

■ What Is the Role of Thermodynamics on Protein Stability?

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Abstract The most challenging and emerging field of biotechnology is the tailoring of proteins to attain the desired characteristic properties. In order to increase the stability of proteins and to study the function of proteins, the mechanism by which proteins fold and unfold should be known. It has been debated for a long time how exactly the linear form of a protein is converted into a stable 3-dimensional structure. The literature showed that many theories support the fact that *protein folding is a thermodynamically controlled process*. It is also possible to predict the mechanism of protein deactivation and stability to an extent from thermodynamic studies. This article reviewed various theories that have been proposed to explain the process of protein folding after its biosynthesis in ribosomes. The theories of the determination of the thermodynamic properties and the interpretation of thermodynamic data of protein stability are also discussed in this article.

Keywords: protein synthesis, stability, protein folding, thermodynamics, entropy, enthalpy

Importance of Thermodynamics on Protein Stability

Thermodynamics is a branch of science dealing with the affiliation between heat and work. The advent of thermodynamics helped in understanding many systems, which in turn had a great impact in chemical engineering. Kinetic and thermodynamic aspects became primary criteria in design of reactors and mass and heat transfer equipment. Till 1950, thermodynamics was not prominent area in biotechnology. The probable reason is the lack of data with respect to biomolecular properties, thermodynamic equilibrium positions, energy efficiency relations and the complexity of biological systems [1]. For this reason, most of the biotechnological processes were not optimized as chemical processes. However, recent developments in instrumentation and experimental techniques had increased the application of thermodynamics in various fields of biotechnology (Fig. 1).

Proteins, polymers of different amino acids joined together by peptide bonds, serve many important physiological functions and have unique structural properties of proteins, especially stability and how to improve these properties is of major concern to biochemists in order to study important mechanisms. The central dogma of molecular biology is the synthesis of proteins from DNA showing the relationship between DNA and protein synthesis. In the last decade, various theories were proposed on the origin of genetic code, protein synthesis, stability, and nucleic acid replication [2-6]. Alberti [6] discussed the problem, various theories and models con-

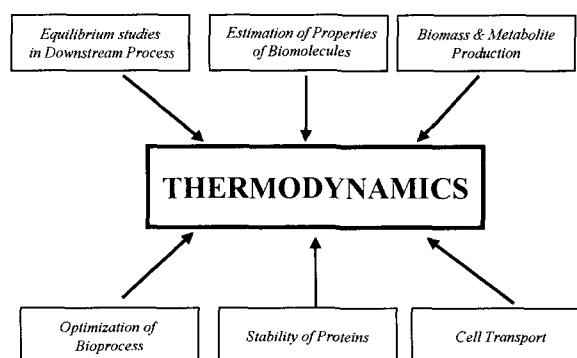


Fig. 1. The applications of thermodynamics in various fields of biotechnology.

cerning the complex problem of how an isolated genetic code could have developed before the appearance of protein synthesis. It has also been reported that structural and thermodynamic determinants of the interaction of nucleic acids with proteins helps in understanding the origin of genetic code and protein synthesis. Recent studies showed that Alzheimer's disease, transmissible spongiform encephalopathies, haemolytic anaemia and certain important diseases arises due to protein conformational disorders suggesting that knowledge on protein folding and unfolding is very important [7,8].

From the early 80's extensive research has been carried out on the relationship between structural conformation of proteins and their stability. A challenging and rapidly emerging field of biotechnology is the tailoring of proteins to carryout unique functions at different physiological and process conditions. The design of

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novel proteins requires a perfect understanding of protein folding and structure-activity relationships. Protein folding is of particular concern in the expression of recombinant proteins or production of industrial biocatalysts where the enzymes are often inactive due to misfolding. Knowledge of kinetics of protein folding and unfolding would significantly help in improving many processes and provide valuable insight into the function relationships of proteins. Another important point to be considered is the stability of biocatalysts. The deactivation of proteins (biocatalysts) is found to be one of the major constraints in the rapid development of biotechnological processes. If one can increase the stability of proteins to some extent, it would be instrumental in the commercialization of many processes [9,10]. In order to understand and get accurate information of protein folding and unfolding it is necessary to have a clear understanding of thermodynamics because thermodynamic properties (enthalpy, entropy and free energy) are helpful in understanding the protein stability.

