



Modalities of Protein Denaturation and Nature of Denaturants

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Received: 31-07-2020; Revised: 03-07-2021; Accepted: 12-07-2021; Published on: 15-08-2021.

ABSTRACT

Denaturation of protein is a biological phenomenon in which a protein loses its native shape due to the breaking or disruption of weak chemical bonds and interactions which makes the protein biologically inactive. It is the process where properly folded proteins formed under physiological conditions is transformed to an unfolded protein under non-physiological conditions. The process of denaturation of proteins can occur under different physiological and chemical conditions. Denaturation can be reversible or irreversible. Denaturation mostly takes place when the protein is subjected under external elements like inorganic solutes, organic solvents, acids or bases, and by heat or irradiations. The denaturing agents or denaturants widely used in protein folding or unfolding experiments are urea and guanidinium chloride (GdmCl). In denaturation, the alpha-helix structure and beta sheets structure of the native protein are disrupted and unfolds it into any random shape. We can also say that denaturation occurs due to the disruption of bonding interactions which are responsible for secondary structure and the tertiary structure of the proteins.

Keywords: Protein Denaturation, Denaturants, Urea, Guanidinium Chloride, Protein Folding.

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DOI:
10.47583/ijpsrr.2021.v69i02.002



DOI link: <http://dx.doi.org/10.47583/ijpsrr.2021.v69i02.002>

INTRODUCTION

Denaturation is a bio-chemical process which can be defined as any alteration in secondary structure, tertiary structure or the quaternary structure of protein molecule which results in disruption of covalent bonds. Almost all of the proteins known to exist, in their native states, are folded into well-defined and are usually essentially rigid and possess three-dimensional structures. The changes occurring in the structure of protein is generally partnered with alterations in properties like chemical, physical and functional properties.¹ It has been found in many of the investigations that few proteins or enzymes lose their activities irreversibly or reversibly when subjects to different natural or man-made conditions. Glyceraldehyde Phosphate Dehydrogenase and Lactate Dehydrogenase are example of two enzymes/proteins that losses their properties when subjected to lower temperature. Denaturation may be easily "reversible" or "irreversible".

The changes associated with denaturation may include ionization of carboxylic group, amino acid group, or the phenolic groups. These may lead to rearrangements of the molecules, followed by the release of sulfhydryl or disulphide groups. These changes can lead to denaturation or disruption of the overall protein molecule. And they may

also result in decreased protein solubility or loss of certain biological activity of the protein.

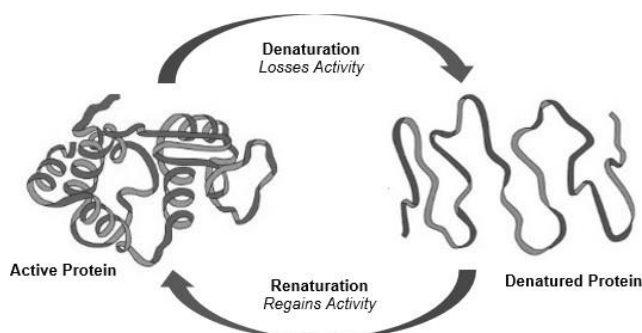


Figure 1: Diagrammatic representation of denaturation of protein.

Denatured states of the protein have become one of the important areas of research in recent years due to their importance in various phenomena like deciphering protein folding problem and the molecular study of many diseases.

Denaturation of protein can be defined as intramolecular change of the protein, which can be studied by studying the displacement and relocation of the constituent groups of the protein and atoms.

Various kinds of denaturation are generally followed the below mentioned changes:

Reduced solubility: The process of protein denaturation is often accompanied by denatured protein precipitation through the addition of smaller amounts of neutral salts. Decline trend in solubility of protein has been studied for many years and is considered as one of the necessary criteria for denaturation.



Decrease in biological activity: Some biochemical activities of the proteins or enzymes may get destroyed due to denaturation. The Proteolytic enzymes when subjected to heat or treated with alkali become inactivated. It is found that viruses lose their proteolytic enzymes to produce disease, and it is also seen that certain hormones lose their certain regulatory functions.

Depletion of crystallizing property: There are many globular proteins which are capable for crystal formation. Proteins lose its ability to crystallize on being subjected to denaturation owing to alteration in structure and shape of protein molecule.

Increased constituent group reactivity: Many alterations in the chemical nature accompany the denaturation of the protein.

