IDENTIFICATION AND STRUCTURAL CHARACTERISATION OF PARTIALLY FOLDED INTERMEDIATES IN SMALL GLOBULAR PROTEINS

THESIS
SUBMITTED IN PARTIAL FULFILMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

BY

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UNDER THE SUPERVISION OF
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BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE
PILANI (RAJASTHAN), INDIA
1996

To
my lovable mammu pyari
whom I love in a special way

BIRLA INSTITUTE OF TECHNOLOGY & SCIENCE
PILANI RAJASTHAN

CERTIFICATE

This is to certify that the thesis entitled "***IDENTIFICATION AND STRUCTURAL CHARACTERISATION OF PARTIALLY FOLDED INTERMEDIATES IN SMALL GLOBULAR PROTEINS***" and submitted by **K. Ravindra Babu** *ID No. 92PHXF006* for award of Ph.D. Degree of the Institute, embodies original work done by him under my supervision.

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ACKNOWLEDGMENTS

I express my deep sense of gratitude to Dr. S. Venkateswaran, Director, B.I.T.S., Pilani and Dr. V.P. Kamboj, Director, C.D.R.I., Lucknow for providing me an opportunity to carry out my research work.

I am thankful to Dr. V.P. Rao, ex-Dean, Practice School, Dr. V.S. Rao, Dean, Practice School and Dr. L.K. Maheshwari, Dean, R&C, B.I.T.S., Pilani for their support and understanding during the entire period.

I acknowledge the help rendered by Dr. G.K. Patnaik, Head, Membrane Biology Division.

There are many feelings, but no words to express them, when I need to acknowledge the support from Dr. Vinod Bhakuni, my Guide. My heartfelt thanks for his friendly nature and I sum up by just saying that it was the most memorable and the most beautiful experience, being with him.

I am indebted to Dr. Rajiv Bhat, J.N.U., New Delhi, Dr. M.V. Jagannatham, B.H.U., Varanasi and Prof. A. Surolia, I.I.Sc., Bangalore for their kind support in carrying out my experiments, without whose help this work would have never been possible.

My special thanks are due to all my labmates who were there always to support and for their good wishes.

I am indebted to all my students, whose company has made my stay at Lucknow an enjoyable and memorable one.

There is a galaxy of friends, whose support needs a special mention, espacially Jagadish, Bharati, Chinnu, Prasad, Suresh, Lali, Sheetij, Vahab, Shashi. Thanks a lot to all of them for their kindness.

The author wishes to express gratitude to Dr. Raja Roy and Dr. Atul Kumar for their moral support.

My deepest sense of gratitude to my family members, Mamma, Papa, Murali, Padma and all kids for their unstinting support which blostered me throughout the course of time, particularly when the going became tough.

I wish to say a word of thanks to all the staff members of Membrane Biology Dept., for their invaluable assistance.

A special mention is required to express my profound gratitude for the love from Singhaji, Mummyji and Uncleji, whose love and kindness made my stay in Lucknow a special one.

I am glad to place on record my thanks to Ashutosh, J.N.U., New Delhi and Edwin, B.H.U., Varanasi for their friendly nature and help during my visits to their labs.

Finally, a word of my loving thanks to the dearest one, nannu, Mitu, PPW, Motu, Chotu, Plyalu for all those sweet moments and to munnu pyari sister for her love.

(K. Ravindra Babu)

TABLE OF CONTENTS

CHAPTER 1

REVIEW OF LITERATURE

1.1 INTRODUCTION

For thousands of proteins that make each of our cells tick, life begins with a challenge. They have to be synthesized as a long floppy chain of amino acids and then transformed into a carefully shaped molecule replete with all the twists, turns and loops it needs to do its job in the cell. Two main processes are involved in the creation of a protein in a living cell: the biochemical and physical. The biochemical process involves the enzymatic synthesis of the desirable amino acid sequence on a ribosome, whereas the physical process involves the folding of the amino acid sequence into a functionally active, 3D structure.

In the late 1950s Christian B. Anfinsen and his colleagues at the National Institute of Health made a remarkable discovery. They were exploring a long-standing puzzle in biology: What causes newly made proteins which resemble loosely coiled strings and are inactive to wind into specifically shaped balls able to perform crucial tasks in a living cell? In the process the team found the answers was simple than anyone had imagined. It seemed the amino acid sequence of a protein, a one-dimensional trait, was fully sufficient to specify the molecule's ultimate three-dimensional shape and biological activity (Proteins are built from a set of just 20 amino acids, which are assembled into a chain according to directions embedded in the genes). Outside factors, such as enzymes that might catalyze folding, did not have to be invoked as mandatory participants.

Many purified proteins can spontaneously refold *in vitro* after being completely unfolded implying that the three-dimensional structure must be determined by the primary structure (1). How this occurs has come to be known as the protein folding problem. It had been primarily of academic interest, but the advent of protein engineering and the ability to produce any protein, often in an insoluble, unfolded, inactive, and useless form, has made it also of great practical importance.

The first question that was explored by the investigators studying the problem of protein folding was that, does a protein fold by a complete search of all possible conformations looking for the structure with minimal energy or does it fall down into one of energy minima (not necessarily the global one) by same mechanistic folding pathway coded with sequence? The first possibility implies that proteins native structure is under thermodynamic control, while the second possibility means that the native structure is the result of some defined pathway i.e. under kinetic control. The obvious difficulty with the first approach i.e. trial and error looks out of the question as it involves very large number of structures have to be searched (2,3). The age of the Universe is short compared with the time it would take even a small protein to sample the many billion possible folds en route to the right one. The obvious difficulty with second approach is that proteins fold into the same structure in quite different situations *in vitro* and *in vivo*.

As suggested above the random search mechanism of the folding seems highly unlikely (4-6) hence much of the efforts of investigation on protein folding are based on the assumption that a limited number of pathways exist between the fully folded and unfolded states (7).

For studying the pathways of protein folding different approaches have been adopted and the problem has been broken down into four related questions:

1. By what kinetic process or pathway does the protein adopt its native and biologically active folded conformation?

2. What is the physical basis of the stability of folded conformations?

3. Why does the amino acid sequence determine one particular folding process and resultant three-dimensional structure, instead of some other ?

4. Given the amino acid sequence of a protein, how can its three-dimensional structure be predicted ?

Considerable progress has been made in recent years in defining the nature of such pathways and in probing the structures and energetics of the intermediates and transition states that lie along them.

1.2 THE STABLE CONFORMATIONAL STATES OF PROTEINS

Different conformations of a protein differ only in the angle of rotation about the bonds of the backbone and amino acid side-chains (Fig. 1.1), although conformations may also differ in their disulfide bonds. The starting- and end-points for virtually all studies of protein folding are those conformational states that are stable at equilibrium and can be characterized in detail.

1.2.1 The fully folded, native state (N)

The native, folded conformations of proteins are complex (8-12), but known in great detail from the structures determined by X-ray crystallography (8,9) and, more recently, by NMR (13-15). The basic unit of protein folded structures is the domain, which has been defined in very many different ways, but is basically a structural unit that could plausibly be imagined to be an independent structural unit and to remain folded in isolation. The interiors of domains consist primarily of elements of secondary structure (α-helices and β-sheets) packed together via their non-polar side-chains. The interactions determining the tertiary structure are largely between residues distant in the primary structure and hence are known as 'long-range' interactions. They contrast with the 'short-range' interactions between groups close in the primary structure, but both types of interaction occur over similar three-dimensional distances.

There appears to be an upper limit to the size of a domain, for they seldom consist of

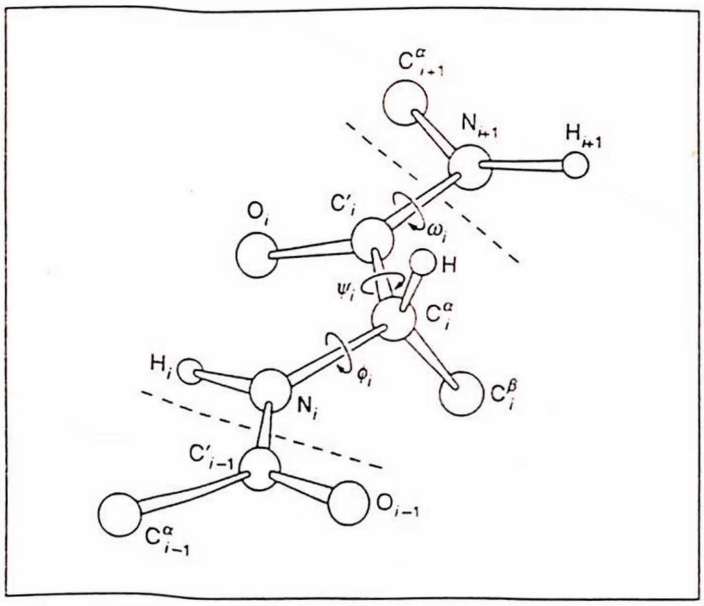

Figure 1.1. Perspective drawing of a segment of polypeptide chain. Only the C^{β} atom of each side-chain is shown. The limits of a single residue (number I of the chain) are indicated by the dashed lines. There is relatively free rotation about the single bonds indicated by the torsion angles ϕ and ψ, but the peptide bond has partial double bond character, so its torsion angle, ω, is limited to values of O° or ± 180°, corresponding to the cis and trans conformations, respectively. The polypeptide chain is shown in the fully extended conformation, with $\phi = \psi = \omega = 180°$.

more than 200 residues, and large proteins are made up of multiple domains or subunits. It is plausible, but unproven, that the domain structure of proteins is a consequence of limitations on the size of the structural unit that can be generated by protein folding (4).

Folded proteins demonstrate varying degrees of flexibility (16), which is of direct relevance to protein folding, in that it reflects the free energy constraints on unfolding and refolding. Nevertheless, the basic architecture of the protein generally stays relatively close to the average structure determined by X-ray crystallography or by NMR; the greatest plasticity of conformation is exhibited by small proteins (17). Certainly no domain is known to adopt alternative fully folded conformations. A domain crystallized in different ways and in different crystal lattices invariably has the same three-dimensional conformation, differing only in surface side-chains and loops. Very similar folded conformations are usually determined by X-ray crystallography and by NMR in solution. The folded conformation of a domain is apparently in a relatively narrow free energy minimum, and substantial perturbations of that folded conformation require a significant increase in free energy.

The folded conformations of proteins are the end-products of the folding process, but they contain remarkably few clues as to how that process occurred. There are only limited tendencies for particular atoms or residues to be in specific positions, so the folding 'rules' must be fairly plastic. The characteristic that is most likely to reflect the kinetic folding process, rather than stability of the folded state, is the tendency for α-helices and β-strands that are adjacent in the sequence also to be adjacent in the tertiary structure (18).

1.2.2 The unfolded state (U)

The ideal unfolded protein is the random coil, in which the rotation angle about each bond of the backbone and side-chains is independent of that of bonds distant in the sequence, and where all conformations have comparable free energies, except when atoms of

the polypeptide chain come into too close proximity. Stearic repulsions are significant between atoms close in the covalent structure, and place limitations on the local flexibility; they would also occur in a fraction of the total random conformations between atoms distant in the covalent structure and thereby exclude these conformations (the excluded volume effect). In spite of these restrictions on the conformational flexibility of a real random coil, there are very many conformations possible with even a small protein. It would therefore be impossible for a fully unfolded protein to encounter on a finite time-scale all its possible conformations (2). Also, each molecule in a typical experimental sample of a fully unfolded protein (likely to contain no more than 10^{18} molecules) will probably have a unique conformation at any instant of time. Consequently, the initial stages of folding must be nearly random, but the native conformation is unlikely to be found by a totally random process.

Unfolded proteins in strong denaturants, such as 6 M-GdmCl or 8 M-urea, and disordered polypeptide copolymers have been demonstrated to have the average hydrodynamic properties expected of random coil polypeptides (19-21). There is a wide variety of evidence, however, suggesting that unfolded proteins are not true random coils under other conditions, such as extremes of pH or temperature in the absence of denaturants (22-25). This is perhaps not too surprising, for in a truly random coil the energetics of interactions between different parts of the polypeptide chain must be exactly balanced by interactions with the solvent. This is virtually impossible with a polypeptide chain composed of 20 different amino acid side-chains, with a diversity of chemical properties. Where interactions between different parts of the polypeptide chain are energetically favored over those with solvent, the polypeptide chain will tend to be more compact and less disordered than expected for a random coil. The opposite will occur where there are especially favorable interactions between solvent and polypeptide. Nevertheless, unfolded states produced under different unfolding conditions, which often have different physical properties, are indistinguishable thermodynamically, so they are probably different sub-sets of the truly random spectrum of non-native conformations (24-26). Most importantly, unfolded proteins do not generally contain co-operative folded

structures.

1.2.3 The compact intermediate (CI), so-called "molten globule", state

A variety of proteins have been observed under certain conditions to exist in stable conformations that are neither fully folded nor fully unfolded. These conformations have sufficient similarities to suggest that they are different manifestations of a third stable conformational state (27, 28). The most common properties are : (1) The overall dimensions of the polypeptide chain are much less than those of a random coil and only marginally greater than those of the fully folded state. (2) The average content of secondary structure is similar to that of the folded state. (3) The interior side-chains are in homogeneous surroundings, in contrast to the asymmetric environments they have in the fully folded state. (4) Many interior amide groups exchange hydrogen atoms with the solvent more rapidly than in the folded state, substantially different from that of the native state. (5) Inter-conversions with the fully unfolded state are rapid and non-cooperative-operative, but slow and co-operative with the fully folded state.

If these observations are applicable to a homogeneous structure, they suggest a collapsed molecule with native-like secondary structure and a liquid-like interior, i.e. a 'molten globule'. Detailed studies of one such protein, α–lactalbumin, however, have demonstrated that portions of the hydrophobic interior are in relatively stable, well-ordered three-dimensional conformations with amide groups highly protected from hydrogen exchange whereas other parts are much less structured (29). Viewing the state as a homogeneous molten globule may therefore is misleading, so the term 'compact intermediate' (CI) will be used here. The CI state appears to be the preferred conformational state of the unfolded protein under refolding conditions, when it is usually only transient. There may be continuum of unfolded conformations, with the CI state at one extreme, fully folded at the other.

1.3 GENERAL PROPERTIES OF PROTEIN FOLDING TRANSITIONS

The native conformational states of proteins may usually be unfolded reversibly by adding denaturants, increasing or decreasing the temperature, varying the pH, applying high pressures. or cleaving disulfide bonds. At equilibrium, unfolding transitions of single domain proteins are usually two-state (26), with only the fully folded (N) and unfolded (U) states populated (Fig. 1.2).

$$N<\!\!-\!\!-\!\!>U$$

This does not mean that partially-folded conformations, with conformational and thermodynamic properties distinctly different from either U or N, do not exist, but merely that they are energetically unstable relative to either U or N under all conditions. It is not surprising that a partially-folded intermediate would be less stable than the fully folded state and would be unfolded under conditions where the fully folded state is only marginally stable. With some proteins, the molten globule state may be present under some conditions, being either a third conformational state or a subset of U (Fig. 1.2).

Multi-domain proteins usually unfold step-wise, with the domains unfolding individually (30,31), either independently or with varying degrees of interactions between them (32,33).

$$N<\!\!-\!\!>I_1<\!\!-\!\!-\!\!>I_2<\!\!-\!\!-\!\!>I_3<\!\!-\!\!-\!\!>U$$

Multi-subunit proteins usually dissociate first, then the subunits unfold, although there are exceptions when the subunits are structurally interdependent (34). The primary consideration

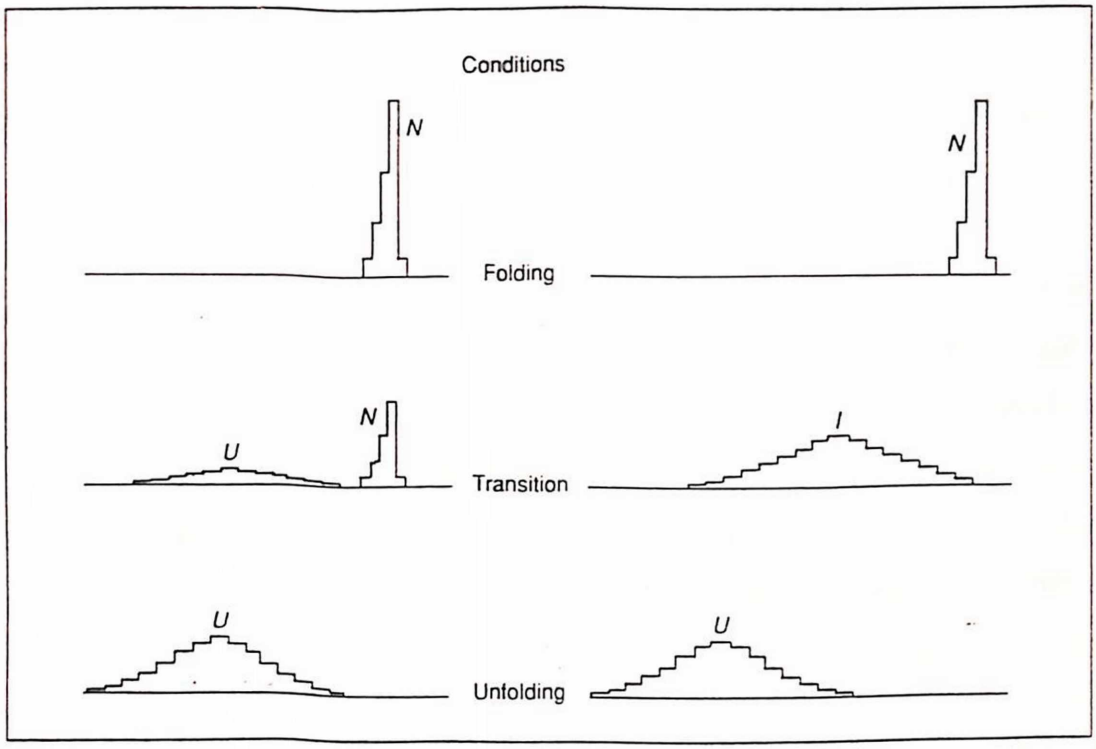

Figure 1.2. Illustration of a co-operative, two-state folding transition (left), as normally observed with single domain proteins, compared with a non-co-operative, multi state transition (right). Histograms of the numbers of molecules (vertical axis) with different degrees of folding (horizontal axis) are illustrated for conditions favoring folding (top) and unfolding (bottom), and for the transition region (middle). The folded state, N, is represented as a narrow distribution of folded conformations, the unfolded state, U, as a broad distribution of less compact conformations. Within the transition region, a two-state transition will have two distinct populations in equilibrium, similar to those at the two limiting conditions of folding and unfolding. In contrast, a non-co-operative transition will have the distribution of the entire population shifted so that most molecules have intermediate, partially-folded conformations (I) within the transition region.

for protein folding is the unfolding/refolding transition of a single protein domain.

1.3.1 Stability of the folded state

Understanding the physical basis of stability of the folded state is crucial for understanding how such a conformation can be acquired. The folded states of proteins are only marginally more stable than the fully unfolded state, even under optimal conditions (26). Typical values of the difference in free energies of the N and U states for small natural proteins are -5 to -10 kcal/mol (-20 to -40 kj/mol), so the equilibrium constant between the N and U states would have a value in the region of 10^4 to 10^7. One consequence of this is that most folded proteins must be spontaneously unfolding completely under all conditions, for the equilibrium constant is the ratio of the forward and reverse rate constants. For example, if a typical protein refolds spontaneously with a rate constant of 1 s^{-1}, its rate of spontaneous total unfolding under the same optimal conditions will be 10^{-4} to 10^{-7} S^{-1}, i.e. half-times of two hours to 80 days. This spontaneous unfolding will normally be only transient, because the protein will promptly refold. Proteins that do not refold spontaneously must be exceptions.

The enthalpies (H) and entropies (S) of unfolding are very temperature-dependent (26, 35) (Fig. 1.3), because the heat capacity of the unfolded state is significantly greater than that of the folded state. The heat capacity results primarily from the temperature-dependent ordering of water molecules around the nonpolar portions of the protein molecules, more of which are solvent accessible in the unfolded state (36).

The large heat capacity change upon protein unfolding causes there to be a temperature at which stability of the folded state is at a maximum (26,35). The stability of the folded state decreases at both higher and lower temperatures. Low-temperature unfolding has changes in enthalpy and entropy that are opposite to those of high-temperature, but is not fundamentally different (37).