Protein Stability *in vivo*

Proteins are synthesized in ribosomes within cells in the form of a linear polypeptide chain. Fig. 2 shows in a simplified way the different stages in the synthesis of a stable protein. The genetic information stored in DNA is transcribed to RNA and translated to linear form (primary structure). The linear form must be converted into a stable 3-dimensional structure. An important factor to be considered during protein folding is whether the process is thermodynamically or kinetically controlled. Are there any intermediates formed during folding of proteins to a stable structure? [11]. It has been reported that the information required for the proper folding of the protein is available in the linear polypeptide chain [12-14]. However, the formation of the 3-D structure of proteins can be affected by miscoding (error in protein synthesis) and/or misfolding (error in protein folding) [12].

The discovery of chaperones, a class of proteins that helps in proper folding of proteins *in vivo* had a significant impact on the existing concepts of protein folding. It has been reported that two classes of chaperones are involved in protein folding. Class I chaperone binds to hydrophobic regions, thereby preventing aggregation and the unfolded protein molecule is transported to various organelles. Inside the organelles class II chaperone helps in correct folding with the aid of various bonds [15-18]. It has also been stated that proteins tend to fold improperly when cells undergo stress, which triggers the synthesis of chaperones [14]. Certain specific peptide sequence in the linear form is necessary for proper folding and it is called "intramolecular chaperones" which could be cleaved by cellular proteases and proper folding is achieved when these peptides are added. With the aid of genetic, biochemical and cytological approaches, it has been shown that the unfolded protein response is simply a regulatory platform for the endoplasmic reticulum chaperones [19]. It is clearly

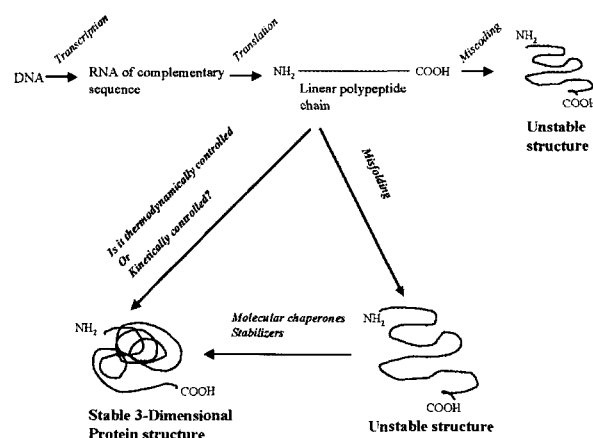


Fig. 2. The different steps involved in synthesis of stable protein.

understood now that the unfolded protein response regulates various genes that affect the cellular physiology. Recent studies demonstrate that when endoplasmic reticulum is under stress, eIF2, a factor that is essential for initiating the translation process will be inactivated by kinase [20]. Insulin producing β cells in the pancreas is destroyed in the absence of this kinase activity. This study showed that glucose metabolism and translations are related with each other. Rizzitello and coworkers showed the genetic evidence for the existence of parallel pathways of chaperone activity in the periplasm of *Escherichia coli* [21]. The loss of either pathway has minimal effect on the cell, while the loss of both pathways results in synthetic phenotypes.

Some chaperones are involved in the formation of disulfide bonds that enhance the stability of proteins. For example, the denatured D-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was assisted by chaperone, protein disulfide isomerase (PDI) [22]. The binding of GAPDH folding intermediates to PDI was favored by large enthalpy decrease with large unfavorable entropy reduction. It has also been explained that the large negative heat capacity is due to hydrophobic interaction. Another molecular chaperone DnaK (~ 70 kDa in *E. coli*) helps in improving the stability of many proteins by peptide-protein interactions. Kasper and coworkers developed a method to determine the interaction energy, which is contributed by nonpolar, electrostatic and entropic contributions [23]. It has been reported that the protein-peptide interaction was mainly controlled by nonpolar interactions for their model system and such models can be used to test various protein-peptide interactions. The activation enthalpy barrier for protein-peptide (DnaK-peptide) interactions can be reduced with the addition of ATP [24]. The peptide used for this purpose was fVSV13 (490-502 amino acids of vesicular stomatitis virus glycoprotein). The results showed that the activation entropy controls the kinetics of peptide binding to DnaK.