“In few cases some of the constituents groups like sulfhydryl, disulphide, or the phenolic groups are detected after denaturation, but in the original protein state these groups are either detected only to a small extent or not detectable at all. The quantitative increase in these groups generally relies on nature of the protein and on degree of denaturation.”²

Alteration in shape of protein molecule: Protein molecules in their native state are equipped with specific molecular dimensions which are expressed as shape and size. This molecular conformation may get affected due to primary denaturation process. Using various methods, it has been found that molar frictional ratio increases when proteins are subjected to denaturation.

Susceptibility to enzymatic hydrolysis: A denatured or unfolded protein molecule can be easily digested as compared to native state of the protein by enzymes like proteinases, which attack peptide bonds. So, from studies it has been found that the process of denaturation makes the protein molecule susceptible to proteolysis or the protein molecule susceptibility increases towards enzymatic hydrolysis.

Denaturation Degree: Characterization of protein molecule is done by considering its amino acid composition and physical configuration. Denaturation of protein is mainly due to the change in its physical conformation of polypeptide chains inside protein molecule. Denaturation Degree can be measured by studying the degree to which the structure of protein has been altered. The denaturation degree in protein structure relays on the nature of protein as well as on the nature of the denaturing agents.

MECHANISM OF PROTEIN DENATURATION

Denaturation is considered to be a tool used for probing the folding properties of the proteins. In recent time, denatured state of proteins has received much importance due to its active participation in study for understanding the protein folding process.³ Denaturing agents mostly used in the folding and unfolding experiments of protein are urea and guanidinium chloride (GdmCl).

The challenges faced in this experiment was whether one can define the denaturation process without denaturants/denaturing agent or not. The experiment done by Privalov and colleagues provided an answer to this. The experiment concluded that thermodynamic properties of unfolding of protein do not depend on the denaturants but structural properties of the protein depends on the denaturants. It can be also mentioned like the net or total enthalpies and entropies of the protein denaturation are intrinsic properties of proteins.

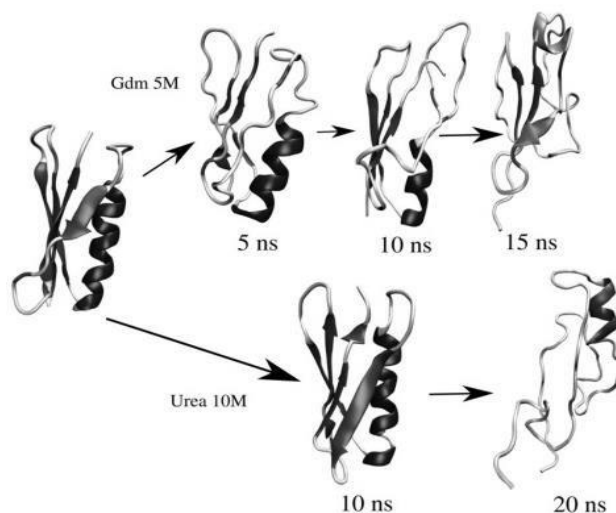


Figure 2: Diagrammatic representation of protein denaturation using GdmCl and Urea as denaturants.⁴

The mechanism of folding and unfolding of protein is generally studied through thorough analysis of structure of the protein and not through direct calorimetric measurements. Like the study of secondary structure is done by circular dichroism, burial of tryptophan is studied by fluorescence, and so on.

The molecular study for protein denaturation using urea and GdmCl as denaturants does not have many strong evidences yet. For this two models was proposed to study. One of the two model is based on a direct, interaction between the denaturant and the protein and the other model is based on the modification of hydrogen-bond structure of water resulting in reduction of strength of hydrophobic interactions.

Protein Denaturation Using Urea: Protein denaturation using Urea as denaturant was first studied in the early years the past century. During 1930s, Urea was most widely and commonly used osmolyte for the study in folding-unfolding of protein i.e., denaturation of the protein.⁵ In research studies, it was found that urea helps in denaturation either by directly interacting with protein initiating solvation of polypeptide chain by water and urea or indirectly by modifying the water molecule structure resulting in changes in the behavior of solvent which weakens or reduces the hydrophobic effect.

Most versions of interaction model states that urea favors the unfolding by binding to the protein, and stabilizes, the denatured state. But the drawback of this interpretation is

that it does not provide explanation for how the protein itself overcomes the kinetic barrier resulting unfolding of the protein.⁶

From a study conducted on the “Kinetics of RNase A at Low Urea Concentrations”.⁵ It was concluded that urea inhibition of RNaseA adheres to uncomplicated competitive model that suggests the probability that additional product is formed by this compound with residues present at active center of the enzyme.⁵ Wu and Wang suggested that the concentrations of urea utilized were very less than pre-transition region concentration for the unfolding. This study also has put light on the fact that in aqueous urea solutions RNase can be inhibited competitively.