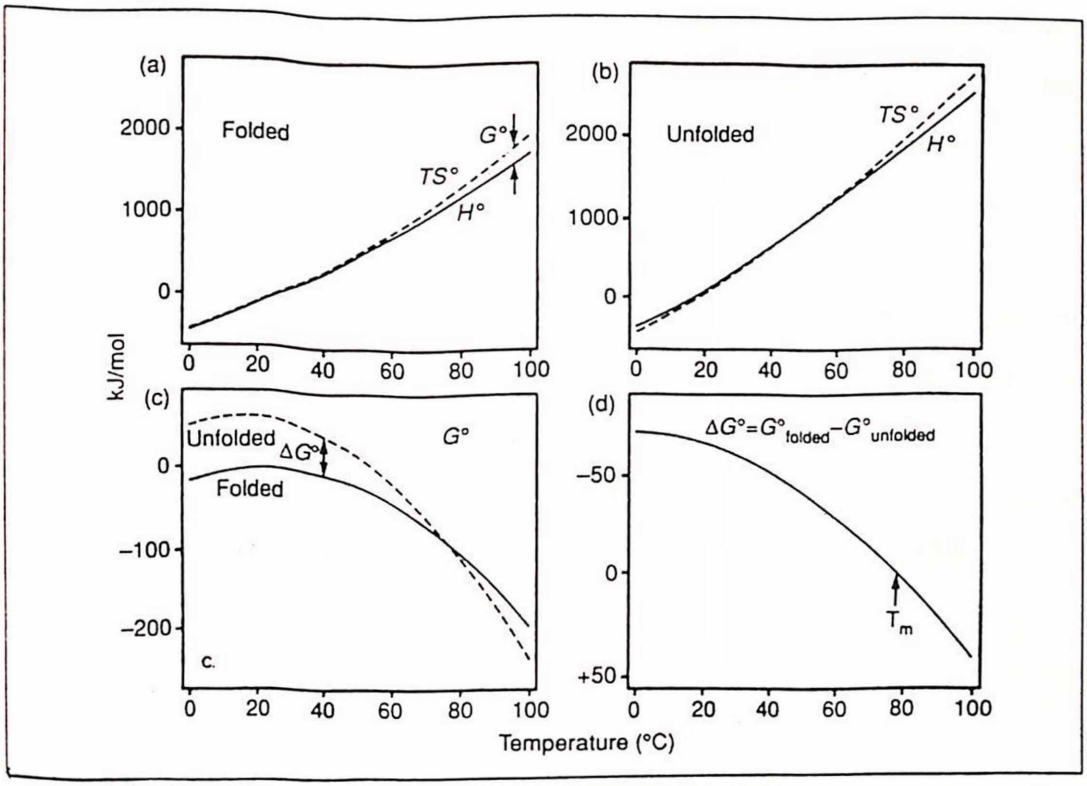

Figure 1.3. Thermodynamic parameters for the folded and unfolded forms of lysozyme in water at pH 7.0 and various temperatures. The enthalpic (H°) and entropic (TS°) contributions to the Gibbs free energies (G°) are plotted as a function of temperature in (a) for the folded state and (b) for the unfolded state. Both vary with temperature because of the substantial heat capacities of both states. The free energies of both states are the differences in the enthalpic and entropic contributions, as illustrated in (a). The values of G° for both states are plotted in © as a function of temperature. The net stability of the folded state (G°) is the difference between the free energies of the two states, as indicated in © and plotted in (d). The melting temperature of native lysozyme is designated by T_m, that temperature where G°=O. Note the change in free energy scales from (a) and (b) to © and to (d); the final G° is a very small difference between the individual enthalpy and entropy contributions. The curvature of the plot of G° in (d) results from the substantial heat capacity difference between the folded and unfolded states.

Rationalizing the stabilities of folded conformations is fraught with danger, for the net effect is a relatively small net difference between much larger individual factors (Fig. 1.3). Nevertheless, it now appears that the two most important stabilizing interactions in the folded state are hydrogen bonds and the van der Walls interactions between non-polar atoms (38-40). The latter term could be replaced by the more frequently quoted 'hydrophobic interactions', but this term means different things to different people and is too often misunderstood. It now seems clear from the study of model systems (39) that the tendency of non-polar atoms to remain in a non-polar, rather than aqueous, environment is due to the more favorable van der Walls interactions they undergo there than when they interact less favorably with water and disrupt the water structure. The contribution of hydrogen bonds to stability of the folded state might seem surprising in view of the fact that the unfolded protein undoubtedly forms an equal number of hydrogen bonds, but with water. Hydrogen bonds within folded proteins are probably the more stable because they are intramolecular, whereas those with water are intermolecular. The former can be much more stable, due to having an entropic advantage (41). This effect probably also accounts for the co-operativity of protein folding.

1.3.2 Co-operativity of folding

The interactions that stabilize folded proteins are individually weak, especially in the presence of competing water (10). That they are able to stabilize folded conformations is probably due to their being many of them possible simultaneously, when they can produce a co-operative system. The two-state nature of protein unfolding transitions indicates that folding is a co-operative process (26). Little happens to the fully folded state prior to complete unfolding. Once unfolding of a domain is initiated, it tends to proceed to completion. Breaking one or more of the stabilizing interactions must destabilize the others, so the free energy increases and the folded conformation becomes unstable.

The simplest example of co-operativity in protein unfolding is pH titration; the

acid-induced unfolding of proteins usually occurs over a much smaller pH range than does titration of a normal ionizing group. This usually arises from the presence of multiple ionizable groups within the protein interior, especially histidine residues, which can ionize only after the protein unfolds (42). Unfolding consequently occurs abruptly, and all the groups ionize in concert.

There are probably two major reasons for the co-operativity of folding transitions. The first concerns unfavorable interactions in the partially-folded states that are not present in either the fully folded or unfolded states. Plausible examples would be the increase in free energy produced by breaking an internal hydrogen bond without supplying comparable hydrogen-bonding partners to the acceptor or donor and by pulling apart two non-polar surfaces sufficiently far that the van der Walls interactions are greatly diminished, but without gaining comparable interactions with other surfaces or with the solvent. Any such conformational strain that is present in partially-folded structures, but not in U or N, should contribute to the co-operativity of folding, but not to the net stability of the folded state.

The second reason for co-operativity is likely to be the presence of many interactions simultaneously in the folded state, and this entropic co-operativity can account for its stabilization by intrinsically weak interactions (10, 41). As described in Fig. 1.4, when the presence of one interaction brings the other groups into proximity and orientation more favorable for interacting, their total contribution to stability may be much greater than that of the sum of the individual interactions. This concept is extended in the simple model illustrated in Fig. 1.5, which indicates that the important problem of protein folding is to build up, through very unstable intermediate states, a folded structure that will be sufficiently co-operative to be stable. The co-operativity of protein folding arises primarily from the long-range interactions between groups distant in the primary structure. Short-range interactions should be largely non-cooperative, depending upon the number of interactions present simultaneously and the interactions between them.

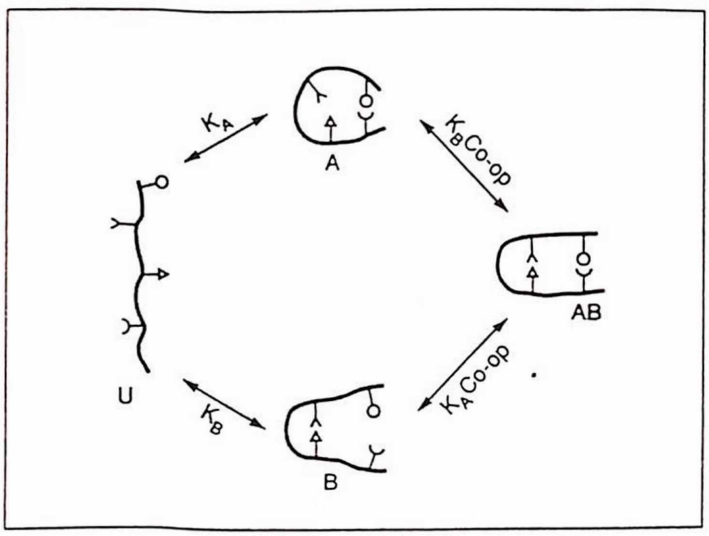

Figure 1.4 Schematic illustration of co-operativity between two interactions, with equilibrium constants K_A and K_B in the unfolded polypeptide chain, U. If both interactions are possible simultaneously, the presence of one will increase the proximity of the other pair of groups, and less conformational entropy of the polypeptide chain will need to be lost for them to interact. Consequently, each interaction will be stronger when the other is also present by the factor Co-op. The effect must be mutual, due to the thermodynamic requirement that there be no free energy change around such a cycle.

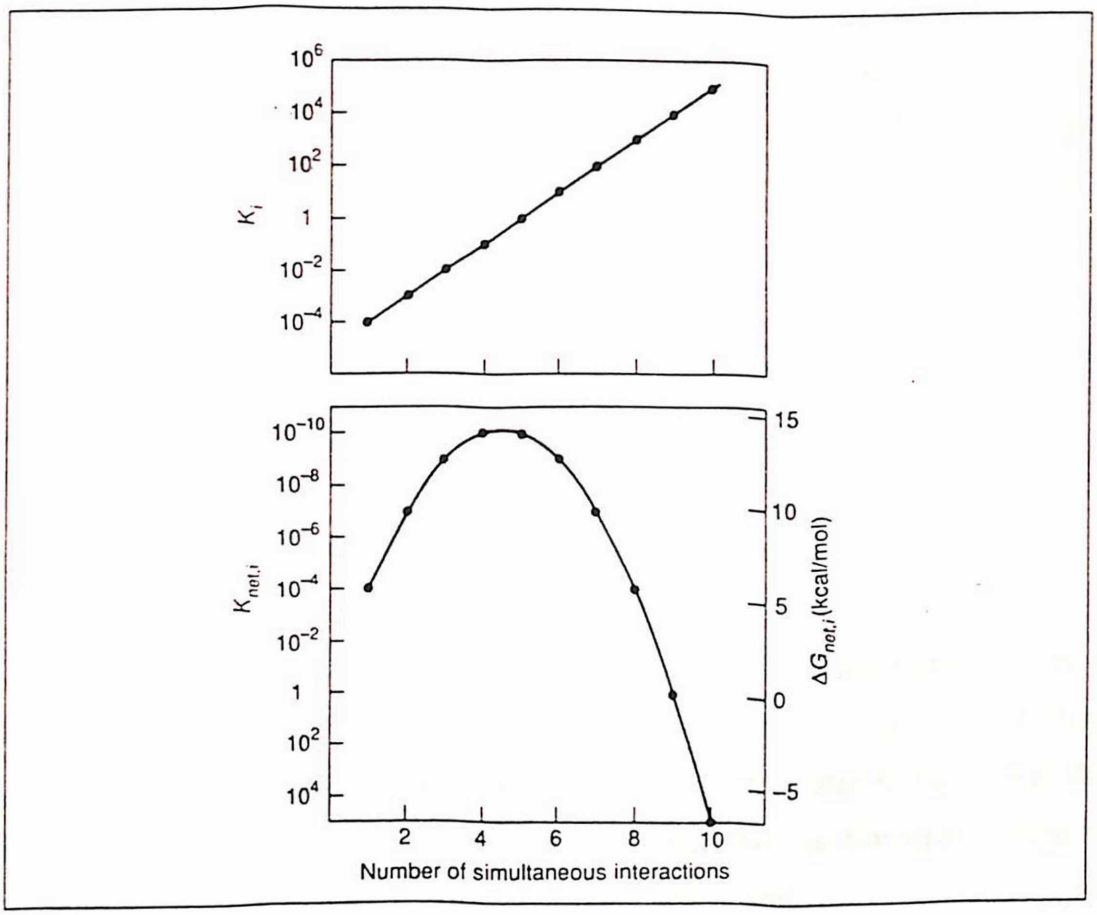

Figure 1.5. Hypothetical illustration of the co-operativity of protein folding produced by multiple weak interactions. The equilibrium constant for forming the ith interaction, K_i, is shown at the top. Individual interactions are postulated to be weak in U, with equilibrium constants no greater than 10^{-4}, each interaction is postulated to be ten times stronger than the preceding one due to entropic co-operativity between them, as described in Fig. 4. The overall equilibrium constant between U with no interactions and each species with I interactions, $K_{net,i}$, is given at the bottom. The value of $K_{net.i}$ decreases initially, as each K_i is less than unity. As K_i becomes greater than unity, the value of $K_{net.i}$ increases. Only with 10 such interactions is $K_{net.i} > 1$ and the folded structure stable. The free energy of each state relative to U is given by $G_{net.i} = - RT \ln K_{net.i}$ with the scale on the right pertaining to 25°C.

1.4 INTERMEDIATE STATES IN FOLDING

The most obvious and general feature of globular proteins is the packing of largely non-polar side-chains to form the 'inside' of the characteristic globule structure. In addition, most proteins contain elements of secondary structure, the polarity of whose surfaces is complementary to the inside-outside polarity of the globule. The regular hydrogen bonding enables the polar backbone groups to be accommodated energetically in the otherwise non-polar interior, thus, purely from looking at folded structures, one is led intuitively to consider the formation of both secondary structure and the globular conformation as important stages in protein folding. The attempt to characterize protein folding pathways has been very largely a search for folding intermediates.

1.4.1 Kinetic folding intermediates

As early as in 1973 Ptitsyn (43) proposed the so-called framework model of protein folding. According to this model (44-46), a protein achieves its native structure, avoiding a large number of alternatives, because it folds step by step in such a way that the results of each step are not reconsidered at subsequent steps, but just fastened by them. More specifically, the folding can start with the formation of the embryos of secondary structure fluctuating around their native positions. Then these embryos can merge into a compact intermediate with a native-like crude 3-D structure (i.e. the native-like folding pattern). Finally, this compact intermediate transforms into the native state with the tertiary structure specified at atomic levels. The main point of this scheme is that three levels of protein structure-secondary structure, folding pattern and tertiary structure-are assumed to be formed as three subsequent stages of protein folding.

At present, all the main points of this scheme have been confirmed by direct experiments. The first ('burst') intermediate predicted by this scheme was revealed

independently in two groups (47-49). The results of studies on 14 different proteins published recently (48) show that this intermediate is formed within a few milliseconds. It has usually 50-100% (or in one or two cases even more than 100%) of native far UV circular dichroism and, therefore, must have a very substantial secondary structure. It binds a hydrophobic probe 1-anilino-naphthalene-8-sulfonate (ANS) (50) which suggests that it has solvent-accessible clusters of non-polar groups. Finally, there is evidence (51-53) that in at least three proteins-immunoglobulin C_L fragment, cytochrome C and ubiquitin-this state is partly compact.

The second intermediate revealed (54,55) usually forms within 1s and accumulates for up to thousands of seconds, when it transforms to the native state. It is almost as compact as the native state (54), has a loosely packed non-polar core (56, 64) and a substantial amount of solvent-accessible clusters of non-polar groups (55, 58). The pulse hydrogen labeling technique elaborated by Baldwin and Roder (60) has shown that this state has many stable α-helices and β-strands (61). Stable secondary structure (detected by hydrogen exchange) forms after fluctuating secondary structure (detected by far UV circular dichroism) in all cases where these rates have been compared (62, 63). Fersht (56) using site-specific mutagenesis in combination with NMR was able to describe this native-like secondary structure at almost atomic resolution (57,64,65). Finally, it was shown (66) that the first native-like epitope is already formed at this state.

1.4.2 Equilibrium folding intermediates

It is interesting to compare these kinetic intermediates with intermediates which can exist at different stages of equilibrium either folding or unfolding. The 'all-or-none' character of the transition means that each molecule undergoes this transition as a whole but it does not necessarily indicate the absence of any other transitions. However, for GdmCl-

induced unfolding of myoglobin and lysozyme there are no intermediates between the native and the completely unfolded states (67, 68). In this case the denaturation of the proteins (monitored by the change in circular dichroism in the aromatic region which reflects the loss of the rigid tertiary structure) is coupled with the 'all-or-none' transition between a compact and a less compact state (monitored by bimodal distribution of elution volumes in size-exclusion chromatography), with real unfolding (monitored by the change of elution volume of a less compact state) and with loss of the secondary structure (monitored by the change in circular dichroism in the peptide region).

However, in a number of cases it is possible to decouple these processes and to see one or even two equilibrium intermediates between the native and the completely unfolded states. For example, for carbonic anhydrase and β-lactamase at room temperature, one can observe two, rather than one, transitions, induced by GdmCl. The first transition is the protein denaturation monitored by the loss of its activity and the rigid tertiary structure. The second transition can be monitored by the change in elution volume and circular dichroism in the peptide region. This indicates the existence of a denatured state with compactness and secondary structure content close to those of the native state but without activity and rigid tertiary structure. The properties of this state coincide with the properties of the molten globule state which has been observed and studied in detail as a pH denatured state of α-lactalbumin (60,70), carbonic anhydrase (71) and many other proteins (28, 72-75). The existence of an equilibrium intermediate state upon solvent-induced unfolding had been observed even earlier by Wong and Tanford (76) and, in particular, by Kuwajima et al. (77). Recent data (67, 68) have shown that this intermediate (like the pH-denatured state) is almost as compact as the native state.

Rather unexpected results have been obtained for the denaturation and unfolding of carbonic anhydrase and β-lactamase at low temperature where the transition between the molten globule and unfolded states becomes very slow (68). In these cases it is possible to

observe three transition curves. The first one is again protein denaturation (usually monitored as the loss of activity and rigid tertiary structures). The second curve is something quite new - it is the 'all-or-none' transition between two denatured states. In fact, in these cases we can observe a bimodal volume distribution between two denatured states-the first being almost as compact as the native one and the second substantially less compact (78). This clearly shows that in addition to the 'all-or-none' denaturation that is observed here another 'all-or-none' transition - the destruction of a compact state was also observed. Finally, the third transition curve shows that even after all the molecules have transformed into the less compact state, they continued to expand. This expansion is much larger than the difference between the compact and the 'less compact' states and much larger than the 'normal' expansion of coils in good solvents, which is well described by Flory's (79) theory. An additional characteristic of transition is the loss of secondary structure which is described by superposition of the second and the third transitions.

The existence of three transitions shows the presence of at least two intermediates between the native and the completely unfolded states, which differ in their compactness. The 'more compact' intermediate is almost as compact as the native protein, has a native-like content of secondary structure and strongly binds ANS. This means that it fulfills all criteria of the molten globule state. The 'less compact' equilibrium intermediate has been observed in the experiments for the first time (68) and has been named as the partially folded state. Its volume is no more than twice as large as that of the native state in contrast with the unfolded molecule whose volume is bigger than the native one by an order of magnitude. This intermediate also has a substantial amount of secondary structure (~50% that of the native state) and weakly binds ANS.

There is strict similarity between the properties of the equilibrium and the kinetic molten globule states. Moreover, the new equilibrium partly folded state also has a number of features similar to the first kinetic intermediate. It appears probable that the kinetic process of

protein folding can be mimicked by the equilibrium folding upon the protein dilution from high concentrations of strong denaturants.

1.4.3 Accumulation of kinetic intermediates and phase transitions between equilibrium states

There is another, probably deeper, similarity between the kinetic and equilibrium intermediates of protein folding. Kinetic intermediates accumulate upon folding which means that they are separated from each other and from the native state by high potential barriers. It has been observed (67, 68) that high potential barriers also separate equilibrium intermediates both from each other and from the native state which leads to the 'all-or-none' character the corresponding transitions. The standard approach to discriminate between 'all-or-none' and other transitions is to compare the mol. wt of a cooperative unit (i.e. the part of the molecule which undergoes the transition as a whole) with the mol. wt of the whole molecule. This approach has been successfully applied to the temperature-induced denaturation (26) but regrettably it is practically impossible to apply it to solvent-induced transitions. Therefore, Uversky amd Ptitsyn have elaborated (70) another approach as discussed above.

It has been demonstrated that the slope of the first order phase transition in small systems is proportional to the number of units in these systems (80). Therefore, it is possible to discriminate first-order phase transitions in proteins from transitions of other types by the mol. wt. dependencies of the slopes of these transitions. This method has been applied to all activity, optical, viscosity. NMR and chromatography data available on the transition between the native and the molten globule states as well as on the transition between the molten globule and the unfolded states. The result was that the slopes of both these transitions are proportional to the protein mol. wt (providing of course, the protein is not very large, as large proteins melt as quasi-independent domains). This means that the molten globule state is separated both from the native and unfolded states by 'all-or-none' transitions which are intramolecular

analogues of the first-order phase transition in macroscopic systems. In other words, this shows that the molten globule state is a third thermodynamic and structural state of protein molecules in addition to two previously known states - the native and the unfolded ones. Size-exclusion experiments (68) suggest that in the case where both the unfolded and the partly folded states can be observed, the 'all-or-none' transition exists between the molten globule and the partly folded states, leaving open the question on the character of the unfolded-partly folded states transition.