Protein misfolding and aggregation can initiate apop-

tosis by proteasomal inhibition and induction of the *N*-terminal kinase dependent pathway [25]. Few reports showed an increase in constitutive levels of chaperones in aging, enabling proper folding of proteins, which in turn increased the life of cells [25-27]. The mechanism by which chaperones prevent membrane damage, RNA misfolding and increase in cell longevity is not clearly known. Further research on this aspect will be highly helpful in determining the critical factors that are essential for proper folding of amino acids to yield a stable protein after synthesis.

How do Proteins Fold to Stable 3-Dimensional Structures?

Many theories on protein folding and stability have been proposed based on theoretical and experimental studies. As described earlier in Fig. 2, the last step was unclear whether protein folding is controlled by thermodynamics or kinetics. It is important to note that the linear polypeptide chain is folded to a stable 3-D structure in a very short time. Considering an example, if a residue has 5 different conformations, then it requires 5^{200} conformational changes for a chain of 200 residues to obtain a stable configuration. Since the time taken for the polypeptide to form stable structure that has minimum free energy is very short, it is not possible for the protein to undergo so many conformational changes within a short time. To explain this, various theories involved in the protein folding are discussed briefly in this section.

Levinthal proposed a theory later called the framework model, stating that the polypeptide chain, does not undergo all possible conformations and that could happen only if the protein folding occurs in a stage wise process suggesting that the process is kinetically controlled [12, 28]. The framework model proposes that secondary structure is formed during the early stages of protein folding. Levinthal paradox can be explained by assuming the formation of simultaneous structured nuclei on polypeptide chains that initiate protein folding, thus avoiding to go through all possible conformations. In 1976, the diffusion collision model was proposed by Karplus and Weaver and was later verified experimentally [29,30]. They proposed that folding occurs via several diffusion-collision steps. By this model, nucleation occurs at different regions of polypeptide chain forming microstructures, which diffuse and coalesce to form substructures with the native conformation. Based on 3-dimensional structure, Wetlaufer proposed the modular model in which he considered the domains as folding units [31]. It has also been suggested that subdomains might also refold to form structural modules as folding intermediates to yield the native protein. Kauzmann suggested that an important factor, which has significant effect on protein stability, is the hydrophobic effect [32]. Based on this, the hydrophobic collapse model was proposed by Dill stating that the initial step in folding is collapse, which is followed by the formation of secondary structure [33].

Based on experimental observations, Ptitsyn proposed that protein folding has three important steps. The initial step is the formation of pronounced secondary structure and occurs very rapidly ($\sim 0.01s$) followed by the formation of the molten globule ($\sim 1s$). The final step is the formation of native protein structure that is a slow step ($\sim 500-2,500s$) [12].

Wolynes and co-workers proposed the folding funnel theory, which could overcome Levinthal's theory accounting for both kinetic and thermodynamic aspects during folding [34,35]. They proposed that the polypeptide chain navigates through a complicated energy landscape. During the process of navigation the protein molecule explores different possible configurations. The width of the folding funnel represents entropy and the depth represents enthalpy. The width of the funnel decreases as the protein molecule navigates indicating the decrease in entropy. The entropy is minimum at the bottom of the funnel thereby reducing the number of conformations. The rate of folding is proportional to the slope of the folding funnel. The authors also stated that the compensation of entropy by enthalpy indicated that proteins behave like fluids at some critical point. It is now widely accepted that 3-dimensional structures of proteins are thermodynamically controlled that can be attained through formation of intermediates (different configurations) which is kinetically governed [11]. It has also been reported that some accessory proteins accelerate the process of protein folding [36]. Another interesting aspect is the effect of glycosylation on protein folding. Glycosylation increases the stability of protein molecule but it does not affect the folding pathway [37]. The analysis of the theories of protein folding clearly indicated that the formation of stable protein structure after synthesis is a thermodynamically controlled process. These theories suggested that the thermodynamic study of protein is important in understanding the complex process of protein folding.