Protein Denaturation by GdmCl: Guanidinium cation was found to be the most effective chemical reagent or denaturant which reduces stability of protein when it is added to the aqueous solvent. Two mechanisms are found by which denaturant can destabilize proteins. One of these mechanisms is indirect method by modifying solvent properties of the water and the other is direct method which takes place by specifically binding with the groups of protein. The indirect denaturation mechanism generally is not applicable for Gdm+.⁷ Various experimental evidence proves that the interaction is very weak between Gdm+ and water molecules. Gdm+ was found to affect the interactions between water molecules in the hydration shell of other solutes. Gdm+ enhances solubility of nonpolar groups in proteins molecule as it lacks hydration shell. Gdm+ denatures proteins by interacting with the protein molecules directly it does not bind peptide groups using hydrogen bond.⁸

METHODS OF DENATURATION USING DIFFERENT TYPES OF DENATURANTS

Physical Agents:

Heat/High Temperature: If any solution of protein is subjected to heat next to its isoelectric point, the protein will coagulate. If the temperature is raised to ten degrees this heat coagulation of protein takes place about 600 times faster. Heat is consider as most familiar denaturing agent for protein. Heat denatured protein has an increased susceptibility for aggregation, depending on pH, dielectric constant and ionic strength of the medium. Acid and alkali dissolve heat coagulated protein and heat coagulated protein can be dissolved even at the isoelectric point by a number of substances such as urea, guanidine hydrochloride, detergents, and salicylate. The protein denatured by heat is slowly regains its original soluble form when cooled. Non-reversible denaturation is caused by heating for longer duration, heating of the protein solution for shorter duration at its isoelectric point or by adding salt. There are many enzymes which shows thermophilic properties i.e. they show stability in higher temperature. “The most common stabilizing agents at high temperatures are probably di-myo-inositol 1,1«-phosphate and cyclic 2,3-diphosphoglycerate. These enzymes are

present in *Pyrococcus woesei* and *Methanothermus fervidus* respectively. These agents increase half-lives of some the enzymes by around 130-fold when subjected to 90 °C in presence of potassium”.⁹ Daniel et al. have studied that the tertiary structure of proteins are not fully stable.⁹ Stability of enzyme at high-temperature was performed by them by subjecting the enzyme to heat then rapidly cooling it and assaying it at lower temperature for residual activity. The stability of the enzyme at higher temperature is studied by heating the enzyme at higher temperature and then rapidly cooling it and then subjecting it to the assaying for residual activity at low temperature.

Pressure: Under high pressure the hydrophobic interaction of the protein weakens and it kicks starts the process of the denaturation of the protein. Moderate pressure cannot drive the unfolding of the protein though some structural changes can occur due to which protein can lose its activity.¹⁰

The effect of pressure on protein denaturation has been studied less. Neurath et al. has studied that denaturation of proteins can occur at high pressures of approximately 6000 kg / cm², and the protein coagulation can take place at the pressures of approximately 10,000 kg / cm².²

Freezing/Low temperature: For many years’ studies have shown that proteins losses the activity when subject to low temperature or stored in refrigerator temperature. The rate and extent of denaturation appear to be affected by salt concentration and pH and also by freezing temperature. This type of cold inactivation or cryo-inactivation has two grounds; first of them is cold dissociation and the other type is cold inactivation of proteins and enzymes. Cold dissociation is generally for the oligomeric proteins. These enzymes when subjected to low temperature and dissociate in the lower association level and the activities of original oligomeric structures are lost. The changes are mostly reversible and therefore incubating at the room temperature for longer duration will make the protein regain activity by restoring native oligomeric structures. Cold inactivation of the enzymes and the proteins, is the process observed in dimeric or monomeric globular proteins. It is also observed in situ, where dissociation process is not involved as the primary step.¹¹ This kind of protein inactivation is also called or termed as cold denaturation. The denaturation of various globular proteins subjected under low temperature have been studied. In a research, it was stated that yeast prion protein Ure2 showed cold denaturation below 35 degree Celsius at 200 Megapascal and was more susceptible to cold denaturation at lower ionic strength. The cold denatured state of the protein is studied by various types of spectroscopic methods, like UV absorbance, fluorescence, IR and NMR, and the scattering methods including SAXS and light scattering.