It follows that the accumulation of two kinetic intermediates has its analog in the existence of two phase transitions between equilibrium states of a protein molecule. It is reasonable to assume that the common reason for these effects is the existence of two levels of protein structure - the folding pattern (i.e. the crude mutual 3-D positions of α– and β-regions) and the tertiary structure on an atomic level. The existence of these two levels was first suggested by Ptitsyn (43) in his 'framework model' of protein folding and later was 'fished out' from the X-ray data on protein 3-D structures (18, 81, 82).

There is growing evidence that the molten globule having lost the detailed native tertiary structure,still preserves many important features of the native folding pattern (83-85). Particularly convincing are the experiments of Peng and Kim (86) which show that disulfide bonds in the helical subdomain of α-lactalbumin are formed between the native pairs of cysteine residues. Therefore, it is quite possible that two large potential barriers in the kinetic folding pathway and two phase transitions between the equilibrium states reflect the independent formation of two levels of protein structure-the large-scale folding pattern which can already exist in the molten globule state and the small-scale detailed tertiary structure which exists only in the native state.

1.4.4 The molten globule as a functional entity

The role of the kinetic molten globule in folding is well established. There are a number of processes in which proteins take part, notably in trans-membrane trafficking and in membrane insertion, where a major conformational change must occur, and there have been suggestions that the molten globule is involved also in these. The background to this hypothesis has been reviewed (87) and a few examples will be outlined here.

1.4.4.1 Protein transport

After their synthesis in the cell, many proteins have to cross membranes in order to reach the site of their function. One such system which has been studied extensively is the transport of proteins into mitochondria (88, 89). In a series of elegant experiments this translocation was shown to take place in two main phases, one a time-dependent adsorption to the membrane, and the other an ATP-dependant process involving other specific protein components. Using dihydrofolate reductase (DHFR) as a model protein, the first phase has been shown to be accelerated if the DHFR is added to the assay system in its unfolded form in urea. This suggests that adsorption requires interaction of the membrane surface with sites on the protein that are more readily accessed from the partially-folded than from the native protein. It also suggests that the time-dependent adsorption from the native DHFR is rate-limited by a relatively slow conformational change to a less folded form, confirmed by the fact that the adsorbed form of the protein is found to be more sensitive to protease digestion. The addition of methotrexate, a ligand that binds strongly to DHFR and stabilizes it against denaturation, essentially inhibits the initial phase of adsorption.

It is suggested that the properties of the molten globule described earlier make its involvement in the process of adsorption both attractive and reasonable. It is less stable than the native state and separated from it by an appreciable energy barrier; it has fewer persistent

tertiary interactions and greater dynamics, allowing it to access a variety of non-native conformations.

1.4.4.2 Membrane insertion

A different example is provided by colicin A, a protein that has to insert into the membrane in order to bring about depolarization. Its pore-forming domain consists of ten well-packed helices, two of which are buried in the non-polar interior of the domain (Fig. 1.6a). This structure contrasts with a proposed membrane insertion intermediate (Fig. 1.6b) in which the protein has been essentially turned inside out and the two hydrophobic helices partitioned from one non-polar environment in the protein to another in the membrane. Again the question is, what sort of intermediate would lower the energy barrier to this massive 'protein quake'?

The process of membrane insertion of the pore-forming domain turns out to be pH-dependent, with an apparent pK of 4.8. The pore-forming domain assumes a classical acid molten globule conformation at pH 2 with a pK for the N-A transition of 2.7. Taking into account the effect of the negatively charged lipid surface on the local pH, a pK of 3 was estimated for the rate of membrane insertion, in reasonable agreement with the molten globule transition (90).

On the basis of the above evidence, it was proposed that, at the surface of the membrane, colicin takes up an acid molten globule conformation in which the barrier to partitioning of the two helices from protein to membrane will be lower. It may well be that the strong electrostatic field also assists the process of distorting the protein conformation. An analogous example is that of tumour necrosis factor (TNF). This three subunit protein forms ion permeable channels in membranes in a pH-dependent manner that mirrors an acid-induced conformational change (91). It is interesting that TNF, a three subunit protein, has been shown to adopt a stable molten globule conformation at acid pH that is trimeric. Again, a molten

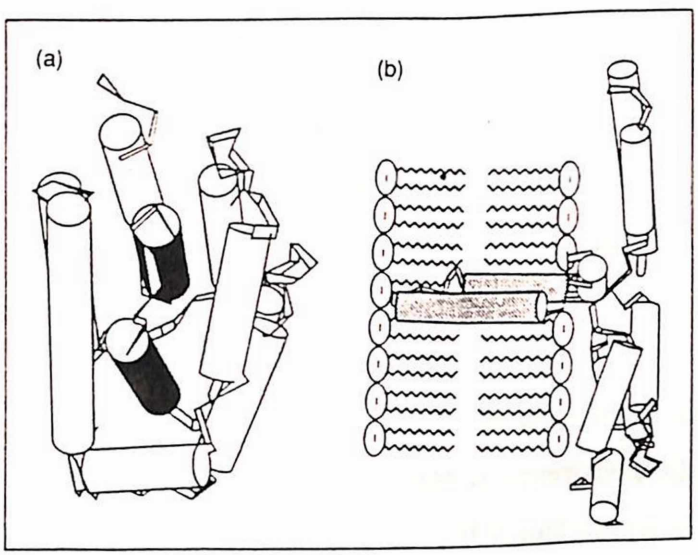

Figure 1.6. Molten globules and membrane insertion. (a) The pore-forming domain of colicin A comprises ten helices arranged in a three-layer sandwich. Two of the helices possess hydrophobic surfaces and are buried in a non-polar environment, whereas the remainder are amphipathic. (b) Evidence supports an 'umbrella' model for membrane insertion in which the first step is turning the protein inside out and transferring the internal helices into a non-polar membrane environment.

globule state is suggested as being involved in membrane insertion.

The existence of either a transient or an equilibrium molten globule intermediate makes a protein more deformable, while preserving the same non-polar internal environment. This suggests the possibility that secondary structure elements may slide relative to one another with rather low energy barriers, allowing the protein to take up quite different conformations as must be required by the models for membrane insertion.

1.4.4.3 Chaperone interactions

There is now recognized to be a wide variety of chaperone proteins, they bind to unfolded or partially-folded proteins, depending on their precise role on the folding pathway. Some of these, GroEL being the most investigated example, bind to the molten globule. This was suggested by the work on the folding and assembly of rubisco (87, 92) and confirmed by more direct evidence that DHFR and rhodanese are in a state similar to the molten globule when binding to GroEL (93).

The functions of chaperone proteins are thought to extend beyond folding. They appear to have a more general role of preserving proteins in a partially-folded state prior to their being translocated across membranes (89). Like GroEL, it would not be surprising if they were found to bind to a molten globule conformation. It is not yet known how GroEL binds to a folding protein. One of the most interesting aspects of the binding, however, is the lack of specificity for type or species of protein. It suggests that the surface of the molten globule must be sufficiently mobile to be deformable to a fairly standard shape and polarity that is appropriately complementary to the chaperone binding surface.

1.5 MODELS OF FOLDING

A vast number of theoretical models for folding have been proposed to explain

how folding could occur rapidly. Although the conformation space of the denatured state is vast, there would be no difficulty in finding the native conformation if each of the amino acid residues could find its correct conformation independently of the others, or if only short-range interactions were involved; folding would then be as rapid as in the case of α-helix formation, which occurs on the 10^{-6}s time-scale (10). It has been argued that folding could occur rapidly by giving each residue in the unfolded chain only a slight energetic short-range bias to the native-like conformation. The difficulty with all such proposals is that they do not consider the necessity of long-range interactions for stability of folded tertiary conformations.

1.5.1 JigSaw puzzle Model

If every molecule of an unfolded protein has a unique conformation at the time of initiating folding, it could be imagined that each molecule would consequently follow a different folding pathway (Fig. 1.7a). There is a useful analogy in the way one puts together a large, complex jigsaw puzzle (94), especially one in which all the pieces have similar patterns and shapes. The pieces are joined together in a different order each time the puzzle is assembled, yet the final result is always the same. If there is a distinctive pattern to the puzzle, certain parts may tend to be assembled before others, but only to this extent would the concept of an assembly pathway be applicable. In order to occur in a short period of time, both for the puzzle and for protein folding, assembly has to occur by fitting together individual pieces only in the same structures they will have in the final structure. On again, local short-range interactions are assumed to determine the final folded conformation. The kinetics of this model were not specified, but it would be very surprising if the different molecules did not require different times for folding.

1.5.2 Nucleation model

Most other models of folding envisage a sequential pathway of folding through

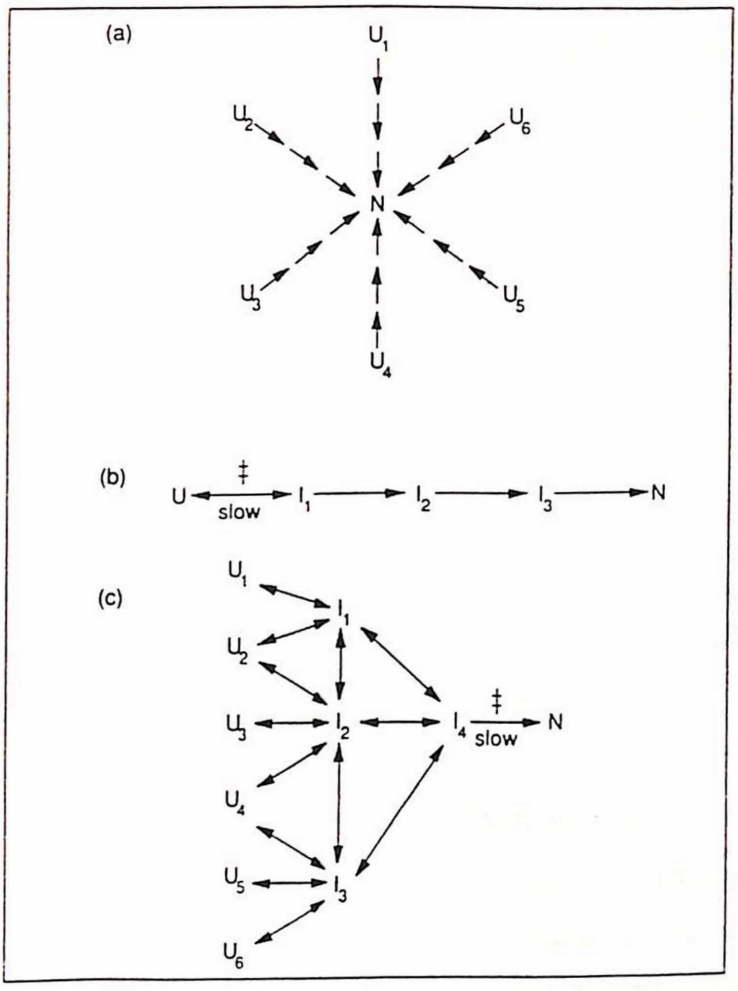

Figure 1.7 Examples of kinetic models for protein folding, in the absence of intrinsically slow isomerizations. U; are various unfolded molecules with different conformations at the start of folding, I_1 are partially-folded molecules, and N is the fully folded protein. All kinetic steps indicated by arrows are rapid, except for those labeled 'slow'; ++ indicates the occurrence of the overall transition state. Single-headed arrows indicate steps that effectively occur only in the indicated direction under conditions strongly favoring folding. (a) The jigsaw puzzle model, in which each unfolded molecule folds by a unique sequence of events. The different pathways converge only at the fully folded conformation. Each pathway would occur with a unique rate and must be essentially irreversible. (b) The nucleation, rapid-growth model, in which a nucleation event in the unfolded protein, here indicated as formation of I_1, is the rate-limiting step. The nucleation event would be very local and occur randomly, so it could occur in all the unfolded molecules. Subsequent steps through various intermediates would be rapid and essentially irreversible under strongly folding conditions. (c)The kinetic model indicated by the experimental data. All the unfolded molecules rapidly equilibrate under folding conditions with a few partially-folded, marginally stable intermediates, which are also in rapid equilibrium. All the molecules pass through a common slow step, which involves going through a transition state that is a distorted form of the native-like conformation. Any intermediates that occur after the rate-limiting step are probably very unstable relative to N.

a limited number of intermediates. An early model (4) imagined the appearance by random fluctuations of a sufficiently small 'nucleus' of structure that would not be stable but would serve as a template upon which the remainder of the polypeptide chain would fold rapidly (Fig. 1.7 b). All molecules might or might not encounter the same nucleus and fold by the same pathway. In this nucleation/rapid growth model, the initial nucleation event would be the rate-limiting step.

1.5.3 Diffusion-collision-adhesion model

In the diffusion-collision-adhesion model (95, 96) individual elements of native-like structure, or 'micro-domains', such as α-helices, β-strands, are recognized as being unstable in the initial unfolded protein and present with only low probabilities. The individual elements will, however, stabilize each other when they interact. The rate constant for formation of such a more stable complex would be given by the rate at which they diffuse together times the probability with which both will be in the appropriate conformation. The dynamics of unfolded polypeptide chains indicate that different portions of the chain encounter each other by diffusion approximately 10^5 times per second (10, so a combined probability of 10^{-5} of both segments being in their appropriate conformations simultaneously would give an observed rate constant for assembly of 1 s^{-1}. If three or more micro-domains are necessary for a stable conformation, the overall kinetics will depend upon the exact stabilities of the partial assemblies (96). Related models envisage two or more 'clusters' or 'embryos' f local structure growing until they merge with each other and form the entire structure (6).

1.5.4 The framework model

This model imagines the folded structure to be formed by packing together pre-existing individual elements of secondary structure (46, 97). The kinetics of this process are not specified, but the elements of secondary structure are imagined to have significant stability

in the unfolded protein and the most difficult step to be their packing together. An α–helix forms in any unfolded polypeptide chain on the microsecond time-scale (10), but it disappears even more rapidly, for the equilibrium constant for its formation is generally less than unity. The most relevant question is at which stage the α-helix becomes stable. But what degree of stability is significant ? Just having relatively stable secondary structure in an unfolded protein does not imply that it is important for the early stages of folding. One of the intrinsically most stable α-helices known is that at the amino-terminus of ribonuclease A, corresponding to the S- and C-peptides. Although well-populated in the unfolded state, this helix is stabilized only at the latest stage of folding (98).

1.5.5 Collapse model

This plausible mechanism imagines the unfolded polypeptide chain to undergo rapid hydrophobic collapse under refolding conditions (99), perhaps to something approximating the molten globule state. Simply constraining the polypeptide chain to be compact might greatly increase the probability of the final folded conformation being encountered (100).

Proteins are synthesized in cells starting from the N-terminus, so an appealing idea has been that folding also starts from the N-terminus (101). Of course, it is now known that the nascent chain interacts with various cellular factors, or chaperones, during biosynthesis *in vivo*. There is little evidence for folding of single domain proteins occurring from the N-terminus *in vitro*, for virtually all the poly-peptide chain corresponding to an active domain appears to be required for it to adopt any stable structure. More pertinently, two proteins with circularly permuted sequences have been shown to fold at nearly normal rates to the same folded conformation, both *in vitro* (102) and *in vivo* (103). Neither the termini nor the order of segments in the polypeptide chain are crucial, so folding need not proceed from the N-terminus. With several multi-domain proteins there is evidence that the individual domains fold

immediately they are completed, while the remainder of the polypeptide chain is being synthesized (104, 105), so *in vivo* folding and *in vitro* refolding are intrinsically different for proteins with multiple domains.

1.5.6 A general scheme for protein folding

The kinetic scheme for protein folding indicated by the available experimental data is illustrated in Fig. 1.7c. The jigsaw model (Fig. 1.7a) probably applies at the initial stage of folding, when the molecules are fully unfolded and have different conformations; however, the result of this folding is not the fully folded protein, but any partially-folded refolding protein, prior to the rate-limiting step. Rapid formation of non-random conformations in the refolding protein may involve nucleation-rapid growth mechanisms (Fig. 1.7b), as in α-helix formation, but not formation of the fully folded protein. Nucleation events seem more pertinent to unfolding than to refolding. The refolding proteins characterized (64, 106) appear to consist of elements of native-like secondary structure interacting and stabilizing each other, as in the diffusion-collision-adhesion and framework models. Both non-polar and hydrogen bond interactions appear to be the major stabilizing forces. The existence of such meta-stable conformations in the refolding protein indicates that folding is not totally co-operative as in the simple scheme of Fig. 1.5. Instead, proteins appear to fold through one or more metastable subdomains. The highest free energy barrier to folding has yet to come, however, with the need to go through a high energy, distorted form of the native conformation. With the current activity in the field, this general scheme will undoubtedly be expanded, or corrected, in the near future.

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CHAPTER 2

THE FOLDING STORY OF LYSOZYME

2.1 INTRODUCTION

Hen Egg-White lysozyme (HEWL) is one of the earliest characterized and most studied globular proteins. Its 129 amino acid residues fold into two structural lobes: the α-domain consists of helices A to D and a 3_{10} helix, the β-domain includes a long triple-stranded β-sheet, a short double-stranded β-sheet, a 3_{10} helix and a long loop (1,2). It was one of the first proteins to be studied by NMR and assignments of ^{1}H NMR resonances of almost all residues of lysozyme have been obtained by sequence-specific methods (3-5). Studies on lysozyme and the homologous protein α-lactalbumin have been particularly important in deciphering the folding pathways of these proteins. A stable, compact molten globule state is observed during equilibrium denaturation of ∝-lactalbumin by contrast, the unfolding transition of lysozyme is a highly cooperative event in which only the native and highly unfolded states are stable at equilibrium (6-8). Kinetics and equilibrium aspects of lysozyme folding have been under extensive investigation (9, 10). For lysozyme, although about 10% of unfolded molecules seems to refold directly to the native state (11), relatively long-lived kinetic intermediates were identified in the early stage (<100 ms) of the refolding pathway (12,2). The ∝-domain of the intermediates appears to have some characteristics of a molten globule, i.e. with an extensive secondary structure but a distorted, far from native, like tertiary structure, while the conformation of the β-domain is still highly labile (2). Structural features of these intermediates are difficult to study because of their transient occurrence due to the presence of high degree of cooperativity in these proteins.

A strategy to overcome the cooperativity of protein folding is to study the protein either under mild denaturing conditions, use of alcohols or to study protein fragments and peptide models (14-16). With the application of structural tools, such as NMR,. hydrogen exchange and scattering techniques to probe different aspects of folding and with the improved precision of the measurements, previously undetected intermediates may also be resolved. The equilibrium unfolding transition of lysozyme, mostly determined from thermal or guanidinium hydrochloride induced denaturation at neutral pH, has been traditionally approximated by a

two-state model (13), absence of stable partially folded intermediates has been inferred from the high degree of cooperativity in unfolding. Recently, stable intermediates have been reported for lysozymes from other species, such as equine and human (17, 18), for a homologous protein α-lactalbumin (8) and for lysozyme using the mild denaturing trifluoroethanol (TFE) at pH 2.0 (19). A partially folded intermediate of lysozyme stabilized by a combination of reduced pH and denaturant has been characterized by small angle x-ray scattering and other techniques (20).

To understand the mechanism of protein folding, it is important to study the intermediate states, as they provide significant information on the folding pathways . Variety of techniques, NMR spectroscopy electrospray mass spectroscopy (ESMS) combined with stopped flow fluorescence experiments and circular dichroism (CD) in near- and far-UV have been extensively used to examine the folding pathway of lysozymes.