Importance of Thermodynamic Studies

The major drawback in expressing recombinant proteins in suitable hosts is that the protein molecules often form inclusion bodies. Recent various methods have been developed for refolding the inclusion bodies. Thermodynamic studies can be used as one of the tool to predict how the protein molecule changes its conformation under various environmental conditions. In other words, the values of thermodynamic parameters are helpful in analyzing the stability of proteins. In addition, protein molecules are deactivated when exposed to different environmental conditions. Joly proposed that the deactivation phenomenon could be considered as a process in which secondary or tertiary structures change without the breakage of covalent bonds [38]. The protein molecule can deactivate either reversibly or irreversibly. The critical factor to be assessed is how much activity is lost and how much can be improved over time. This aspect is particularly important for both industrial and biologically important proteins. This problem could

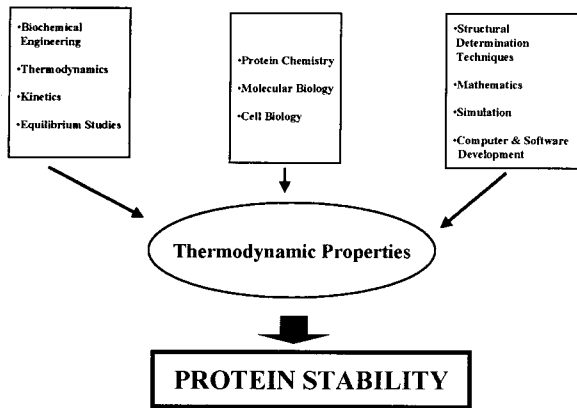


Fig. 3. Integrating various aspects to predict protein stability efficiently.

be tackled by thermodynamic studies of protein molecules and the requirements to conduct thermodynamic studies are given in Fig. 3. The theory involved in the determination of thermodynamic properties is discussed in the following section.

Theory

The enthalpy of system (H) is correlated to internal energy (U) by the following relationship

$$H = U + PV \quad (1)$$

where P and V are the pressure and volume of the system

$$\text{By the first law of thermodynamics } dU = dq - dw \quad (2)$$

where dq and dw represents the differential change in heat and work done by the system combining with equation 2, equation 1 becomes

$$dH = dq - vdp \quad (3)$$

Since most of the proteins deactivations occur at constant pressure, the equation 3 becomes

$$dH = dq \quad (4)$$

equation 4 implies that the heat absorbed by the process at constant pressure is the enthalpy of the system. When the protein folds/unfolds from its stable structure, entropy, the randomness of the system changes which can be expressed in terms of specific heat of protein molecule

$$\Delta S^\circ(T) = C_p \ln(T_2/T_1) \quad (5)$$

where C_p is the specific heat capacity at constant pressure, T_1 & T_2 refer to the initial and final absolute temperatures.

The Gibbs free energy function is a composite quantity,

defined as a trade-off of $\Delta H^\circ(T)$ and $T\Delta S^\circ(T)$ terms. The Gibbs free energy function may be expressed as:

$$\Delta G^\circ(T) = \Delta H^\circ(T) - T\Delta S^\circ(T) \quad (6)$$

with proper substitution of the Kirchoff's law

$$\Delta H^\circ(T) = \Delta H^\circ(T_0) + \int \Delta C_p^\circ dT \quad (7)$$

$$\Delta G^\circ(T) = \Delta H^\circ(T_0) - \int \Delta C_p^\circ dT - T \int (C_p^\circ/T) dT \quad (8)$$

$\Delta G^\circ(T)$ displays an interesting variety of behavior patterns as the temperature changes. $\Delta G^\circ(T)$ can change the sign (K_{eq} from <1 to $K_{eq} >1$ or vice versa) only if $\Delta H^\circ(T)$ and $\Delta S^\circ(T)$ remain of the same sign. That is to say:

- if ΔH° is (+) and ΔS° (+) then ΔG° goes from (+), unfavorable to (-) favorable (from <1 to $K_{eq} >1$)
- if ΔH° is (-) and ΔS° (-) then ΔG° goes from (-), favorable to (+) unfavorable (from >1 to $K_{eq} <1$).