Irradiation: If the protein molecules are exposed to ultraviolet light it causes coagulation of the protein. Coagulation of the proteins using ultraviolet radiation consists of mainly two methods: 1) proper light



denaturation, 2) photochemical reaction which does on depend on temperature and then it is followed by the flocculation of denatured protein. This coagulation of protein has high temperature coefficient. Irradiation can cause change in the state of protein solutions aggregation which can lead to loss of biological activity and also the pH of solutions tends to shift towards the isoelectric point of protein.

Sound waves: When protein molecules are subjected to the mechanical vibrations of high intensity, produced by ultrasonic or sonic waves, the enzymes losses their activity and proteins are coagulated.

Surface Forces: If the proteins are spread over an aqueous surface, or in an interface, protein denaturation takes place by protein molecules unfolding into structures which resembles fully uncoiled polypeptide chains.

Chemical Agent:

Acid/Low pH: It has been found that some proteins retain their native conformations at very low pH if the temperature is kept low whereas the others undergoes unfolding. The decrease in pH/low pH can cause conformational change in the second type of proteins. Many proteins lie intermediate between these extremes. Lysozymes is one of the protein which remain unaffected in low pH at room temperature. β -lactoglobulin and ribonuclease are also the examples of protein resistant to acid denaturation. Yeast glyceraldehyde-3- phosphate dehydrogenase is a protein which undergoes significant change in enzymatic activity, molecular weight at low pH and low temperature. It shows transition between pH4-pH 11 but highly cooperative transition occurs below pH 4, it results in loss of enzymatic activity, dissociation into subunits, and thorough disorganization of the conformation of the subunits themselves. Ferrimyoglobin is highly unstable at low pH. When it is subjected to pH 5 and 4 at room temperature undergoes a transition. The denatured state is probably the same as that of the product of the reversible thermal transition of this protein, a conclusion based primarily on the thermodynamic analysis of Acampora and Herman (1967).¹²

Alkali/High pH: Some proteins shows denaturation at alkaline pH is like that at acid pH while in other proteins the course of alkaline denaturation is different. Hemoglobin and Myoglobin stable toward alkaline pH than toward acid pH. The denaturation of proteins at alkaline pH is complicated for proteins that contain thiol groups or disulphide bonds. Apart from chemical modification, the products of alkaline denaturation are undoubtedly as diverse as the products of acid denaturation. Because buried tyrosyl residues tend to become exposed above pH 10 or 11, there may be less tendency to retain residual structure at very high pH than at very low pH, but meaningful experimental studies are difficult to make because of the prevalence of chemical instability.

Organic Solvents: Denaturation or coagulation of protein could be seen when alcohol or acetone are added to

protein solution aqueous in nature in region of isoelectric point. This procedure is dependent on the temperature and not appears to happen at a quantifiable rate at temperatures underneath - 15°C.

Studies had reported that proteins which belongs to group prolamins like hordein, secalin and kafirin need alcoholic medium for disintegration of protein while no indication of denaturation getting obvious.

It is likely that organic solvent denaturation is associated with the impact of conclusion on dielectric constant of medium; however there is an incomplete definitive proof concerning the underlying mechanism.

Alcohols and glycol can be good denaturant. The branching of hydrocarbon in alcohol tends to decrease their denaturing properties. As denaturants, glycols are less efficient than the corresponding alcohols. It suggests that increased polarity or hydrogen-bonding capacity is of secondary importance when compared with the effects of increasing hydrocarbon content.¹³

Organic solutes: There are several outstanding organic compounds acts as denaturing agents. Chemical and physical method has been deliberately studied to observe the activity of acetamide, urea, formamide, and guanidine salts present in concentrated solutions.

The above-mentioned organic denaturants have increased the dissolvability of the denatured protein, subsequently offering especially appropriate conditions for examining the procedure healthy by aggregation or flocculation.

Impact of organic denaturing compounds on the solubility of denatured protein could be observed, subsequently provides appropriate conditions to study procedure unimpaired by flocculation or aggregation of denatured protein.

The recent investigation on protein denaturation has been conducted on studying the denaturing effects of the synthetic detergents. As per studies conducted on the anionic detergents like alkyl sulfonates and alkyl sulfates, alkyl sulfosuccinates and mixed alkyl-aryl sulfonates, and cationic detergents like alkyl-aryl-substituted ammonium halides were efficient denaturants.² The powerful denaturing action of these denaturants surpasses even the denaturing activity of GdmCl of equimolar concentrations.