2.2 Hydrogen exchange labeling, NMR and mass spectrometery.

In principle, it can be imagined that the structural transitions occurring during folding might be monitored simply by carrying out NMR experiments while the protein refolds from a denatured state to the native conformation. Although this approach is possible in principle, in practice this is not the case, primarily because the time scale of the folding process is normally much too fast (seconds) to permit even one spectrum to be accumulate before folding is complete. However, NMR methods have been developed that allow such fast reactions to be studied and these have been applied successfully to the study of the folding of lysozymes (7, 21-23). These experiments are performed under conditions in which the native and denatured states are interconverting at equilibrium, such as at the midpoint of the thermal denaturation transition. This however leads to a further problem: folding tends to be highly cooperative under equilibrium conditions so the partially folded intermediates whose structure are sought are rarely observed. One of the approaches is to study the partially folded states of proteins generated under conditions such that they are stable (8). The other is to monitor indirectly the structural changes occurring as the protein folds, of which the most sophisticated approach

involves hydrogen exchange labeling in combination with two-dimensional NMR. The basis of this method is the protection from exchange occurs only when structure is formed during folding (22). If such structure is persistent the amides remain protected during subsequent folding steps and are ultimately trapped in the native state. The proton occupancies in the native state resulting from labeling at various stages of refolding can then be monitored conveniently by NMR and interpreted on a residue-by-residue basis in terms of the structural transitions experienced by the protein during folding. In the case of lysozyme, this technique has proved particularly powerful because more than 50 amides of the polypeptide chain can be followed, giving rise to a very detailed view of the development of stable structure throughout the folding of protein (2). These studies revealed two classes of amides with distinct protection kinetics for lysozyme. About half of the amides monitored are virtually completely protected from exchange within about 200 ms, while the other half are not completely protected until more than 1 s (ignoring about 20% of molecules which fold much more slowly-probably as a consequence of cis proline isomers in the denatured state (25). The more rapidly protected amides are almost exclusively within the domain containing the four ∝-helices, whereas the more slowly protected amides are in the domain containing the β-sheet. This, therefore, gives rise to the idea that the structural domains in the native protein are also distinct 'folding domains'. Furthermore, the rates of protection of different amides within each domain, in general, are similar, suggesting that the formation of each domain is a highly cooperative process. The protection kinetics do not simply reflect the differences in the type of secondary structure in each domain since, for example, the two 3_{10} helices are protected at very different rates: in each case, the rate of protection is similar to those of other protons within the domain in which each helix is located. But the folding pathway appears to be more complicated than a simple mechanism involving sequential protection of the ∝- and β-domains since the amides in both domains showed a biphasic kinetics of protection. The fast phase has a time constant of about 5-10 ms for each domain and an average amplitude of 40% and 25% for the ∝- and β-domains, respectively. The slow phases have average amplitudes and time constants of 45% and 65 ms, and 55% and 350 ms for the ∝- and β-domains respectively. More detailed analysis suggests that this behavior is a result of multiple pathways of folding,

and it is clear from their amplitudes and time-scales that this does not arise merely as a consequence of cis-trans isomerism of the two proline residues in the primary sequence.

While the NMR analysis of hydrogen exchange labeling is unique in that it reveals residue-specific information about the folding process, it cannot tell us the extent to which formation of the two domains is cooperative in different molecules, thus leaving significant ambiguities in terms of the overall folding mechanism.

A new approach involving hydrogen-exchange labeling and ESMS (26), has recently been employed to study the folding of lysozyme. The basis of this experiment is that the exchange of deuterons for protons results in a change of mass of the protein. ESMS allows the mass of an intact protein to be determined to an accuracy of about 0.005% (27). This approach is used to analyze the distribution of molecular masses in samples generated in the same manner as in the pulse-labeling experiments described above. For lysozyme these studies revealed three well defined species with different masses (and hence different extent of protection from exchange). One of them has a protection between that of denatured and the native states suggesting that it is associated with major folding intermediate in which the α-domain is formed in the absence of β-domain.

2.3 Stopped flow optical studies.

Despite the wealth of detail derived from the hydrogen exchange labeling studies, there are still several key issues about which little is understood. For example, these experiments cannot reveal the specific factors responsible for protection of a particular amide. Even the identity of hydrogen-bond acceptors cannot be determined, although synchronous protection of groups of amides corresponding to native-like structural elements often provides strong circumstantial evidence that these structures are formed in the intermediate detected. Furthermore, studies of peptide fragments of lysozyme in aqueous solution suggest that secondary structure alone is not sufficient to account for the exchange protection observed in these experiments.

Stopped-flow CD is of particular value in conjunction with the pulse-labeling experiments because it can provide an indication of the overall extent of structural organization in the

folding molecules, both in terms of secondary structure (from the far UV region) and tertiary structure (from the near UV). In the case of lysozyme studies in the far UV (2, 29 , 31 , 32) indicate that a large complement of secondary structure is formed within the first few milliseconds of folding. A detailed analysis of the spectrum of the intermediate at this time has suggested that the overall structural content may indeed be close to that of the native state (29). The rate of secondary structure formation is, therefore, much faster than the rate at which exchange protection develops, suggesting that protection probably arises from stabilization of already formed helical structure. Later excursions in the far UV CD are thought to reflect the changing contribution of non-peptide chromophores, a possible candidate being disulphide bonds in non-native conformations (29). Interestingly, the near UV CD spectrum develops as a single exponential function with kinetics similar to those of protection of amides in the β-domain. The fact that the ∝-domain is protected from exchange faster than the near UV CD develops (2, 32), even though the majority of aromatic residues concerned are contained in this domain, indicates that it cannot have a wholly native-like conformation in the partially folded intermediate as the aromatic residues are not yet fixed in their native orientations. In fact, this state has many characteristics of a 'molten globule', now observed to be formed for an increasing number of proteins under mild denaturing conditions (30).

The intrinsic fluorescence of the six tryptophan residues of lysozyme provides further insight into the behavior of side-chains during the folding process. A change in the environment of some or all of the six tryptophan residues was found to occur in early collapse, in later intermediates and on formation of the native structure. The quenching of fluorescence by iodide was also used as a measure of the exposure of the fluorescing indole groups to bulk solvent. The results again show that there is a marked decrease in the susceptibility to fluorescence quenching in the dead time of the experiment, providing compelling evidence that there is substantial exclusion of water, even in the very early stages of folding. Thereafter the accessibility decreases further and reaches a native-like value by around 100 ms. At later times there is little further change in the accessibility to the quenching agent, suggesting that the slow phases observed by total fluorescence intensity result from rearrangement of the local environment of tryptophan residues without substantial alteration of their solvent exposure.

Hydrophobic clustering during refolding of lysozyme was studied by monitoring the binding to the fluorescent dye 1-anilino naphthalene sulphonate (ANS). This dye binds to hydrophobic regions with a concomitant increase in its fluorescence quantum yield and has often been used to identify partially folded intermediates (30). Emission from this probe in the lysozyme folding experiments is maximal in the very early stages of the reaction, supporting the idea that a relatively loosely packed, condensed state exists at these times. Subsequently, the intensity drops indicating, in accord with the fluorescence quenching experiments, that in later intermediates hydrophobic surfaces are more excluded from solutes. Only in the slowest phase of folding is the fluorescence enhancement finally abolished, suggesting that at least part of the hydrophobic core is not organized until this late stage.

While all of these probes monitor aspects of the structure of the refolding molecule, one of the fundamental outstanding questions is the time scale of formation of the native functional enzyme. This can be determined directly by monitoring the evolution of binding to a fluorescent sugar inhibitor and therefore, the kinetics of formation of the active-site cleft (25). These studies show that formation of the active-site cleft occurs in a single kinetic event with a rate essentially identical to (1) the slow step of protection of amides in the β-domain, (2) the development of the near UV CD, and (3) the slowest steps observed in the fluorescence intensity and far UV CD experiments. This shows that the active site, which is situated between the ∝- and β-domains, does not require further structural rearrangements following the formation and docking of the structured domains in the slow (-350 ms) folding phase. By contrast, molecules observed by ESMS to be protected in both domains in the fast (5-10 ms) phase cannot be entirely native since the active-site cleft is formed much more slowly than this as judged by the inhibitor binding experiment.

2.4 The nature of the folding intermediates.

The combination of techniques used to characterize the refolding of lysozyme has allowed two distinct types of folding intermediates to be identified. The earliest detectable species can be described as a partially collapsed fluctuating state, with substantial secondary structure, but which has few stable tertiary interactions. The second type of intermediate is

populated in the next stage of folding in which persistent (protective) structure evolves. The major one of these has amides protected from exchange in only the ∝-domain; the β-domain is still unstable and rapidly fluctuating, and does not significantly protect amides from exchange. A second protected intermediate appears to be present; this intermediate is relatively minor but is particularly interesting, however, in that both the ∝- and β-domains have formed presumably native-like structure and yet this species is not the fully organized native enzyme. By about 200 ms into the folding process, a single major intermediate remains in which the ∝-domain has formed a highly stable structure with a hydrophobic core which is well developed, but which is still not completely ordered. The native conformation of the enzyme forms in the slowest step observed, with a time constant of about 350 ms. In the majority of molecules this occurs concomitantly with the formation of stable structure in the β-domain. In all cases this involves organization of the fine details of the tertiary structure both within the hydrophobic core of the ∝-domain and in the alignment of the two folding domains.

2.4.1 Stable intermediates:

Both CD and hydrogen exchange data suggest there is some similarity between the stable molten globule state in a lactalbumin and the major kinetic intermediate of lysozyme which has persistent structure in the ∝-domain alone (2, 8, 28). NMR studies of the molten globule state of ∝-lactalbumin reveal a high degree of disorder in the majority of side chains, although conformational preferences are evident in some regions of the structure (33). However, it seems likely, from the greater magnitude of the amide protection factors and the relatively diminished fluorescence enhancement on ANS binding that the lysozyme kinetic intermediate is more highly structured than the equilibrium molten globule state of ∝-lactalbumin (34, 35). The stable molten globule state may, however, have a greater resemblance to intermediates populated in the dead time of the lysozyme refolding experiments. A stable, partially folded state of lysozyme has been generated in 50% trifluoroethanol (TFE) at pH 2.0 (19). This state at lest superficially resembles the major kinetic intermediate in that amides in the ∝-domain are protected from exchange in the absence of a stable β-domain. Interestingly, the protein appears to contain at least a native amount of helical structure as viewed by far UV CD, but only those

regions of the structure that are helical in the native state are substantially protected from exchange. This suggests that there may be substantial structural preferences inherent in local sequence elements which could be important in directing the early stages of folding.

2.5 Determinants of folding.

The results of a variety of experimental approaches focussed on a single experimental system, reveals the events, occurring during the folding of lysozyme. By combining both experimental and theoretical approaches (36, 39), one can speculate on the crucial question - how is the folding of a protein directed to the native state ?

2.5.1 Collapse and secondary structure formation

Very rapidly after refolding is initiated, secondary structure forms. The overall content may be close to that of the native protein but the persistence of specific interactions seems to be limited at this stage. Few specific tertiary interactions are formed but there is evidence that a significant amount of bulk water is already excluded, to form a relatively condensed structure. This may provide the driving force for formation of the hydrogen-bonded secondary structure. Whether or not hydrophobic collapse precedes secondary structure formation is still under debate (38, 39). Despite this, it is conceivable that, as the hydrophobic interior develops in the collapsing protein, specific types of secondary structure could be stabilized in sequences with particular patterns of hydrophobic and hydrophilic residues. An amphipathic helix is a particularly clear example of this as it will be stabilized at an interface between an aqueous surface and a hydrophobic interior. Thus, although the protein structure undoubtedly remains fluid and fluctuating at this stage, the formation of a compact state could enhance or amplify intrinsic preferences for secondary structure, as well as restricting the conformational space that needs to be explored to further the folding process (40).

2.5.2 Stabilization of secondary structure.

Within this initial collapsed state, regions of secondary structure then coalesce resulting in their mutual stabilization (41). It is at this stage that protection from hydrogen exchange is

first observed. Provided that native-like secondary structure predominates in the collapsed state, there should be a higher probability that native-like assemblies will result. Selection of native structure may be further enhanced if side chain complementarily is greater in this state rather than in non-native, structures. Indeed, the greater the degree of cooperativity (as seen, for example, in the protection kinetic of the individual domains of lysozyme), the easier it may be for folding to be directed towards native-like rather than non-native-like structures. It seems likely that the order of stabilization of secondary structural elements will vary in detail between individual protein molecules within a folding population and between closely related proteins. For example, the folding pathways of hen and human lysozymes differ in the rate and manner in which elements of secondary structure are assembled, even though the fundamental characteristics of their folding pathways are very similar.

2.5.3 Multiple pathways:

Given that the order of assembly of secondary structural elements may differ in different molecules, there is no reason that only a single pathway should exist (42, 44), indeed, this seems not to be the case for lysozyme. In addition, given that the preference for certain types of secondary structure over other types is generally limited, even if this is amplified by hydrophobic collapse, it is likely that unproductive pathways will be explored and 'mistakes' will need to be rectified (43). It is possible that this is the origin of the multiple folding pathways found for lysozyme. Here, the faster phases might represent the formation of molecules with 'correct' interactions, while the slower phases may represent molecules misfolded in previous events. Further experiments will need to be performed to explore this possibility further.

2.5.4 Towards the native structure:

With the interactions that stabilize segments of the structure in place, folding can proceed towards the native structure. These later steps in folding are, in general, slower than the early events, and it is only in these final stages that the specificity of side chain packing is developed. As the native structure is approached, barriers to rearrangement are established as molecules become more and more closely packed. Only at these late stages of folding is it likely that

conformational entropy is sacrificed and is compensated by the favorable noncovalent forces within the ordered native state. Contrary to early speculation, it appears not to be the extent of conformational space that limits the rate of folding but rather the rearrangement of near-native conformational states to the native structure.

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CHAPTER 3

IONIC STRENGTH DEPENDENT TRANSITION OF HEN EGG WHITE LYSOZYME AT LOW pH TO A COMPACT STATE AND ITS AGGREGATION ON THERMAL DENATURATION.

3.1 INTRODUCTION

The extent of unfolding of denatured states of proteins under different conditions has long been of interest because of the possible relevance of their conformations to the protein folding pathways. Studies have been focussed on the conditions which disrupt the cooperative folding of the protein but allow substructures to persist. Recently, it has been demonstrated that residual structural preferences, ranging from local clusters of side chains to highly ordered side chains to highly ordered subdomains, persist in denatured states of proteins (1-7, 35). Hence, there has been a growing recognition of the importance of the compact denatured and partially folded states of proteins, as determination of their structure and thermodynamic properties may provide critical insight into the mechanisms of protein folding. The characterization of these structures and the factors involved in their stability would provide important insight into the interactions responsible for their formation as well as their role in protein folding.

The importance of electrostatic interactions in determining the stability of a protein has long been recognized (8). pH is known to influence the stability of a protein by altering the net charge on the protein. and many proteins denature at extreme pH because of the presence of destabilizing repulsive interactions between like charges in the native protein (9, 10) . Extremes of pH do not always completely denature the proteins but result in denatured states that are partially unfolded as compared to the fully unfolded ones observed in presence of high concentrations of guanidine hydrochloride (11, 12). This is maybe due to the fact that the driving force for acid-unfolding of proteins are repulsive forces between ionized residues in the protein which may fail to overcome the interactions such as hydrophobic forces, salt bridges, etc. that favor folding. The exact behavior of a given protein at low pH is a complex interplay between a variety of stabilizing and destabilizing forces, some of which are sensitive to the environment. Salt-dependent conformational transition at acidic pH's from a largely unfolded state to an intermediate conformational state have been reported for several proteins (13-16), suggesting that salt-dependent conformational transitions at acidic or alkaline pH

regions may be a general property of many proteins.

Denaturation of c-type lysozyme occurs without any thermodynamically stable intermediate forms (8, 17, 18). The thermodynamic and folding behavior of c-type lysozymes under equilibrium conditions have found to accord well with a cooperative two-state model (19). However, under conditions far from equilibrium, existence of a transient species has been suggested for hen egg lysozyme on the basis of refolding studies (20). Furthermore, at least one, and possibly more, intermediate states with characteristics of molten globules, notably ordered secondary structure without specific tertiary interactions as monitored by near- and far-UV CD, were reported to be formed during folding of c-type lysozyme (19, 20). Recently, the existence of an equilibrium partially folded state of human lysozyme at low pH has been reported (21). The population of this intermediate state was found to be strongly dependent on pH and temperature and had at least some characteristics of the molten globule state of homologous α -lactalbumin and of kinetic intermediates observed in the folding of c-type lysozymes. No such partially folded intermediate at low pH has been reported for hen egg lysozyme (18, 21). This prompted us to look into the structural details of hen egg lysozyme at low pH.

In the present work, we have carried out a comparative study on the structural as well as thermodynamic properties of hen egg lysozyme at pH 7.0 and 1.5. At pH 1.5 hen egg lysozyme has a substantial secondary structure, a large solvent exposure of non-polar clusters and a significantly disrupted tertiary structure. Furthermore, a large enthalpy is associated with conversion of acid denatured lysozyme to a fully unfolded state. These observations suggest the presence of an equilibrium partially folded intermediate of hen egg lysozyme at low pH and this intermediate seems to be more ordered than most other molten globules discussed in the literature. Studies on the effect of ionic strength on the partially folded intermediate showed its ionic strength dependent transition to a compact conformation and aggregation on thermal denaturation.

3.2 MATERIALS AND METHODS

Materials.

All the chemicals used were purchased from Sigma Chemical Company, St. Louis, USA and were of the highest purity grade. Hen egg white lysozyme was purchased from Sigma Chemical Company, St. Louis, USA. The purity of it was checked by SDS-PAGE followed by silver staining. It was found to be > 95% pure.

Circular Dichroism (CD).

CD measurements were made on a Jasco J-500C spectropolarimeter at 27 ^{0}C. Cuvette of 0.1- and 1-cm pathlength were used for the ranges of 190-250 and 250 to 350 nm, respectively. The solvent spectrum was determined and subtracted from the protein spectrum. The concentrations of hen egg lysozyme samples was typically 2-4 uM for far-UV and 20-40 uM for near-UV. Molar ellipticities, θ (in degrees centimeter squared per decimoles of residues), are reported.

Differential scanning calorimetry.

All calorimetric scans were performed with Microcal MC-2 differential scanning calorimeter. The calorimetric unit was interfaced to an IBM PC microcomputer using an A/D converter board (Data Translator DT-2801) for automatic data collection and analysis. The protein concentration of each sample used for the calorimetric experiments was about 2-3 mg/ml; 1.3 ml of sample was introduced into the sample cell and a similar amount of buffer into the reference cell. Sample were scanned at a rate of 60 ^{0}C/h, or otherwise mentioned. The samples were degassed for 15 min at room temperature prior to being scanned in the calorimeter. Data reduction and analysis was performed with software developed by Prof. Ernesto Freire (22).

ANS fluorescence measurements.

Fluorescence spectra were recorded with Perkin-Elmer LS5B spectroluminescencemeter in a 5mm path length quartz cell. The aliquots of protein with final protein concentration of 50 uM were equilibrated in the desired buffer for 30 minutes at 25 ^{0}C. They were then mixed with concentrated stock solution of ANS dissolved in the same buffer. The excitation wavelength was 365 nm and the emission was recorded either from 420 to 520 nm or at fixed wavelength at 480 nm. The final ANS concentration was 125 uM. The values were normalized by subtracting the baseline recording for ANS alone.

3.3 RESULTS

pH induced structural perturbations in hen egg white lysozyme .

Secondary and tertiary structure (far- and near- UV CD).

The effect of lowering the pH on the secondary and tertiary structure of native hen egg lysozyme was studied by recording the nea`r- and far-UV CD spectra at pH 7.0 and 1.5. The CD ellipticities at 222 and 270 nm are commonly used as a measure of the extent of secondary and tertiary structure respectively, in proteins. Figure 3.1A, shows the near- and far-UV CD spectra of hen egg lysozyme at pH 7 and 1.5. For hen egg lysozyme at pH 1.5, a spectrum with significant negative ellipticity was observed in the far UV-CD, but the observed ellipticity at 222 nm was significantly less as compared to that observed for the native protein. Similarly, in the near-UV region also a significant lowering of ellipticity for acid denatured lysozyme as compared to the native lysozyme was observed. These observations suggest that lowering of pH to 1.5 results in partial unfolding of hen egg lysozyme, as a decrease and not complete disruption of both secondary and tertiary structure was observed.

Solvent-accessible non-polar clusters (ANS binding).