The Gibbs energy can be determined from van't Hoff equation

$$\Delta G^\circ(T) = \Delta H^\circ(T) - T\Delta S^\circ(T) = -RT \ln(K_{eq}) \quad (9)$$

where K_{eq} is equilibrium constant, $\Delta G^\circ(T)$ is absolute free energy, $\Delta H^\circ(T)$ absolute enthalpy and $\Delta S^\circ(T)$ is absolute entropy.

So far, the thermodynamic properties discussed are absolute properties, i.e., change in property (enthalpy, entropy, specific heat, free energy) of a system when taken from absolute zero to a particular temperature. For protein systems, these properties are always with reference to standard state (stable state). Hence, it should be carefully noted that the change in thermodynamic properties during folding and unfolding refer to relative properties or changes in thermodynamic properties with respect to standard state.

Consider the scheme, $P_s \rightarrow P_{den}$ where P_s is protein at stable configuration and P_{den} is protein at denaturated state or unfolded state. This model is known as single step two-stage theory and is most commonly used for many proteins. This theory assumes that there are no intermediates formed during unfolding. This is treated as obeying first order kinetics.

$$-(dP_s / dt) = k_d P_s \quad (10)$$

where k_d is the deactivation rate constant

Integrating and rearranging the above equation gives

$$\ln(P/P_s) = -k_d t \quad (11)$$

k_d can be calculated from the plot of $\ln(P/P_s)$ vs. t . The enthalpy and entropy change during deactivation can be estimated by the use of absolute reaction states [39]. The deactivation rate constant can be expressed in terms of enthalpy and entropy by the following equation

$$k_d = (\kappa T/h) \exp(\Delta S_{den}/R) \exp(-\Delta H_{den}/RT) \quad (12)$$

where κ is Boltzmann's constant and h is Planck's constant

The plot of $\ln(k_d/T)$ vs. $1/T$ gives slope of $(-\Delta H_{den}/R)$ and intercept of $\ln(\kappa/h) + (\Delta S_{den}/R)$ from which the enthalpy and entropy values can be calculated. Another thermodynamic parameter, which is often used to calculate deactivation, is the Arrhenius energy. By Arrhenius law

$$k_d = A \exp(-E/RT) \quad (13)$$

where E is Arrhenius energy and A is Arrhenius constant or frequency factor. The values of E and A can be calculated from the slope and intercept of plot of $\ln(k_d)$ vs. $1/T$. Apart from this, various different models for denaturation are explained in detail by Sadana [9].

Interpretation of Protein Stability from Thermodynamic Parameters

The thermodynamic parameters estimated from the above equations are highly helpful in predicting protein stability. Entropy in particular can be considered the most useful parameter in understanding the stability of proteins because when a protein molecule is deactivated the randomness of the system increases which is a direct measure of entropy. A large number of reports are available on the role of thermodynamics in protein stability. Some of the important reports are discussed below.

The thermodynamic stability of proteins can be studied by determining the thermodynamic parameters. The heat capacity and absorbance data for plastocyanin, a single strand protein (~10.5 kDa) that plays an integral part of electron transport in plant photosynthesis showed irreversible and kinetically controlled deactivation mechanism in aqueous environment [40]. This protein has a central copper atom and surrounded by two nitrogen and sulfur bonds resulting an unusual tetrahedron structure. A structural change of copper environment was observed during denaturation and the lower stability of plastocyanin has been concluded due to structural factors associated with the native protein based on thermodynamic data. Reilly and co-workers studied the stability of thermosensitive glucoamylase (GA) in both wild and mutant strains [41-43]. It has been found that wild-type GA expressed in *Saccharomyces cerevisiae* was more glycosylated and more stable than the native *Aspergillus awamori* GA. The free energy of inactivation of mutants was found to be less than wild type GA indicating that mutants are less stable than the wild-type enzyme [41]. A 2.5 fold decrease in the first-order thermal deactivation rate constant of mutant GA below the wild type has been observed at most widely used industrial conditions [42]. This is in agreement with the enthalpy and entropy values of deactivation. This was achieved by single Asn182-Ala mutation by site-directed mutagenesis. They also improved the stability of GA by replacing the helical glycine residues and found that nearly 3-fold increase in stability has been achieved by mutating Gly 137 residue [43].

