associated with the endotherm. While in the equilibrium method, transition is followed by measuring a suitable structural property as a function of temperature and analysis of the resulting transition curve (sigmoidal) for the equilibrium constant (KD). Hence this method is also called equilibrium method.

Since, temperature is a thermodynamic property; temperature dependence of equilibrium provides an access to the enthalpy (ΔH), entropy (ΔS), and heat capacity ΔCp) components of the Gibbs free energy (ΔG_D). These parameters can be used to establish the relative stability of different proteins or mutants or to estimate the size of the cooperative units of different proteins [91]. When the protein unfolds, the buried

non-polar side chains come in contact with water. In order to accommodate these side chains, cages of water molecules surround them so that the extent of hydrogen bonding is increased. The heat capacity of unfolded protein is greater than that of folded protein because now these cages must be melted in order to raise the temperature of the protein solution. Thus the same effect that gives rise to hydrophobic interaction also gives rise to larger ΔC_p. Privalov studied the effect of melting temperature (T_m, the midpoint of transition) and enthalpy of denaturation of a number of proteins. It was observed that the plots of enthalpy versus the corresponding melting temperature were linear for all proteins studied. It is concluded that in these experiments ΔH_m, the value of ΔH at T_m is a direct function of temperature. ΔH_m = Constant + $\Delta C_p T_m$, where T_m is the temperature at which $\Delta G_D = 0$ and ΔH_m is the value of ΔH at T_m. The apparent dependence of ΔH_m on pH results from the fact that pH changes T_m; the temperature at which ΔH is evaluated [91]. The heat capacity is the temperature derivative of basic thermodynamic function, the enthalpy. Therefore denaturation heat capacity increment determines the temperature dependence of the enthalpy and hence of the entropy on temperature i.e., the parameter that determines the native state stability.

The two-state heat-induced denaturation curve is also sigmoidal (Figure 5), which is used to derive the thermodynamic parameters T_m (midpoint of denaturation), ΔH_m (van't Hoff enthalpy change at Tm) and ΔCp (constant pressure heat capacity change), associated with it. The entire data points obatained are fitted to the equation (10) or (11) using softwares like Origin or Sigmaplot to obtain the thermodynamic paramaters [89, 92].

$$y(T) = \frac{y_N(T) + y_D(T) Exp\left[\frac{-\Delta H_m^{van}}{R}\left(\frac{1}{T} \quad \frac{1}{T_m}\right)\right]}{1 + Exp\left[\frac{\Delta H_m^{van}}{R}\left(\frac{1}{T} \quad \frac{1}{T_m}\right)\right]} \quad (10)$$

$$y_{obs} = \frac{(y_n + m_n T) + (y_d + m_d T) \quad \exp\{([\quad C_p \quad ((T_m/T \quad 1) + \ln(T/T_m))] \quad [\quad H_m \quad (T_m/T \quad 1)])/R\}}{1 + \exp\{([\quad C_p \quad ((T_m/T \quad 1) + \ln(T/T_m))] \quad [\quad H_m \quad (T_m/T \quad 1)])/R\}} \quad (11)$$

Where, yobs is the experimentally observed optical property of the protein at temperature T (K), yn and yd are the optical properties of the native (N) and denatured (D) molecules at same temperature, and R is universal gas constant. It should be noted these equation assume that the heat-induced denaturation is a two-state process.

The thermodyanamic values thus obtained can be put in Gibbs–Helmholtz equation (12), to calculate the Gibbs free energy change (ΔG_D^o)

$$\Delta G_D(T) = \Delta H_m\left(\frac{T_m \quad T}{T_m}\right) \quad \Delta C_p\left[(T_m \quad T) + T ln\left(\frac{T}{T_m}\right)\right] \quad (12)$$

The dependencies of pre- and post-transition baselines in a thermally induced transition curve can be analyzed by three different methods; like linear model, mixed model and parabolic model. It has been shown that if analysis is carried out assuming that the temperature dependence of pre- and post-transition baselines is described by a parabolic function, there exists an excellent agreement between ΔH_m values of all proteins obtained from equilibrium and calorimetric methods [93,94].

There are several methods for the determination of ΔC_p from conformational transition curves. The earlier method involves the estimation of the values of ΔH_m^{van} as a function of temperature from vant's Hoff analysis of thermal denaturation curves measured at different pH values or chemical denaturation [95]. A second approach involves the measurement of ?Hmvan and Tm from thermal-transition curves obtained at different pH values and estimation of ΔC_p from plot of ΔH_m^{van} versus Tm [82]. Another approach developed by Swint and Robertson [96], ΔC_p is determined from the fit of the entire transition data (y,T) in equation that includes the temperature-dependence of ΔH_D (equation (3) in Swint and Robertson. Singh et al. [42] developed a non-calorimetric method for measurement of ΔC_p. This method involves the use of thermodynamic data obtained from isothermal GdmCl (or urea)-induced denaturation and heat-induced denaturation in the presence of the chemical denaturant concentration at which significant concentrations of both native and denatured states exist. The method involves the determination of ΔH_m (0) (enthalpy change in absence of denaturant), T_m (0) (mid-point of denaturation in the absence of the denaturant) and ΔG_D^o (Gibbs energy change in absence of denaturant). ΔH_m and Tm can be obtained from the measurement of effects of temperature on the equilibrium constant, KD. For this denaturation curves of the protein are measured in the presence of transition region concentration of the chemical denaturant by following changes in optical property as a function of temperature.

Conclusion

Predicting 3-D structure of a protein from its sequence is still a fascinating field of modern biology and biophysics and at the same how does a protein fold, the pathway involved and the thermodynamic aspects has remained and is still an area which has puzzled scientists over the decades. Still at present, there are unanswered questions to be answered and puzzles to be solved like, predicting the tendency of a protein to aggregate or to misfold, explaining the reason why cellular proteome doesn't precipitate because of so much crowding, preventing and intervening in misfolding diseases, function of intrinsically disordered proteins (IDP's) and so on. Thus, the future of proteins sciences is as persuasive as its past.

Conflict of Interest

The authors declare no conflict of interest.

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