The solvent exposure of non-polar clusters in the acid unfolded hen egg lysozyme at pH 1.5, was studied by ANS binding. ANS binds to solvent-accessible clusters of non-polar

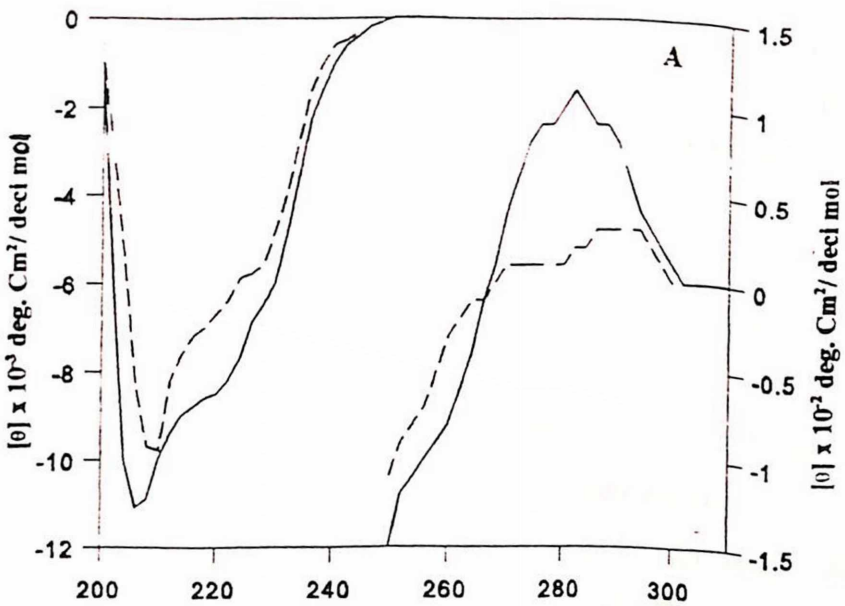

Figure 3.1 A: Far-UV and near-UV CD spectra of hen egg lysozyme at pH 7.0 and 1.5 at 27 ^{0}C. The solid line represents the curve for hen egg lysozyme at pH 7.0 and the dashed line represents the curve for lysozyme at pH 1.5. Protein concentrations were about 0.35 mg/ml and 1.23 mg/ml for far- and near UV-regions respectively. The spectra were collected after substraction of solvent baseline.

groups in proteins. The fluorescence emission of ANS is known to increase on binding to hydrophobic clusters of a protein (23). Figure 3.2 shows the fluorescence spectra on binding of ANS to native and acid denatured hen egg lysozyme. At pH 7.0, no significant ANS fluorescence was observed, suggesting a very low binding of ANS molecules to native lysozyme. But for acid denatured lysozyme at pH 1.5, a very large increase in ANS fluorescence intensity, to about 30 fold, was observed. Furthermore, the emission maximum also shifted from 515 to 480 nm, indicating the burial of ANS molecules in a hydrophobic environment under these conditions (24). The physical reason for these observed behavior may be the formation of solvent-accessible non-polar clusters on acid-induced partial unfolding of protein; these clusters are screened from the solvent in the native protein.

Structural perturbations in partially folded intermediate induced by ionic strength.

Ionic strength induced perturbations in the secondary and tertiary structure of hen egg white lysozyme at pH 1.5, was studied by far- and near-UV CD studies in presence of increasing concentration of NaCl. Figure 3.1B shows the far- and near- UV CD spectra of hen egg lysozyme at pH 1.5 in presence and absence of 100 mM NaCl. No significant changes in the far UV-CD spectra characteristics of acid denatured lysozyme was observed in presence of 100 mM NaCl. Contrary to this, in the near-UV CD spectra, addition of 100 mM NaCl resulted in a large increase in the ellipticity at 270 nm, indicating that NaCl induces a significant alteration in the tertiary structure. Further increase in NaCl concentration upto 500 mM, showed no significant changes either in the far- or near-UV CD characteristics (data not shown). These observations suggest that increase in ionic strength of hen egg lysozyme solution at pH 1.5 has no significant effect on the secondary structure of protein , but has a pronounced effect on the tertiary structure.

Effect of ionic strength on the solvent exposure of non-polar clusters present in the acid denatured lysozyme was also studied. As shown in figure 3.2, addition of 100mM NaCl to hen egg lysozyme at pH 1.5, resulted in a significant drop of about 16 fold, in the ANS fluorescence intensity. No further drop in ANS fluorescence intensity was observed on

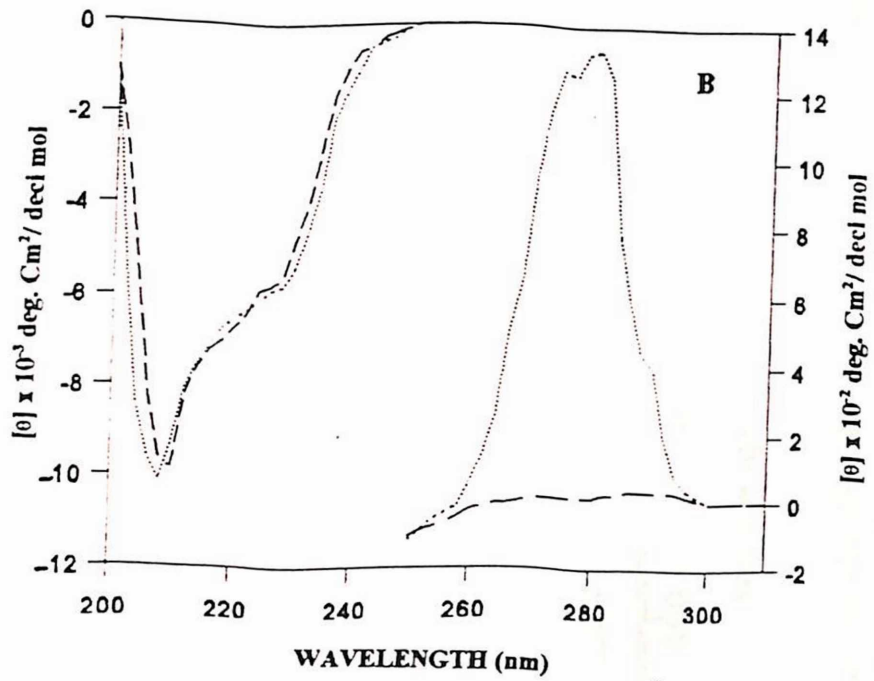

Figure 3.1B: Far-UV and near-UV CD spectra of hen egg lysozyme at pH 1.5 and in presence of 100 mM NaCl at 27 ^{0}C. The dashed line represents the curve for lysozyme at pH 1.5 and the dotted line represents the curve for lysozyme at pH 1.5 in presence of 100 mM NaCl. Protein concentrations were about 0.35 mg/ml and 1.23 mg/ml for far- and near UV-regions respectively. The spectra were collected after substraction of solvent baseline.

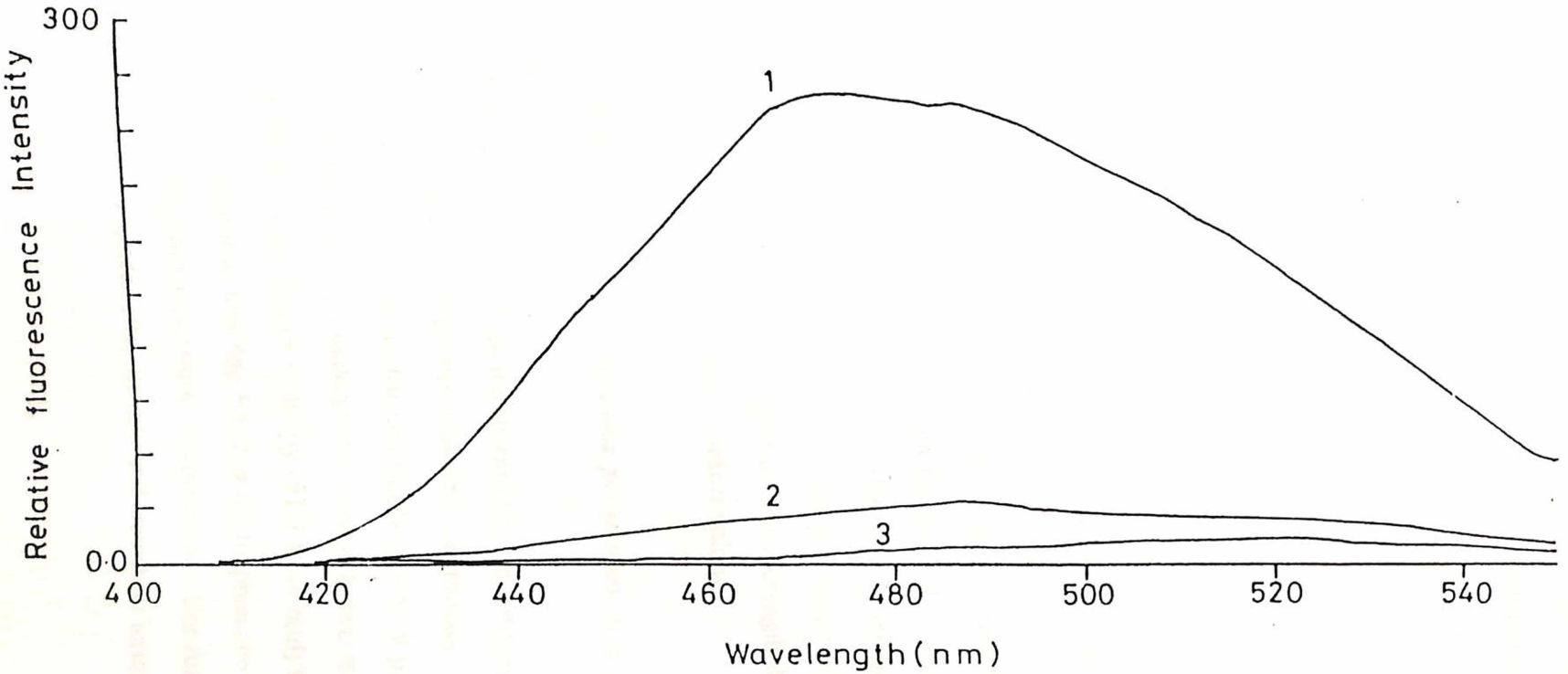

Figure 3.2: Fluorescence spectra of 125 uM ANS in the presence of 5uM hen egg lysozyme at pH 7.0 and 1.5 at 27^{0}C. Curve 1 is for protein at pH 1.5; Curve 2 for protein at pH 1.5 in presence of 100 mM NaCl and curve 3 for protein at pH 7.0

increasing the NaCl concentration upto 500 mM (Table 3.1). These observations suggest that addition of NaCl to lysozyme solution at pH 1.5, induces a conformational transition such that the solvent-exposed non-polar clusters present in the acid denatured lysozyme get buried, as is the case in the native protein.

Compactness.

Recent studies have shown that, size-exclusion chromatography permits the characterization of native, unfolded and intermediate states (26, 27). Hence, the effect of ionic strength on the molecular size of acid denatured hen egg lysozyme was studied by gel filtration chromatography. Figure3.3 summarizes the retention time observed for hen egg lysozyme at pH 1.5 in presence of increasing concentration of NaCl. For acid denatured hen egg lysozyme at pH 1.5, a retention time of 21.40 minutes was observed. Addition of 100 mM NaCl to this lysozyme solution resulted in an increase of 3 minutes (24. 43 min.) in retention time, indicating that addition of NaCl to acid dentured lysozyme results in a decrease in its molecular size . Further increase in NaCl concentration resulted in a significantly smaller increase in retention time. These observations suggest an ionic strength dependent transition of acid denatured hen egg lysozyme to a compact conformation.

Effect of ionic strength on the thermodynamic properties of acid induced partially folded intermediate.

The effect of ionic strength on the thermodynamic properties of acid denatured lysozyme at low pH was studied by differential scanning calorimetry. Figure 3.4 shows the excess heat capacity versus temperature curves for hen egg lysozyme at pH 1.5 in presence and absence of NaCl. Lysozyme at pH 1.53 undergoes a reversible two state transition centered at 53.3 ^{0}C. This suggests that a substantial enthalpy (91.7 k cal/mol) is associated with the thermal unfolding of acid denatured hen egg lysozyme. In presence of 100 mM NaCl, a decrease of about 3 °C in transition temperature was observed. For further increase in NaCl concentration upto 400 mM, a similar DSC profile and transition temperature was observed

TABLE 3.1

Effect of ionic strength on the ANS binding of hen egg lysozyme at pH 1.5.

[NaCl] mM	*ANS FLUORESCENCE **
0	*350*
50	*88*
100	*36*
200	*26*
300	*19.3*
400	*19.4*
500	*19*

* Fluorescence was measured at 480 nm, with excitation at 360 nm.

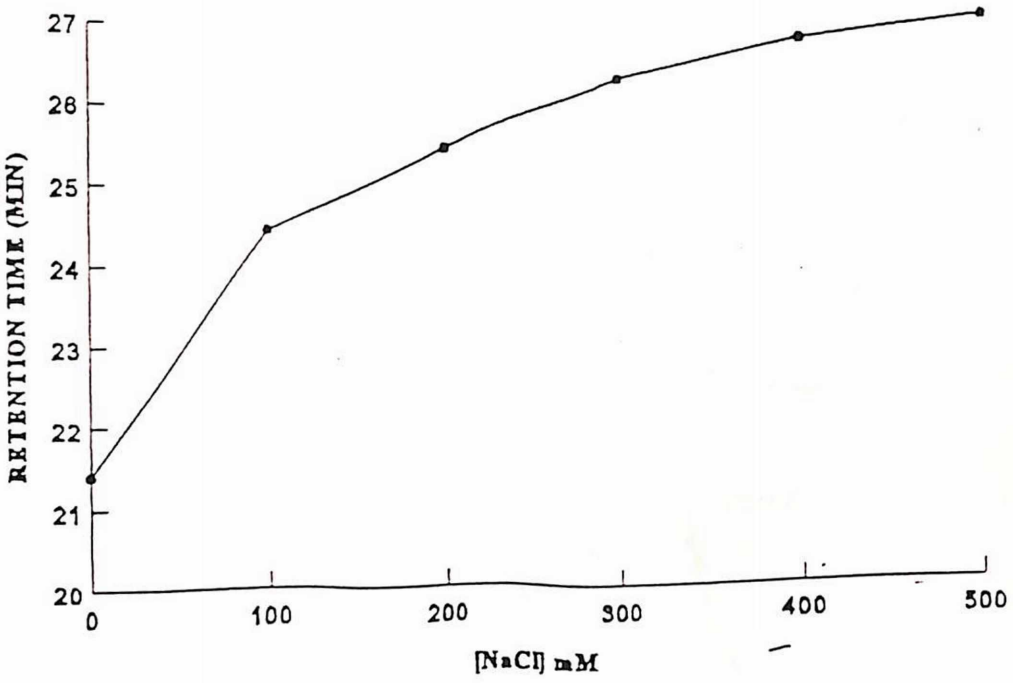

Figure 3.3: Retention time of hen egg lysozyme at pH 1.5 as a function of NaCl concentration on TSK G3000 SW column at 27 ^{0}C. The stock solutions of proteins were loaded into the column which was equilibrated with the same buffer. The flow-rate was 30 ml/h. The stock solutions of proteins were loaded into the column (Pharmacia Ultrapac TSK G3000 SW) which was equilibrated with the same buffer.

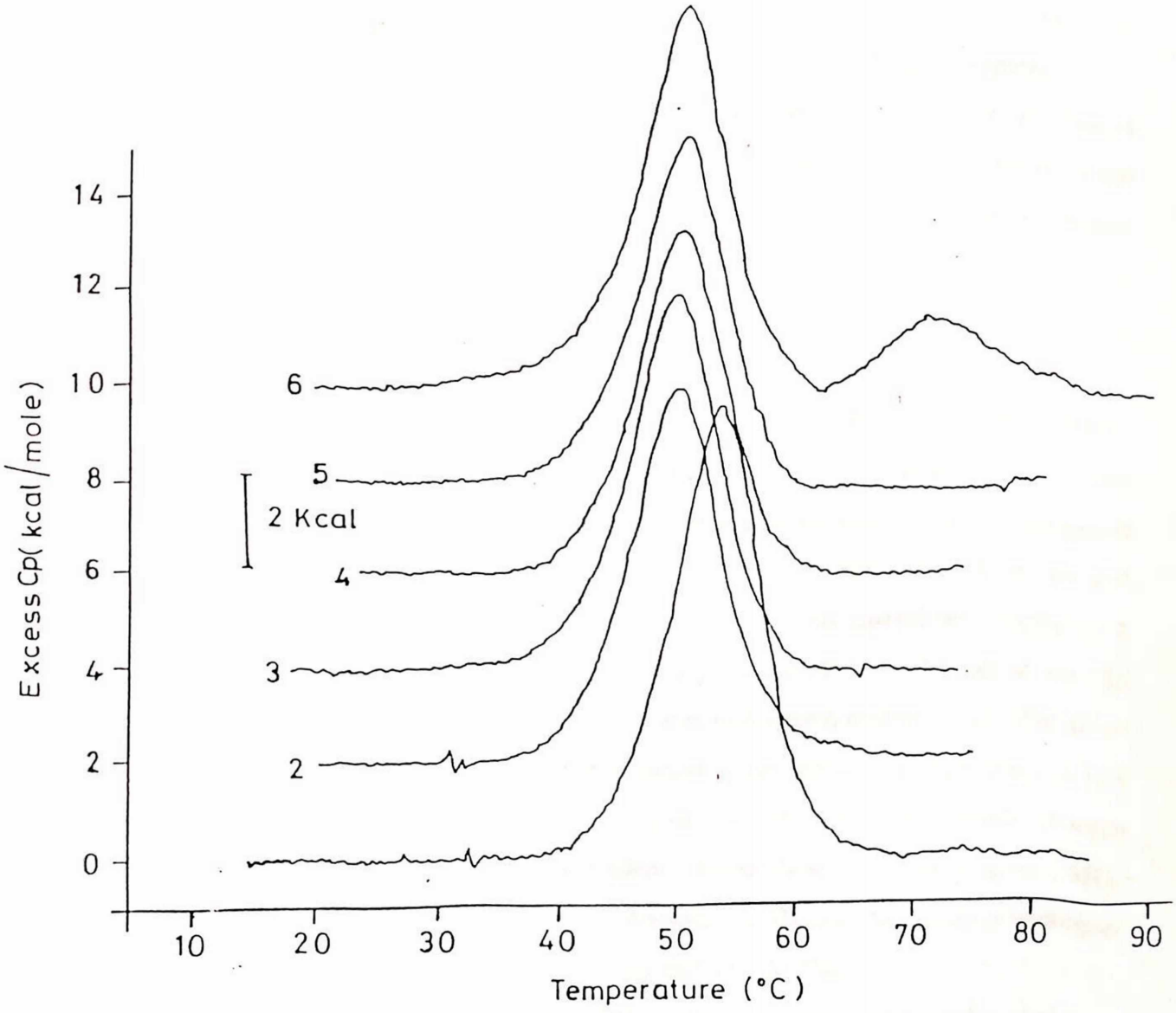

Figure 3.4: Excess heat capacity versus temperature curves for hen egg lysozyme at pH 1.5 as a function of NaCl concentration. The calorimetric scans were performed with a protein concentration of 3.5 mg/ml at a scan rate of 60°C/h. Curve 1 is hen egg lysozyme at pH 1.5, curve 2 to 6 are hen egg lysozyme at pH 1.5 in presence of 100 to 500 mM NaCl, respectively. The reversibility of all the scans, except for in presence of 500 mM NaCl, was better than 95%. Excess heat capacity function was obtained after subtracting the baseline from the heat capacity function.

(Figure 3.4). But, in presence of 500 mM NaCl, two independent transitions centered at 50 (corresponding to the thermal denaturation of acid denatured lysozyme under these conditions) and 72 °C, respectively, were observed . The thermodynamic parameters obtained from DSC studies are summarized in Table 3.2. All the transitions except for the higher temperature one observed in presence of 500 mM NaCl, were found to be following the two state process as indicated by ratio of vant Hoff to calorimetric enthalpy; unity or near-unity values for this ratio provide a necessary and sufficient criterion for a two-state process (22). For the higher temperature transition observed in presence of 500 mM NaCl, the ΔHvh/ ΔHcal ratio was found to be 5.1, suggesting the presence of aggregated state.

Ionic-strength dependent aggregation of acid induced partially folded intermediate.

As mentioned above the higher temperature transition observed in the DSC scan of lysozyme at pH 1.5 in presence of 500 mM NaCl suggested the presence of an aggregated state of lysozyme solution on thermal unfolding. In order to confirm this , scan rate and protein concentration dependent studies under these conditions were carried out. Figure 3.5A shows the DSC scans of lysozyme at pH 1.5 in presence of 500 mM salt at varying concentrations of protein. As seen in the figure, the area under the transition centered at about 71 °C, was found to increase with increasing protein concentration. Furthermore, at low protein concentration, a shift in the transition temperature was also observed. Similar changes were observed for studies with scan rate dependence, under these conditions (Figure 3.5B). These observations suggest that acid denatured lysozyme on thermal denaturation undergoes self association leading to aggregation, in presence of 500 mM NaCl.