Renaud *et al.* studied the inactivation and substrate binding of human cytochrome P-450 3A4 that was expressed in yeast [44]. The thermodynamic parameters were estimated by the principal component analysis technique and were compared with the values of cytochrome P-450 2B4. The enthalpy and entropy change for spin transition of substrate free form and for substrate binding to low-spin were found to be similar for the two cytochromes. But the spin transition of the substrate complex of 3A4 has lower values than that of 2B4. They concluded that this might be because substrate binding might affect haem-protein interactions in 3A4 differently from that for 2B4. Interestingly, the entropy values were found to be negative for both cytochromes during substrate binding and in free state (at both high and low spin states). Interestingly, the entropy change for substrate binding (for both high and low spin state) was found to be negative and the significance for negative entropy was not explained. The measurement of entropy change during unfolding of protein molecule is very much helpful in enhancing the thermostability of proteins of known 3-dimensional structure. This can be achieved by selective amino acid substitutions, which decreases the configurational entropy change of unfolding thereby increases the stability of the protein molecule [45]. The authors introduced Xaa--- Pro and Gly--- Xaa substitutions in phage T4 lysozyme. These substitutions stabilize the protein toward reversible and irreversible denaturation at physiological pH and it has no effect on enzyme activity. They also showed that stabilization of protein could be achieved by forming disulfide bonds [46]. The formation of disulfide bonds also decreases the configurational entropy change of the unfolded polypeptide.

The stability of activated thrombin active fibrinolysis inhibitor (TAFIa) was compared with plasma derived TAFI (pTAFI) and recombinant TAFI (rTAFI) [47]. The enthalpy (~45 kcal/mol) and entropy (~80 cal/mol/K) of inactivation was determined by transition theory. They concluded that inactivation was not enthalpically favored, because more energy was required to break noncovalent bonds. This conclusion was made based on the interpretation of thermodynamic parameters, however the structural basis for instability of TAFIa is not clearly understood. These studies showed that determining entropy change is highly helpful in understanding the mechanism of inactivation and protein stability. Interestingly, negative entropy has been observed during deactivation of many proteins. Masullo *et al.* produced two truncated forms of *Sulfolobus solfataricus* elongation factor 1 α (SsEF-1 α), one deprived of C and M domain Ss(G)EF-1 α and the other deprived of C domain Ss(GM)EF-1 α , expressed in *E. coli* [48]. The thermal inactivation studies showed that both the truncated forms are less stable than the intact protein due to deletion of domains. This was also proved by values of entropy, enthalpy and activation energy of inactivation by transition state theory and Arrhenius theory. Interestingly, the entropy values were found negative and the authors did not explain the significance of negative en-

tropy.

Similar negative entropies were observed during pH and thermal deactivation of pectolytic enzymes produced from *Aspergillus niger* [49,50]. The deactivation process was modelled as first order kinetics and the deactivation rate constant was found to be minimum at pH 2.2 and 23°C respectively for polymethylgalacturonase, 4.8 and 28°C respectively for polygalacturonase and 3.9 and 29°C respectively for pectinase. Thermodynamic parameters like ΔG^* , ΔH^* , ΔS^* , and activation energies were evaluated for crude and partially purified enzymes. It was found that entropy values are negative for all the three enzymes. The negative entropy values were also found for *Trichoderma harzianum* chitinase [51]. The stability of cytochrome C decreases as it changes from oxidized state to reduced state [52]. The authors found that entropy change for both wild type and Tyr67Phe variant was found to be -43 and -53 J/mol.K respectively and the enthalpy values were identical. It has also been reported that the entropy values provides information regarding the relative degree of solvation, likely the degree of compactness.