THERMODYNAMICS OF DENATURED STATE LOOP FORMATION

A simple loop is the most common structure obtained from the disordered or denatured protein. The conformational constraints that helps in loop formation and also control the folding efficiency of protein; because of this they can provide information on basis of misfolding diseases. Till date various methods have been devised to study loop formation kinetics. The evidences from the previous studies were used to explain a regulatory check for protein folding. In a research study it was found that loops are formed under denaturing conditions when at sixth



coordination of heme site is taken by unique histidine.¹⁴ The below diagram is taken from the research study on “Thermodynamics of denatured state loop formation” by Bowler.¹⁵

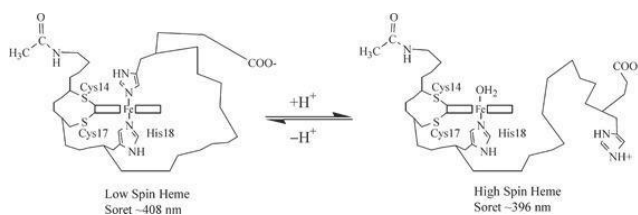


Figure 3: Diagrammatic representation of loop formation of histidine and heme.¹⁵

Simple pH titration is used to measure stability of loop. Altering the sequence position in engineered histidine can control the size of the loop subjected under denaturing conditions

Dependence of stability of loop on loop size can be used to evaluate factors like stiffness of chain and degree of representation of chain as a random coil. In many research studies for DNA loop formation large scaling exponents have also been observed. The thermodynamic methods employed for studying the formation of denatured state loop have provided important information about the conformational constraints. It is evident from kinetic methods that smaller loops form faster, equilibrium studies have proved that the smallest loops are always not most stable and thus not necessarily that it will lead to a productive folding.

CONCLUSION

The mechanism of protein denaturation has been studied in this review paper. It would enhance our knowledge on the events of protein folding and unfolding by giving detailed analysis of the steps of this fundamental process associated with the structural stability of the proteins.⁵ For analyzing or deep understanding the denaturation of proteins various types of spectroscopic methods, like UV absorbance, IR, fluorescence, Nuclear Magnetic Resonance and the various scattering methods includes SAXS and light scattering can be used. These methods are known to scrutinize the behaviors of different segments and the different levels of protein denaturation.

Uncovering residual/unused structure of the protein in its denatured states have been playing a vital role in research studies for characterization of thermodynamic significance of the residual structure. Thermodynamic methods are vital for studying how the protein folding process is impacted by factors like compactness of protein structure, excluded volume and internal friction present in the protein chain.

It has been mentioned in research papers of different researchers that relation between the kinetics of protein folding and stability of the denatured protein state structure is an integral part to examine how the protein folding efficiency is impacted by the denatured state of the protein.

Thus, denatured states of protein possess various interesting as well as unusual characteristics and properties which are very important to understanding folding of protein molecules and its stability.

Several future insights and directions are very much evidential from above mentioned text. Studying the role of non-native against native protein is important for conducting various studies. The quantitative measurements for strength of electrostatic interactions in denatured state have been conducted. Likewise, measurements of hydrophobic interactions are also required, particularly for those which are modulated by aromatic molecules like tryptophan.

In the field of protein engineering, the techniques for combining native and denatured state would be useful in protein stabilization for development of hyper stable proteins. The recent advancement in research studies of the thermodynamics of denatured state provides an excellent foundation many future researches.

Denatured state of protein or the denatured protein is biological state which consists of the distribution of various molecular conformations and the average of these are analyzed or quantified by performing various experiments. There are many evidences which states that even in highly effective denaturants like 6M GdmCl and 9M urea, some structure may continue to exist in its native protein chains. It is studied under the physiological conditions, denatured states of almost all proteins appear to be very compact with large number of secondary structure. Various theoretical as well as experimental studies has suggested that entropies of chain conformational, hydrophobic interactions and electrostatic forces plays a vital role in determining the structure. The process of protein denaturation in urea or GdmCl could be modelled as a two-state transition between the original or native structure and a relatively compact denatured state. It experiences a gradual increase in radius if denaturant is added to it further. When a protein gains large net charge in acids or bases, it tends to exists in the two stable denatured form, one compact structure and other one is extensively unfolded.

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Source of Support: The author(s) received no financial support for the research, authorship, and/or publication of this article.

Conflict of Interest: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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