The ionic strength dependent aggregation of thermally denatured acid induced partially folded intermediate of hen egg lysozyme was further confirmed by turbidity measurements and proton NMR studies. Figure 3.6 shows the aggregation monitored by turbidity measurements at 340 nm for hen egg lysozyme at pH 1.5 in presence of increasing NaCl concentration at 50 ^{0}C. As shown in the figure no change in the absorbance of the protein solution was observed for acid denatured lysozyme in presence of NaCl upto 400 mM,

TABLE 3.2

Thermodynamic parameters obtained from differential scanning calorimetric studies on the ionic strength dependence of hen egg white lysozyme at pH 1.5.

NaCl[mM]	*Tm °C*	*ΔHcal(kcal/mol)*	*ΔHvh(kcal/mol)*	*ΔHvh/ΔHcal*
0	*53.33*	*91.7*	*87.9*	*0.96*
100	*50.03*	*79.6*	*82.7*	*1.04*
200	*49.79*	*80.1*	*83.4*	*1.04*
300	*50.17*	*72.1*	*85.2*	*1.18*
400	*50.32*	*71.6*	*86.4*	*1.2*
500	*50.7*	*80.7*	*84.6*	*1.05*
	2.33	*16.3*	*88.4*	*5.42*

* Two independent transitions in the Cp versus temperature scan were observed.

The lysozyme concentration for these studies was 0.407 mm (5.8 mg/ml); and scan rate was 60 °C/hour.

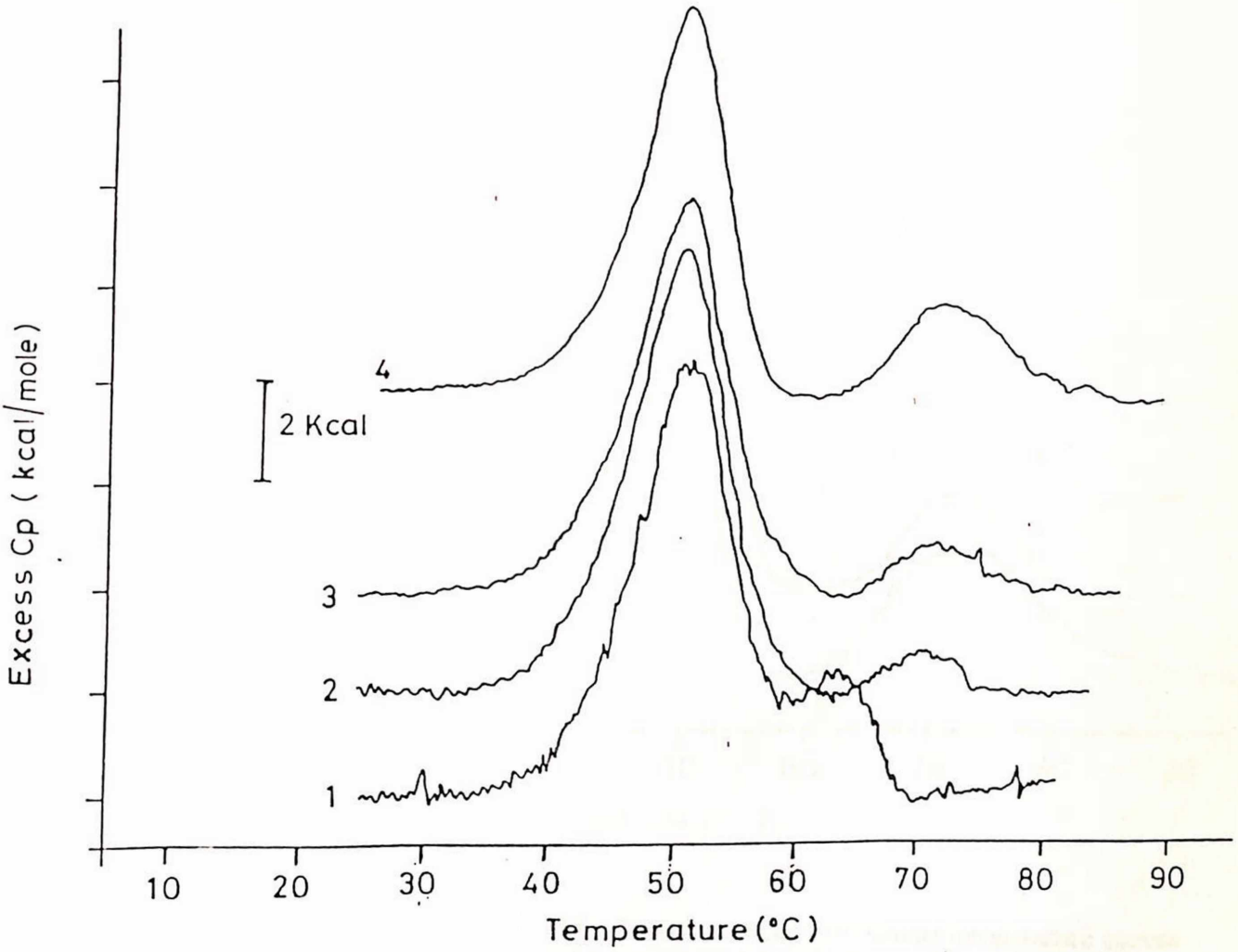

Figure 3.5 A: Protein concentration dependent excess heat capacity versus temperature curves for hen egg lysozyme at pH 1.5 in presence of 500 mM of NaCl. The calorimetric scans were performed at a scan rate of 60^{0}C/h. The concentration of hen egg lysozyme for various scans were as follows: Curve 1: 0.112 mM; curve 2: 0.24 mM; curve 3: 0.358 and curve 4: 0.479 mM. Excess heat capacity function was obtained after subtracting the baseline from the heat capacity function.

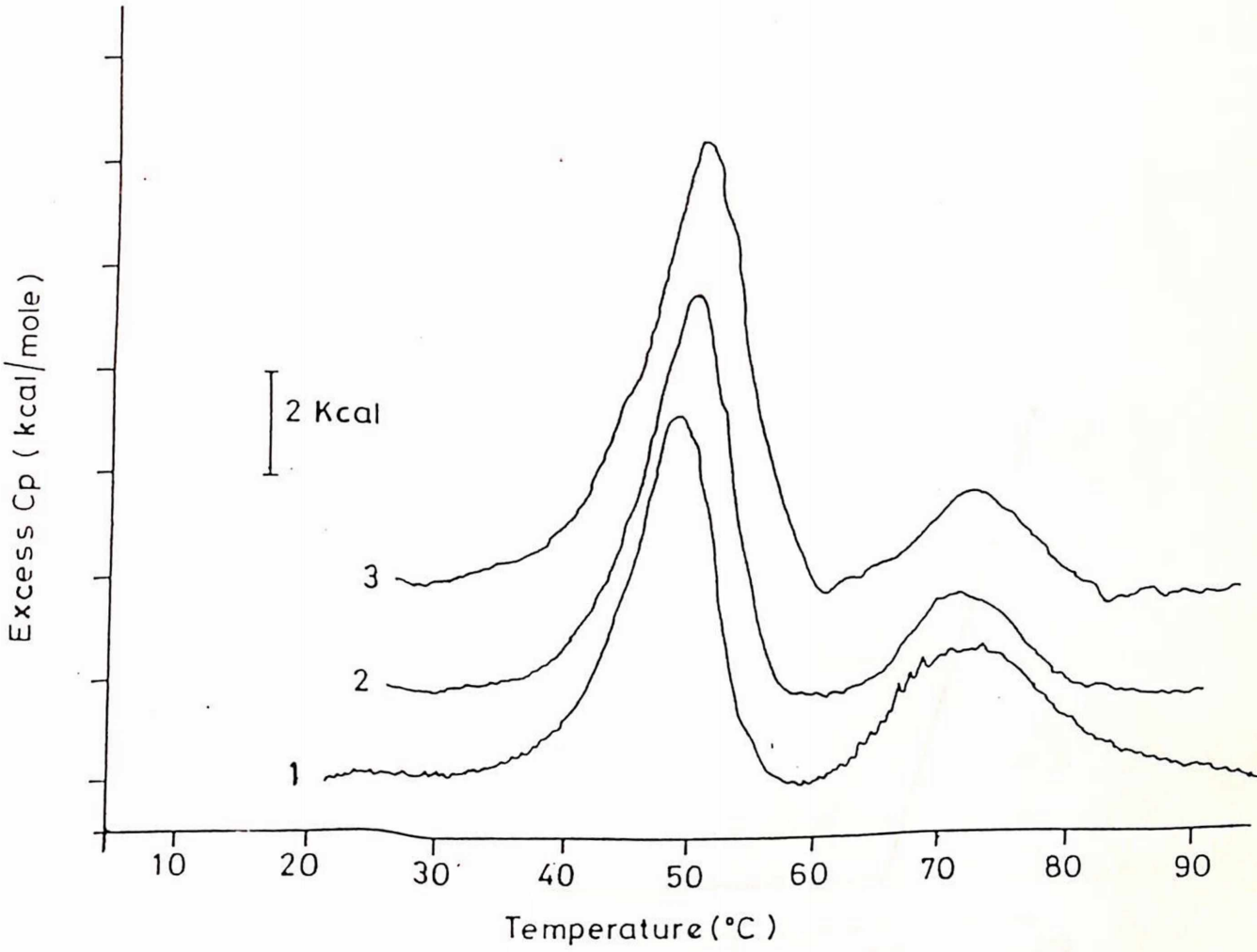

Figure 3.5B: Dependence of scan rate on the excess heat capacity versus temperature curves for hen egg lysozyme at pH 1.5 in presence of 500 mM NaCl. The calorimetric scans are : curve 1, 30 ^{0}C/hour; curve 2: 60 ^{0}C/hour and curve 3: 90 ^{0}C/hour . The protein concentration used was 0.479 mM. Excess heat capacity function was obtained after subtracting the baseline from the heat capacity function.

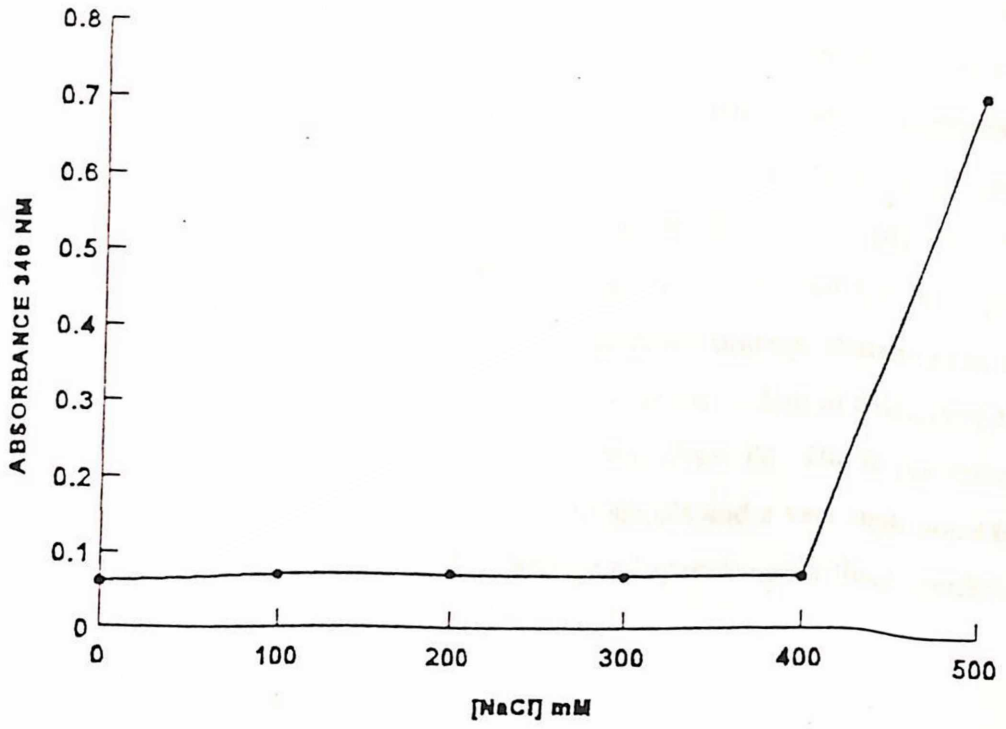

Figure 3.6: Turbidity measurements at 340 nm for hen egg lysozyme at pH 1.5 in presence and absence of NaCl at 50 ^{0}C. No absorbing species are present at this wavelength, so the light beam is attenuated by scattering from the aggregated protein particles. Samples were prepared and transferred to a cuvette, and the OD at 340 nm was monitored.

under these conditions. But in presence of 500 mM NaCl, a large increase in absorbance was observed. These observations suggest that acid dentured lysozyme in presence of 500 mM NaCl at about 50 ^{0}C undergoes aggregation. The temperature dependent aggregation of lysozyme in presence of 500 mM NaCl at pH 1.5 was further confirmed by the thermal unfolding studies using NMR. Figure 3.7 shows the NMR spectra of hen egg lysozyme at low pH and in presence of 100 and 500 mM NaCl at 25 and 50 ^{0}C. At 25 ° C, a very good dispersion of the aromatic and the amide protons was observed , both in presence and absence of NaCl suggesting the presence of a well structured protein. The addition of 100 or 500 mM NaCl showed no major changes in the spectra observed for acid denatured lysozyme. However, several changes in chemical shifts were observed which can be attributed to local changes in the environment associated with the ionization of individual acidic residues in lysozyme. Similar observations have been reported earlier for lysozyme at pH 3.8 in presence of 500 mM NaCl (27). Even at 50 °C, lysozyme at pH 1.5 showed a good dispersion of aromatic and amide signals, suggesting that protein does not undergo denaturation upto this temperature. In presence of 100 mM NaCl, a spectra corresponding to a denatured state as observed by the loss of several well defined signals, was observed. But in presence of 500 mM NaCl at 50 ^{0}C a significant broadening, loss of signals and a very high noise to signal ratio was observed, suggesting the presence of an aggregated species under these conditions and support the DSC data presented earlier.

3.4 DISCUSSION

Structural properties of the equilibrium partially folded intermediate of hen egg lysozyme and its similarity with kinetic folding intermediate.

The far- UV CD spectrum of hen egg lysozyme at pH 1.5 was characterized by a significant negative ellipticity at 222 nm, although this was significantly lesser as compared to that observed for native protein. Ellipticity at this wavelength reflects predominantly the α-helical structural content. Hence the above observations indicate that lowering of pH of hen

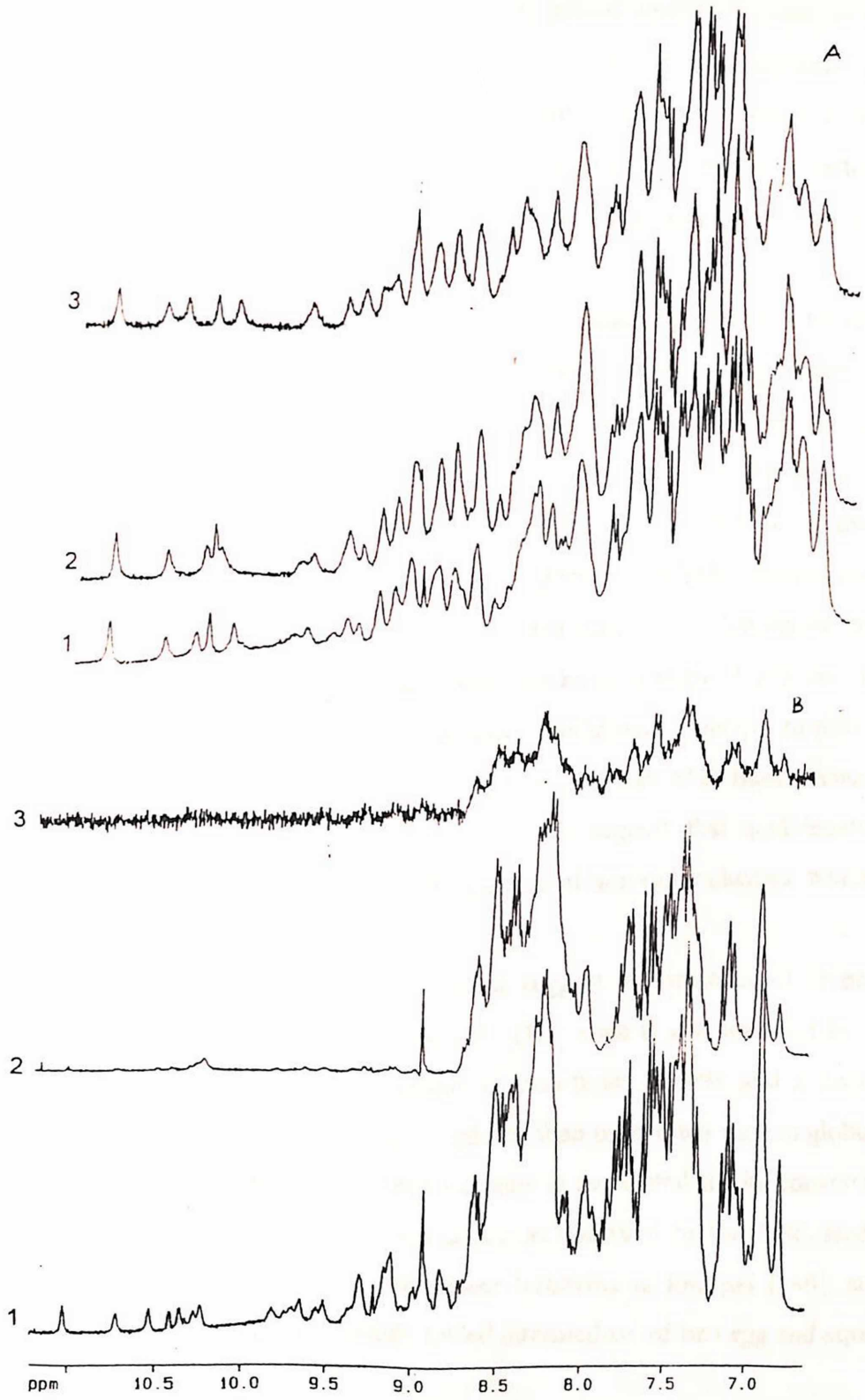

Figure 3.7: One-dimensional 1H-NMR spectra of hen egg lysozyme in the amide and aromatic region at pH 1.5 and in presence of 100 and 500 mM NaCl at (A) 25 ^{0}C and (B) 50 ^{0}C. In both the figures, Curve 1 is lysozyme at pH 1.5; Curve 2 is lysozyme at pH 1.5 in presence of 100 mM NaCl and Curve 3 is lysozyme at pH 1.5 in presence of 500 mM NaCl. Spectra were recorded on Bruker Avance DRX 300 spectrometer operating at 300 MHz, using a digital resolution of 0.182 Hz/point. Concentration of protein used was 30 mg/ml in D_2O buffer at pH 1.5. All spectra are referenced to an internal dioxane standard at 3.743 ppm.

egg lysozyme solution results in a partial loss of α-helical content of native protein. Similarly, a significant loss in the ellipticity was observed at 270 nm for acid denatured lysozyme, indicative of a large disruption of tertiary structure on acid denaturation at pH 1.5. These observations suggest that lowering of pH of hen egg lysozyme results in partial unfolding of lysozyme structure, as a significant secondary and partial tertiary structure was observed for acid-denatured lysozyme.

Semisotonov *et al.* (28, 29) have shown that a specific tool for characterization of intermediate states in protein folding is the binding of 8-alininonaphthalene-1-sulphonate (ANS). Binding of ANS to the equilibrium and kinetic intermediates is usually much stronger, resulting in a strong increase in fluorescence maxima, as compared to that in the native or fully unfolded states. The physical reason for this behavior has been attributed to the formation of solvent-accessible non-polar clusters in the intermediate state which remain screened from the solvent in the native and do not exist in the unfolded state. ANS binding studies reported in this study showed that ANS molecules does not bind to native lysozyme, as a very low fluorescence intensity was observed under these conditions. Contrary to this, a very strong ANS binding, as indicated by about 30 times increase in ANS fluorescence intensity for lysozyme at pH 1.5 was observed . These observations suggest that acid denaturation of hen egg lysozyme results in a significant solvent exposure of non-polar clusters which are shielded in the native lysozyme.