By second law of thermodynamics, the entropy of the system is positive but the negative values of entropy are often encountered in biological systems, especially in the case of proteins. Interpretation in mechanistic terms of these thermodynamic parameters is very complex. Foster suggested that two major complex effects contribute to the numerical values of these parameters [53]. The two complex effects are: (i) solvent effect- arising from surrounding water molecules as a result of burying/exposing hydrophobic groups and (ii) structural effect- the conformational changes occurring in the enzyme molecule (folding/unfolding). It has also been reported the negative entropies changes during inactivation are consistent with the compactation of the enzyme molecule, but equally such changes could arise from the formation of charged particles and the associated gain and the ordering of solvent molecules [53]. Since water is the environment in which the linear polypeptide chain exists and interacts, the interaction between the non-polar amino acids with water is very important in forming stable structure. This effect is called the hydrophobic effect. Hydrophobic interactions result in the burial of the hydrophobic residues in the core of the protein. Water tends to form ordered clathrate structures around the non-polar molecule and this leads to a decrease in entropy (negative entropy). The sequestering of non-polar groups is energetically favorable from both entropic and enthalpic point of view. This is not true at higher temperatures, where cages are weaker than bulk water, and the entropy change will be positive (unfavorable). Since, the temperature dependence of entropy and enthalpy are not the same, there is some temperature at which the hydrophobic effect is strongest, and the effect decreases at temperatures above and below this temperature. Fernandez reported that few hydrogen bonds in native protein structure are poorly protected from water, which can be protected by desolvation shell [54]. An approach by which solvent-structuring moiety of a binding partner

should contribute significantly to enhance the stability.

The thermal stability of proteins can be increased in the presence of monomeric amino acids. Taneja and Ahmad worked on the thermal stability of cytochrome c in the presence of various amino acids [55,56]. Based on the Gibb's free energy and entropy values the effect of amino acids was studied. It was found that arginine and histidine affect the stability of proteins while valine and less hydrophobic amino acids stabilize the protein. Proteins can also be denatured by decreasing the temperature known as cold denaturation. The decrease in the strength of the hydrophobic effect with decreasing temperatures is probably the major cause of cold-denaturation in proteins. Thermodynamic studies were applied to cold denaturation of proteins and many studies have been carried out on important proteins like ubiquitin and *Streptomyces* subtilisin inhibitor (SSI) [57-59]. Thermodynamic studies have also been used in understanding the stability of many proteins by binding with metal ions like Ca^{2+} and Sr^{2+} [60,61].

It has been reported that increasing the salt concentration can increase the stability of the protein [62]. The free energy of unfolding of barstar increased in the presence of 1 M KCl and MgCl_2 . The increase in free energy was found to increase with increase in salt concentration, which supported the hypothesis, that stabilization of native state occurs primarily through a Hofmeister effect. A number of organic solvents of different physico-chemical characteristics were found to stabilize the protein. The non-polar solvents utilized the ability to enhance thermal stability of proteins [63-65]. Various reports are available on the effect of various co-solvents such as, n-dodecane, dimethylformamide, trifluoroethanol and detergents on the stability of various proteins and peptides [66-69]. The different methods by which the stability of proteins can be increased include increase in the hydrophobic interactions, cross-linking and prevention of irreversible processes like aggregation and incorrect folding. Improving the properties of industrial enzymes like cellulases, pectinases, amylases etc., is very important from an economic point of view. It is possible to obtain these thermostable proteins from thermophiles, which are reviewed by many researchers [70,71]. These reviews also suggested that thermodynamic studies are highly helpful in understanding the structure and improvement of the functional stability of many proteins.

Different Models Used to Predict the Protein Stability

Since proteins are used for different operations in many unusual environments, the stability of protein is important. According to phase equilibrium model, protein was modelled as hydrophobic phase and the thermodynamic parameters of unfolding as average properties of the protein phase [72]. This thermodynamic approach does not require any sequence information or crystallographic structures but it requires only the amino acid composition of independently folding domains. This model was especially used to predict the behavior of high temperature enzymes. The authors stated that