The above reported observations suggest the presence of a partially folded intermediate of hen egg lysozyme at low pH. This state is characterized by a significant secondary structure, a significant exposure of non-polar clusters and a disrupted tertiary structure, suggesting that this state is more ordered than most other molten globules discussed in the literature. Furthermore, a large enthalpy is associated for its conversion to a fully unfolded state (i.e. thermally denatured state), as observed by the DSC studies. Similar properties have been reported for the equine lysozyme at low pH (30), suggesting the structural similarity between the partially folded intermediate of hen egg and equine lysozyme at low pH..

Kinetic refolding of hen egg lysozyme has shown that the protein does not become organized in a single cooperative event but various parts become stabilized with very different kinetics. At least one, and possibly more, intermediate states with characteristics of

molten globule, notably ordered secondary structure without specific tertiary interactions as monitored by near and far-UV CD and exposure of non-polar clusters have been reported to be formed during the folding of hen egg lysozyme (19, 20). The equilibrium partially folded intermediate at low pH, reported in this paper also has similar structural characteristics, implying the possibility of structural similarity between the kinetic and the equilibrium intermediates.

Structural and thermodynamic characterization of salt-dependent compact conformation.

Studies on the effect of ionic strength on the secondary and tertiary structure of hen egg lysozyme at low pH was carried out by far- and near-UV CD spectroscopy. Increase in ionic strength did not show any significant effect on the secondary structure of acid denatured lysozyme as observed by the far-UV CD spectra, but a significant enhancement in the elipticity at 270 nm was observed, suggesting that presence of NaCl affects the tertiary structure of acid denatured lysozyme. The other significant effect of NaCl on the structural property of acid denatured lysozyme that was observed was a substantial decrease in the solvent-accessible non-polar clusters. In fact the solvent-accessible non-polar clusters observed in presence of 100 mM NaCl was similar to that observed for native lysozyme, suggesting that binding of Cl ions to the acid denatured lysozyme leads to a conformation in which the hydrophobic portion of the protein is buried inside, as is the case in native protein.

Regarding the thermodynamic properties, no significant effect on cooperativity or enthalpy of the heat induced transition was observed in presence of 100 to 500 mM of NaCl, although a slight decrease in Tm , about 3 ^{o}C, compared to the acid denatured state was observed . These results suggest that increase in ionic strength of hen egg lysozyme solution at pH 1.5 does not affect the secondary structure or the thermodynamic parameters of acid denatured lysozyme but affects the hydrophobic exposure and the tertiary structure significantly.

The main forces that are important for protein structures are the hydrophobic interactions, the valence forces, the dispersion forces and the repulsive forces (31). Under conditions of extreme pH's the main forces to unfold the protein are the repulsions between charged groups on the protein molecule. The stabilizing or destabilizing effects of salts on

proteins will thus arise either by effect on hydrophobic interactions, or by interactions with charged groups. The net effect of salt on protein stability, depends critically on the charge distribution on the native protein as well as on salt effects on its denatured state. The results presented in this paper suggest that increasing the ionic strength of pH 1.5 buffer by addition of NaCl results in a transition of partially unfolded protein to a compact conformation as observed by decrease in ANS fluorescence and an increase of retention time in HPLC studies. Hen egg lysozyme has a net charge of about 6 to 7 over the pH range 4-7 (32). Furthermore, it has also been suggested that for hen egg lysozyme, on an average, nearly one Cl^- ion binds for each proton bound below pH 6 over a wide range of ionic strength (32). Hence, it seems that addition of salt to the acid unfolded state of hen egg lysozyme would decrease the electrostatic free energy of the acid denatured state due to the fact that, in presence of NaCl the positively charged sites of the acid denatured protein would attract the counter-ion, Cl^- from both NaCl and HCl, and consequently the repulsion is reduced, resulting in a decrease of electrostatic free energy which may result in an increased compactness of this conformation.

Salt-dependent conformational transitions at acidic pH's from a largely unfolded state to an intermediate conformational state has been reported for a number of proteins (13, 15, 16). These reports along with the results presented here suggest that salt-dependent conformational transition at acidic or alkaline pH regions may be a general property of many proteins, however the mid salt concentration of the transition will depend on protein studied and other conditions.

Ionic-strength dependent aggregation of thermally denatured acid induced partially folded intermediate.

Based on several *in vitro* studies, it has been concluded by some investigators that folding intermediates rather than native or denatured proteins are generally involved in aggregation. While other investigators believe that intermediates, while possibly important for the aggregation of some proteins under certain solvent conditions, do not generally have to be invoked to explain protein aggregation. For proteins consisting of a single domain, there are only a few cases where intermediates have been shown to be involved in the aggregation of the protein (33, 34). The observations presented in this paper suggest that increase in ionic strength of lysozyme at pH 1.5 does not show any signs of aggregation. But temperature dependent

studies suggest that the thermally unfolded form undergoes self association leading to aggregation in a salt dependent manner, favoring higher salt concentration , >400 mM NaCl. These studies prove that thermally denatured state of lysozyme is involved in the ionic strength dependent aggregation. Furthermore, removal of NaCl from protein samples containing 500 mM NaCl at pH 1.5 showed a ^{1}H-NMR spectra identical with those before NaCl addition. This observation supports a close relation between aggregation and conformational change of the protein with salt.

Taken together, the results presented in this paper demonstrated the presence of an equilibrium partially folded state of hen egg lysozyme at low pH. The presence of an equilibrium intermediate state in a protein supposed to be a prototype of cooperative two-state unfolding is of great significance as it indicates the general occurrence of folding intermediates in the pathways of protein folding.

3.5 SUMMARY.

Equilibrium acid-induced unfolding of hen egg white lysozyme has been investigated by a combination of optical methods, size exclusion chromatography and differential scanning calorimetry. The results showed a presence of partially folded state of hen egg lysozyme at pH 1.5, characterized by a substantial secondary structure, a large solvent exposure of non-polar clusters and significantly disrupted tertiary structure. A large enthalpy was also found to be associated with the conversion of acid-unfolded state to a fully unfolded state. Size exclusion chromatography and ANS binding studies showed an ionic strength induced transition of the partially folded state to a compact conformation. Furthermore, an ionic strength dependent aggregation on thermal unfolding of the partially folded intermediate was also observed. These observations provide insights into the possible features responsible for the stabilization of intermediates in folding of hen egg lysozyme.

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CHAPTER 4

CHARACTERIZATION OF PROPANOL-INDUCED PARTIALLY FOLDED EQUILIBRIUM INTERMEDIATE OF HEN EGG WHITE LYSOZYME.

4.1 INTRODUCTION

It is generally accepted that folding of globular monomeric proteins occur through fixed pathways defined by the partially folded intermediates. The characterization of these partially folded intermediates and of features which give rise to their stability are of critical importance for the elucidation of the mechanism by which protein folds [1-4]. Recent studies of the *in vitro* mechanisms of protein folding have substantiated the significance of kinetic and thermodynamic intermediates for understanding how protein folds.

The process of protein folding is characterized by a high degree of cooperativity. Hence, almost negligible population levels of partially folded stable intermediates are observed during folding/unfolding transitions. For most small globular proteins (M_r< 20,000), the population of partially folded intermediates never exceeds more than 5% [5]. One of the recent approach for generating partially folded intermediates of proteins is to make use of non-aqueous solvents. Addition of alcohols is known to destabilize the tertiary structure [6-8] and stabilize the helical structure [9-11] of a protein. This mixed property of alcohols has been exploited for the induction of partially folded intermediates of proteins. The nature and degree of structures observed in these states and their relation to the transient folding intermediates from kinetic studies have become central to many investigations to protein folding. The alcohol denatured states of a number of proteins have been found to have some properties similar to those of molten globules [9,10,12].

We have studied the effect of propanol on the structural, functional and thermodynamic properties of hen egg white lysozyme under native conditions. The differential scanning calorimetric studies along with the tryptophan fluorescence studies showed the presence of a propanol induced transition of hen egg lysozyme at pH 7.0. A propanol induced equilibrium intermediate at 15% propanol was identified. The structural and functional characterization of this equilibrium intermediate showed that it had a native like secondary structure, enhanced tertiary structure, and a significant loss of catalytic activity. Furthermore,

it was found to undergo a non-cooperative thermal transition.

4.2 MATERIALS AND METHODS

Materials.

All the chemicals used were purchased from Sigma Chemical Company, St. Louis, USA and were of the highest purity grade. Hen egg white lysozyme was purchased from Sigma Chemical Company, St. Louis, USA. The purity of it was checked by SDS-PAGE followed by silver staining. It was found to be > 95% pure. Propanol was purchased from Aldrich Chemical company, USA.

Differential Scanning Calotimetry.

All calorimetric scans were performed with Microcal MC-2 differential scanning calorimeter. The calorimetric unit was interfaced to an IBM PC microcomputer using an A/D converter board (Data Translator DT-2801) for automatic data collection and analysis. The calorimetric scans were performed with a protein concentration of 3.5 mg/ml; 1.3 ml of sample was introduced into the sample cell and a similar amount of buffer into the reference sell. Samples were scanned at a rate of 60^0C/h. The samples were degassed for 15 min at room temperature prior to being scanned in the calorimeter. Data reduction and analysis was performed with the software developed by Prof Ernesto Freire [13]. Excess heat capacity function was obtained after subtracting the baseline from the heat capacity function.

Tryptophan Fluorescence.

Fluorescence spectra were recorded with Perkin-Elmer LS 5B spectroluminescencemeter in a 5mm path length quartz cell. Stock solution of hen egg lysozyme was made in 50 mM Tris-HCl, pH 7.0. Samples containing different percentages of

propanol were equilibrated at desired propanol concentration for 30 minutes at 25 ^{0}C before recording. The excitation wavelength was 290 nm and the emission was recorded from 300 to 500 nm. Protein concentration was 7uM for all experiments.

CD Measurements.

CD was measured with JASCO J500 spectropolarimeter calibrated with ammonium d-10-camphorsulfonate. Cuvetts of 0.1- and 1.0-cm path length were used for the ranges of 190-250 and 250-350 nm, respectively. The solvent spectrum was determined and subtracted from the protein spectrum. Protein concentrations were about 0.35 mg/ml and 1.23 mg/ml for far- and near-UV regions, respectively. The results are expressed as the mean residue ellipticity [θ]. which is defined as [θ]=100x θobs / (lc), where θobs is the observed ellipticity in degrees, c is the concentration in residue mol l^{-1}, and l is the length of light path in centimeters. Spectra were obtained in 50 mM Tris-HCl, pH 7.0.

Catalytic Activity.

The catalytic activity of hen egg lysozyme was studied by the decrease in absorbance at 450 nm of *M. lysodeikticus* cell suspension in 0.1 M potassium phosphate, pH 6.2 [14]. 0.5 ug protein was used for the experiments. The specific catalytic activity was evaluated from initial velocity of the lysis of *M. lysodeikticus* cell. The value obtained for native lysozyme was taken as 100% activity and the values relative to this were calculated.

Limited Proteolysis by Trypsin.

Lysozyme was incubated at 25^{0}C for 1h with trypsin (1:100 by weight) in 50 mM Tris-HCl, pH 7.0 and different concentration of propanol 0, 5, 10, 20, 30, 40, 70 % by volume. The reaction was stopped by adding soya bean trypsin inhibitor (1:5 trypsin:trypsin inhibitor by weight) at desired time. The sample buffer was added to this and the SDS PAGE

was carried out. Same pattern of digestion was observed even after 24 hour incubation with trypsin.

4.3 RESULTS

Propanol-Induced Transition.

Tryptophan fluorescence of hen egg white lysozyme was obtained in water-propanol mixtures with different concentrations of propanol (from 0 to a 70% by volume) at pH 7.0. Figure 4.1 illustrates the propanol dependence of fluorescence intensity at the maximum of the fluorescence spectrum as well as the wavelength of maximum emission. The figure shows that the increase in fluorescence consists of two stages. The increase observed in the first stage at low propanol concentration (0-20 % propanol) is much less as compared to that observed for the second stage at high propanol concentration (at 70% propanol). Furthermore, the first stage is accompanied by a negligible shift of wavelength maxima (336 nm) as compared to the native protein (336.5 nm), while the second stage is coupled with a large red shift of wavelength maxima to 340.5 nm (at 70% propanol).

The existence of propanol-induced transition of hen egg white lysozyme at pH 7.0 was further confirmed by differential scanning calorimetric studies. Effect of propanol on the stability and thermodynamic properties of native lysozyme was studied using differential scanning calorimetry (DSC). Figure 4.2 A shows the excess heat capacity versus temperature scan for lysozyme at pH 7.0 with increasing propanol concentrations. The thermodynamic parameters obatined from the DSC studies are summarized in Figure 4.2B. Figure 4.2 B (ii) illustrates the dependence of propanol on the entahlpy (ΔHcal) associated with the temperature dependent denaturation of hen egg lysozyme. ΔHcal shows a complex dependence on propanol composition, an initial increase followed by a decrease and finally pleatueing at higher propanol concentration. The main transition observed for the ΔHcal versus propanol concentration almost coincides with the first transition observed in tryptophan fluorescence

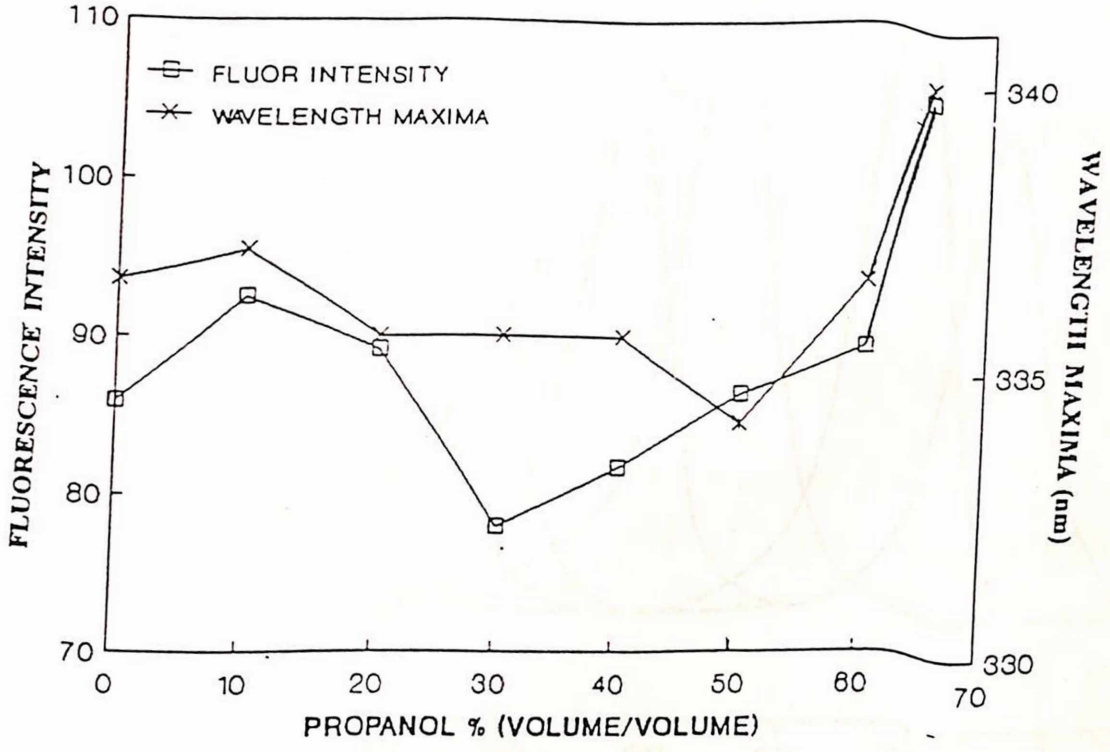

Figure 4.1: Dependence on propanol concentration (% by volume) of the tryptophan fluorescence intensity and wavelength of hen egg lysozyme in 50 mM Tris-HCl, pH 7.0 and 25^{0}C.

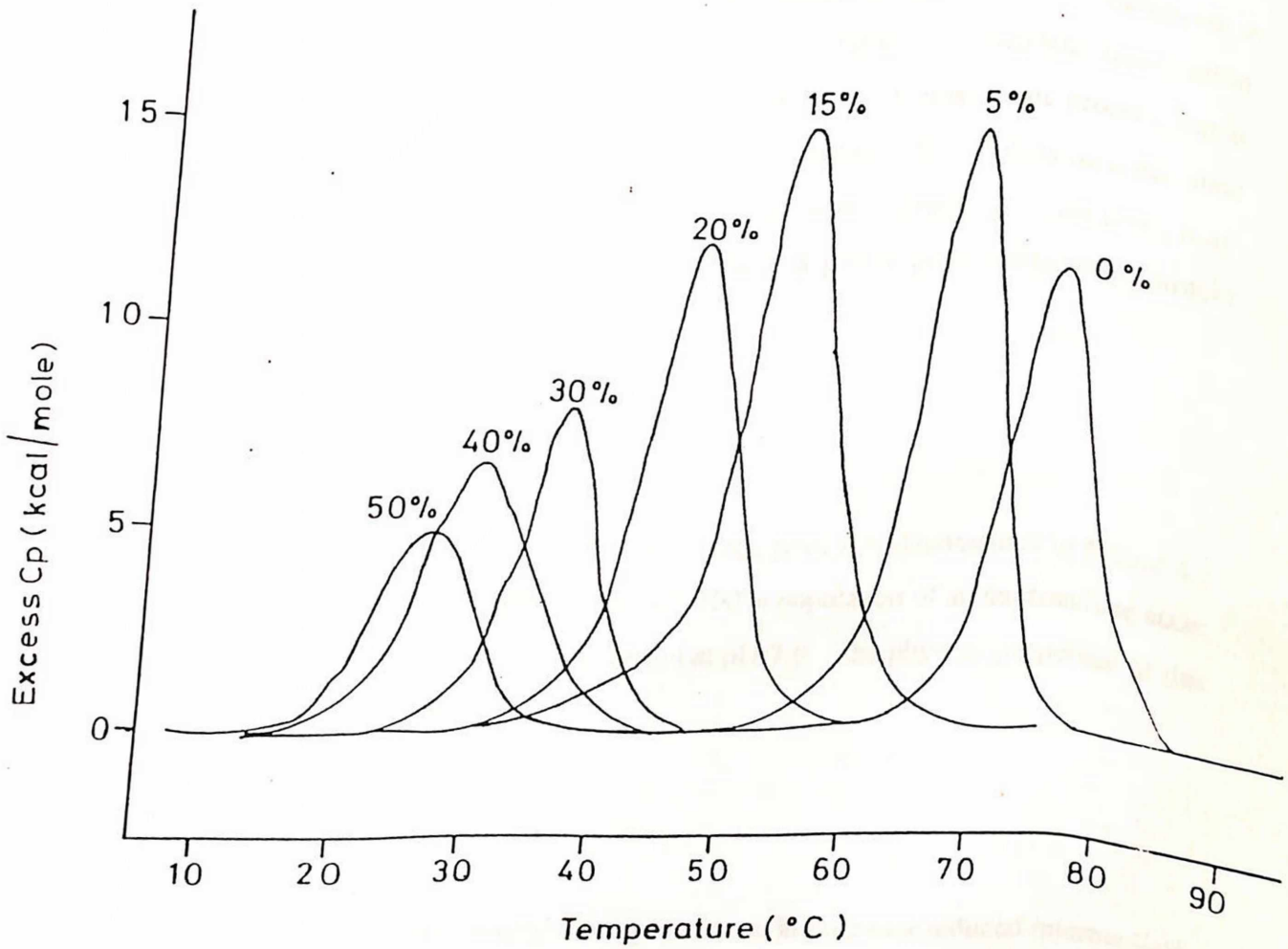

Figure 4.2 A: Excess heat capacity versus temperature curves for hen egg lysozyme at pH 7.0 as a function of propanol concentration. The numbers refer to propanol concentration in percentage by volume.

studies at low propanol concentration.

The most interesting observation from differential scanning calorimetric studies was the effect of propanol on the cooperativity of hen egg lysozyme on thermal denaturation. This is determined by the $\Delta H_{vh}/\Delta H_{cal}$ ratio from DSC data and summarized in Figure 4.2B (iii). Unity or near-unity values for this ratio provide a necessary and sufficient criterion for a two-state process [13]. At low propanol concentration the temperature dependent denaturation of hen egg lysozyme showed a shift from two-state-process to a non-two state process, but at higher propanol concentration the melting process was again found to be following a two state process, thus indicating that addition of lower concentrations of propanol brings about non-cooperativity in the native protein which otherwise is a prototype of cooperative protein [15 16].