this model successfully predicted the stability of proteins under various environmental conditions. The main advantage of this model is that it requires only information about amino acid composition of independently folding domains and does not require sequence information and x-ray structures. Introduction of disulfide bond was found to enhance the stability of the protein under various conditions. An approach was made to increase the stability of subtilisin by incorporation of disulfide linkages, electrostatic interaction and hydrophobic contacts. The location of the disulfide bonds was determined using computer program, which scored various sites according to the amount of distortion that a disulfide bond would introduce into X-ray model of wild type subtilisin [73,74]. The authors showed that free energy change of unfolding due to individual change in variants are additive so that the total change is equal to the sum of the parts. The decrease in entropy of the unfolded state, which had disulfide bonds, was lower than the decrease in the entropy of the unfolded state that did not contain cross-links. This clearly showed that addition of disulfide bonds increases the stability of the protein. The authors pointed out that the change in the free energy of unfolding due to adding a disulfide bond can be approximated by 1.6 kcal/mol. Breslauer *et al.* used the thermodynamic data of 19 DNA oligomers and 9 DNA polymers to predict the temperature dependency of the DNA duplex structure based on the base sequence [75]. This method was successfully used to predict the transition enthalpies and free energies for a series of DNA oligomers. This allowed predicting the stability and melting behavior of a DNA duplex from its primary sequence. Development of model based on such approach to protein system will be very much helpful in understanding the stability of proteins. They also reported that this could be used in various important applications such as predicting stability of probe gene complex, selection of optimum conditions for hybridization and to reduce the length of the probe.

Noncovalent binding is also considered as one of the important factor that influences the stability of the protein. Statistical thermodynamics of noncovalent binding has been topic of interest for researchers because very few methods of computing binding affinity are derived explicitly [76]. The central basis for this review is the derivation of standard free energy of binding. This derivation also makes it possible to define clearly the changes in translational, rotational, configurational and solvent entropy upon binding.

Akiyama *et al.* developed a continuous flow-mixing device to investigate the protein folding dynamics in terms of compactness [77]. They showed that Cyt c folding proceeds with a collapse around a specific region of the protein followed by a cooperative acquisition of secondary structure and compactness. Studies carried out by Akiyama and co-workers provided a new insight in examining and understand the mechanism of folding pathway. Examining folding from different perspective will help our knowledge in understanding the protein folding [78]. Bakk and coworkers constructed a Hamil-

tonian for a single domain protein where the contact enthalpy and entropy decrease linearly with the number of native contacts [79]. The hydration effect upon protein unfolding is included by modeling water as ideal dipoles that are ordered around the unfolded surfaces. The model simulation is in good accordance with the experiments on myoglobin. Konig and Dandekar developed a model that describes the solvent entropy as the driving force in protein folding [80]. They modeled hydrophobic and hydrophilic residues on a lattice and two- and three-dimensional simulations are compared. Considering the solvent entropy alone, they showed that native fold could be achieved by an entropy-driven simulation on its own. They also showed that the combination of energy and entropy driven search gives the best simulation. He and co-workers developed a new structure template library that represents 358 distinct folds where each model was mathematically represented as Hidden Markov model [81]. Since large number of models in the library can delay the prediction, they used a new triage method for fold prediction. They showed that this triage method gave more predictions as well as more correct predictions when compared to simple prediction methods.

CONCLUSION

Thermodynamics has an important role in the synthesis of a native, stable protein molecule. The different theories support the hypothesis that the formation of a stable protein molecule within a short time is a thermodynamically controlled process. Recently it has been found that various diseases such as Alzheimer's disease, Huntingto disease, cystic fibrosis and diabetes type II arises from protein conformational disorders [7]. The importance of protein folding and misfolding on protein conformational diseases is extensively reviewed [7,8]. These recent studies showed the importance of synthesis of a stable protein, protein folding and the biological functions of a protein.

The probable mechanism of deactivation and stability can be predicted to an extent easily from thermodynamic studies of protein molecules. Thermodynamic studies are helpful in improving the stability of proteins. The exact mechanism of protein deactivation cannot be determined from the thermodynamic studies, but it can be used as one of the efficient tools to understand and predict the stability of proteins, which can then be confirmed through experimental data. The significance of negative entropy is one of the interesting aspects in protein stability; however, there is not much information available on the exact interpretation of negative entropy with structural conformations. Such studies would lead to understand the interesting aspects of protein folding and unfolding. Complex models have to be developed involving various factors influencing the protein stability such that the models can be used to predict the stability more precisely with the aid of thermodynamic data and powerful mathematical packages. This could be achieved

by integrating protein engineering; biology and biochemical engineering and such a cumulative research would be a powerful resource to understand the protein stability through a wide perspective (Fig. 3). Research in these aspects has great potential and the outcome will be highly helpful in the future to solve unanswered questions in our understanding of protein stability and treating conformational disorders.

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