Intermediate State.

The propanol-induced transition in hen egg lysozyme documented in Figure 4.1 is well resolved, suggesting the presence of almost 100% population of an intermediate state. This 100% population corresponds to 15% propanol at pH 7.0. The physical properties of this intermediate state are as follows:

Secondary structure.

The far-UV CD spectrum of hen egg lysozyme in propanol-induced intermediate state is compared with the spectra of native protein and at high concentration of propanol in Figure 4.3. As shown in the figure, in presence of 15% propanol far UV-CD spectra similar to that observed for native hen egg lysozyme was observed. But, at high propanol concentration (70% by volume) a significant enhancement of α-helical content was observed.

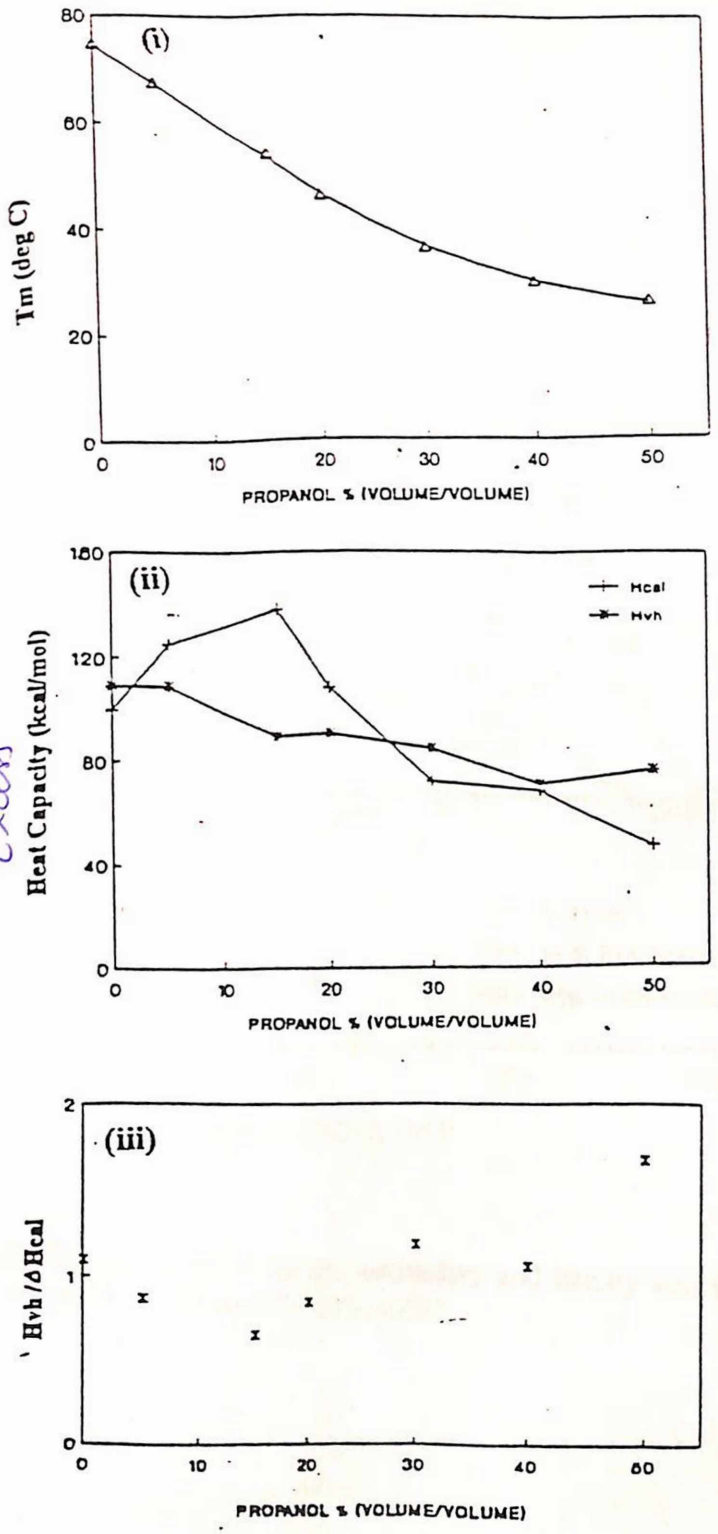

Figure 4.2B: Propanol concentration dependence of (i) transition temperature, (ii) calorimetric and van't Hoff enthalpy changes, and (iii) van't Hoff to calorimetric enthalpy ratio as associated with the thermal unfolding of hen egg lysozyme at pH 7.0.

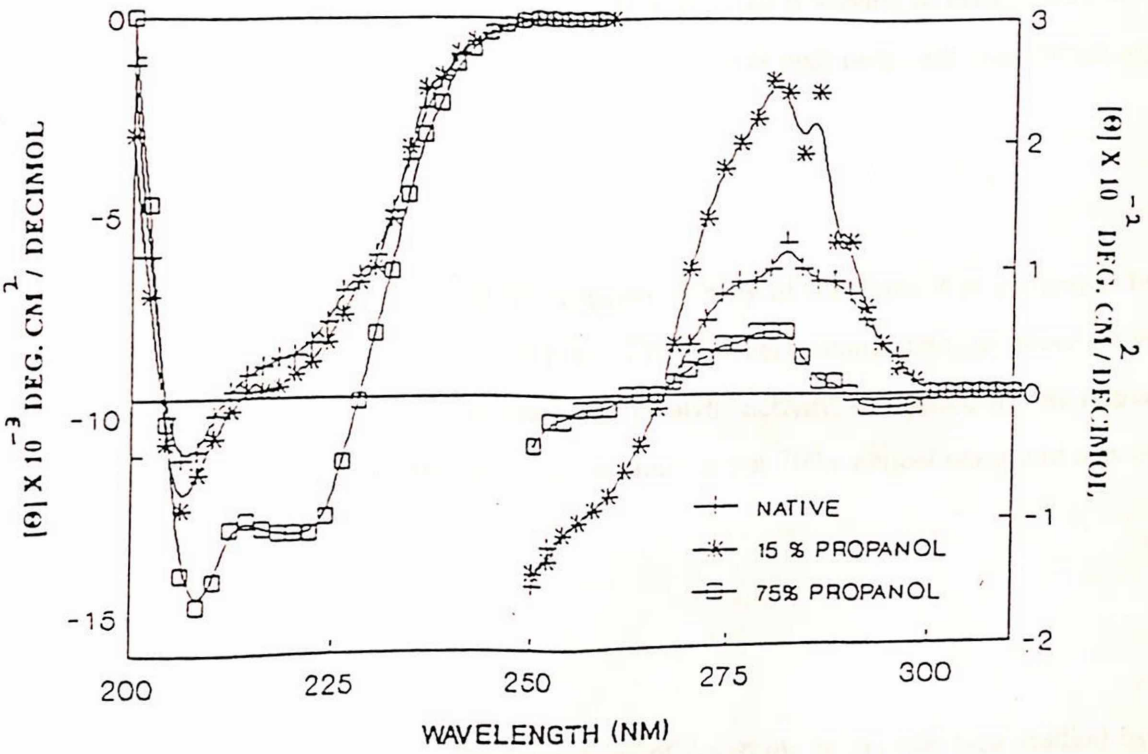

Figure 4.3. *Dependence of propanol on the secondary and tertiary structure of hen egg lysozyme as measured by far- and near-UV CD at 25°C.*

Tertiary structure.

Figure 4.3 presents the comparison of the near-UV CD spectra for the intermediate state in 15% propanol with spectra of native protein and at high propanol concentration. A significantly higher ellipticity at 280 nm as compared to the native protein was observed for the intermediate state, suggesting the presence of a more ordered structure for the intermediate as compared to the native protein. Contrary to this, a significant decrease in the ellipticity compared to the native was observed for hen egg lysozyme in presence of 70% propanol indicating a significant disruption of tertiary structure at high propanol concentrations.

Catalytic activity.

The effect of propanol on the catalytic activity of lysozyme was evaluated by following the lysis of *M. lysodeikticus* cells [14]. For the intermediate state (in presence of 15% propanol by volume), a 50 percent loss in the catalytic activity, compared to the native protein, was observed. At higher propanol concentration,about 70%, almost complete loss of activity was observed.

Limited proteolysis by Trypsin.

Effect of propanol on the proteolysis of lysozyme by trypsin was studied by varying the alcohol concentration, protease concentration and time of incubation. The results of the SDS/PAGE analysis as summarized in Figure 4.4 shows a propanol concentration dependent cleavage of lysozyme by trypsin. As shown in the figure the digestion of lysozyme by trypsin occurs at propanol concentrations exceeding approximately 30% by volume, indicating that only above this concentration of propanol lysozyme acquires a new conformational and dynamic state prone to attack by trypsin.

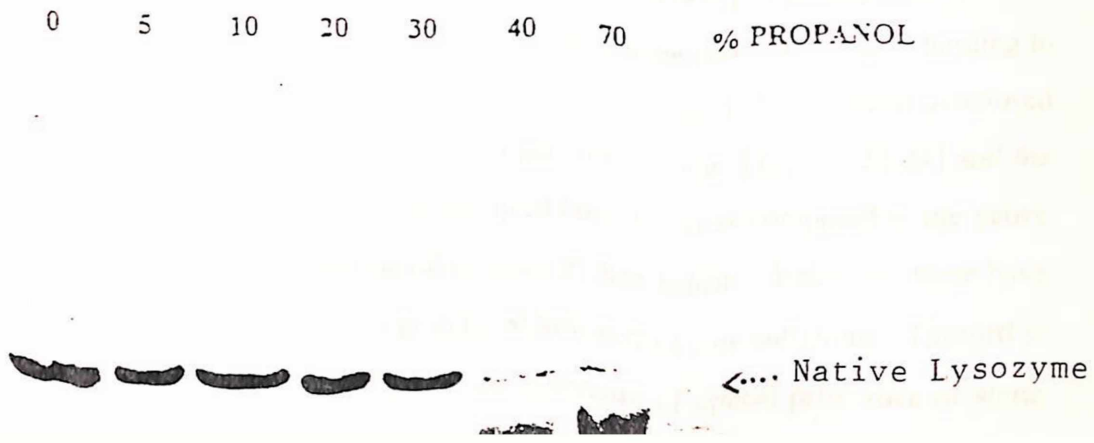

Figure 4.4: SDS/PAGE analysis (reducing conditions) of the proteolysis of hen egg lysozyme by trypsin. The details are give under materials and methods section.

4.4 DISCUSSION

Cosolvents, such as alcohols , are commonly used to induce partially folded states in proteins. The basic physical mechanism by which alcohols influence protein structure is still unsolved. The various physico-chemical properties of proteins that have been reported to be affected by alcohols are that, alcohols strengthen hydrogen bond interactions in the protein resulting in an enhanced α-helical content in the protein [17-19]. Furthermore, alcohols acts to influence the hydrophobic interactions within the protein either by directly binding to the hydrophobic regions [20] or by causing changes in water structure [21]. Proteins denatured by high alcohol concentration have been studied by many investigators [10, 11, 22-24] and the general result is that all of them have more pronounced far-UV CD as compared to the native or molten globule state, suggesting a substantial increase in their helicity. Relatively there have been a few reports on the denaturation of proteins at low alcohol concentrations. Tanford *et al* [25,26] have demonstrated the existence of an extremum of optical properties of some proteins at moderate alcohol concentrations. But the detailed structural or thermodynamic characterization under these conditions was not studied. Recently methanol induced denaturation of cytochrome C at low pH has been studied[27]. A two-stage transition of cytochrome C by methanol under these conditions was observed.

In the present study we have demonstrated that at low concentration of propanol an equilibrium intermediate is formed. This intermediate form is characterized by a secondary structure similar to the native, an enhanced tertiary structure and significant loss of catalytic activity. The most significant observation was that propanol induced intermediate of hen egg lysozyme showed a non-cooperative melting transition. The thermodynamic and folding behavior of c-type lysozymes under equilibrium conditions have been found to accord well with a cooperative two-state model [15,16]. However, under conditions far from equilibrium, existence of a transient species has been suggested for hen egg lysozyme on the basis of refolding studies [28]. Furthermore, at least one, and possibly more, intermediate states with characteristics of molten globules, notably ordered secondary structure without specific tertiary

interactions as monitored by near- and far-UV CD, were reported to be formed during folding [15,28]. Hence the identification of a propanol-induced intermediate at 15% propanol, having a non cooperative melting transition under equilibrium conditions of hen egg lysozyme, which is supposed to be a prototype of cooperative two-state unfolding, is of great significance as it indicates the general occurrence of folding intermediates in the protein folding pathways.

4.5 SUMMARY

Effect of propanol on the thermodynamic, structural and functional properties of hen egg white lysozyme at pH 7.0 was investigated by the combined use of differential scanning calorimetry, optical methods, proteolytic digestion and catalytic activity. A propanol-induced transition of hen egg lysozyme was observed. The results indicated that low propanol concentration induce a non-cooperative disorganization of native lysozyme molecule on thermal denaturation. A propanol-induced equilibrium intermediate at 15% propanol by volume was identified, which was characterized by a native like secondary structure, enhanced tertiary structure, and a significant loss of catalytic activity. Furthermore, this intermediate was found to undergo a non-cooperative melting transition.

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CHAPTER 5

ALCOHOL-INDUCED PARTIALLY FOLDED INTERMEDIATES OF HEN EGG WHITE LYSOZYME: *A Structural and thermodynamic characterization.*

5.1 INTRODUCTION:

One of the recent approach to generating partially folded proteins is to make use of water-miscible solvents, especially alcohols (1-6). It is known that addition of certain organic solvents to an aqueous protein solutions induce alterations in protein structure. Residual structural preferences persists in denatured states of proteins and in polypeptides in aqueous - organic solvent mixtures, ranging in characteristics from local clusters of side chains to highly ordered subdomains (1-7). The extent and order of structures detected in these states, intermediate between the native folded conformation and fully unfolded chain, have become central to many investigations of protein folding. The characterization of these structures and of features which give rise to their stability are likely to provide important insights into the interactions which are responsible for their formation as well as their possible role in protein folding.

Addition of alcohols such as methanol. ethanol, propanol, trifluoroethanol and 2-chloroethanol . is known to induce non-native partially folded states in protein and to increase the helical content of short peptides (8-12). Alcohol-induced structures in proteins have been shown to have high α-helical content and are usually characterized by the absence of specific tertiary interactions (6,7,13). The type of unfolding process one can visualize for alcohol denaturation of proteins has been summarized as follows:

N <=====> I <=====> H

Native Partially unfolded Helical

According to this model, the native folded protein N, is first converted to the partially folded non-native intermediate I in which the buried hydrophobic amino acid residues in the interior part of the native protein become largely exposed, and then converted to a nonglobular form H richer in α-helical content. The ordered and usually more helical conformation observed at higher alcohol concentrations, is represented by a greater number of segments having helical folding. Studies on optical spectroscopy and hydrodynamic measurements (14-16) have shown that, at high methanol, ethanol and 2-chloroethanol concentrations, globular proteins as a rule tend to assume more helical conformation relative to native proteins. Furthermore, in presence of monohydric alcohols, the temperature of thermal denaturation of proteins decreases linearly

with increasing alcohol concentration suggesting that alcohols effect the stability of native proteins (17,18).

Although lot of work has been carried out on the structural details of the alcohol induced denaturation of globular proteins nevertheless, no comprehensive work has been carried out in order to correlate the effect of alcohol on stability of protein and its structural features. These studies are of importance in order to assess that whether the helical conformation observed at high alcohol concentration is due to the effect of alcohols on the native or unfolded protein. Such an information will be of great importance in understanding the mechanism of alcohol-induced denaturation of globular proteins as well as in understanding the pathways of protein folding.

The present study was undertaken to correlate the thermodynamic properties with the structural features observed on alcohol induced denaturation of lysozyme in trifluoroethanol (TFE) and methanol. The results presented in this paper suggest that alcohol decrease the stability of proteins as reflected by the decrease in mid point of the melting temperature of lysozyme observed in presence of increasing concentration of alcohols. In presence of high alcohol concentration in buffer-alcohol system, the melting transition temperature of lysozyme was found to be below 5°C thus suggesting that under these conditions the proteins are in thermally unfolded state even at room temperature. Temperature dependent CD studies and limited proteolysis studies suggested that the increased helical open structure observed at high alcohol concentration is due to the effect of alcohol on the unfolded (thermally denatured) state rather than the native state of the protein.

5.2 MATERIALS AND METHODS

Materials:

All the chemicals used were purchased Sigma Chemical Company, St. Louis, USA and were of the highest purity grade. Hen egg white lysozyme was purchased from Sigma Chemical Company, St. Louis, USA. The purity of it was checked by SDS-PAGE followed by silver staining. It was found to be > 95% pure.

Circular Dichroism (CD).

CD measurements were made on a Jasco J-500C spectropolarimeter at 27 °C unless otherwise specified. Cuvette of 0.1-cm pathlength was used and scanned for the ranges of 190-250 nm. The solvent spectrum was determined and subtracted from the protein spectrum. The concentrations of hen egg lysozyme samples was typically 2-4 uM. The results are expressed as the mean residue ellipticity [θ], which is defined as $[\theta]=100x\ \theta_{obs} / (lc)$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in residue mol l^{-1}, and l is the length of light path in centimeters. Spectra were obtained in 50 mM Tris-HCl for pH 7.0 and in 50 mM glycine-HCl for pH 2.0.

Differential scanning calorimetry.

All calorimetric scans were performed with Microcal MC-2 differential scanning calorimeter. The calorimetric unit was interfaced to an IBM PC microcomputer using an A/D converter board (Data Translator DT-2801) for automatic data collection and analysis. The protein concentration of each sample used for the calorimetric experiments was about 2-3 mg/ml; 1.3 ml of sample was introduced into the sample cell and a similar amount of buffer into the reference cell. Samples were scanned at a rate of 60 °C/h, or otherwise mentioned. The samples were degassed for 15 min at room temperature prior to being scanned in the calorimeter. Data reduction and analysis was performed with software developed by Prof. Ernesto Freire (19). Excess heat capacity function was obtained after subtracting the baseline from the heat capacity function.

Limited proteolysis using thermolysin.

Lysozyme was incubated at 25°C and 4 °Cfor 24h with thermolysin (1:20 by weight) in 50 mM Tris-HCl containing 10 mM $CaCl_2$, pH 7.0 and different concentration of TFE 0, 10, 20, 30, 40, 50 % by volume. The reaction was stopped by adding EDTA at desired time. The sample buffer was added to this and the SDS PAGE was carried out.

5.3 RESULTS:

Effect of alcohols on the thermodynamic properties of native and acid denatured lysozyme on thermal denaturation.

Differential Scanning calorimetry.

Figure 5.1 and 5.2 , are representative of the DSC scans for hen egg-white lysozyme in TFE-water mixtures at pH 7.0 and 2.0, respectively. The heat absorption curves shown correspond to thermal denaturation of lysozyme under these conditions in increasing methanol and TFE concentrations. It can be observed that the temperature of maximum heat capacity (Tm) decreases with increasing alcohol concentration, while the peak area becomes smaller and the peak width slightly larger. All the transitions were also found to be reversible. The thermodynamic parameters obtained through analysis of these DSC curves are summarized in Figures 5.3 to 5.5.

Effect of alcohol on the Tm.

Figure 5.3 shows the dependence of alcohol concentration on the temperature mid point of thermal denaturation. A clearly decreasing trend of mid point of thermal transition with increasing alcohol concentration was observed irrespective of the pH and alcohol studied. A decrease in Tm of thermal denaturation of protein suggests the destabilization of native protein by the additive, hence the above observation indicates that alcohols destabilize the native lysozyme. Furthermore, the slope of the Tm versus alcohol concentration curve for TFE, both at native and acidic pHs for lysozyme was found to be significantly more steeper as compared to that observed for methanol under similar conditions. These observations suggest that TFE is much more effective in destabilizing protein conformation as compared to methanol.

Depression in denaturation temperature of proteins by alcohols has been reported earlier for several proteins using spectroscopic studies of thermal denaturation. In the earlier reported differential scanning calorimetric studies on lysozyme at pH 2.0 in presence of monohydric alcohols it has been shown that the effectiveness of monohydric alcohols in decreasing the

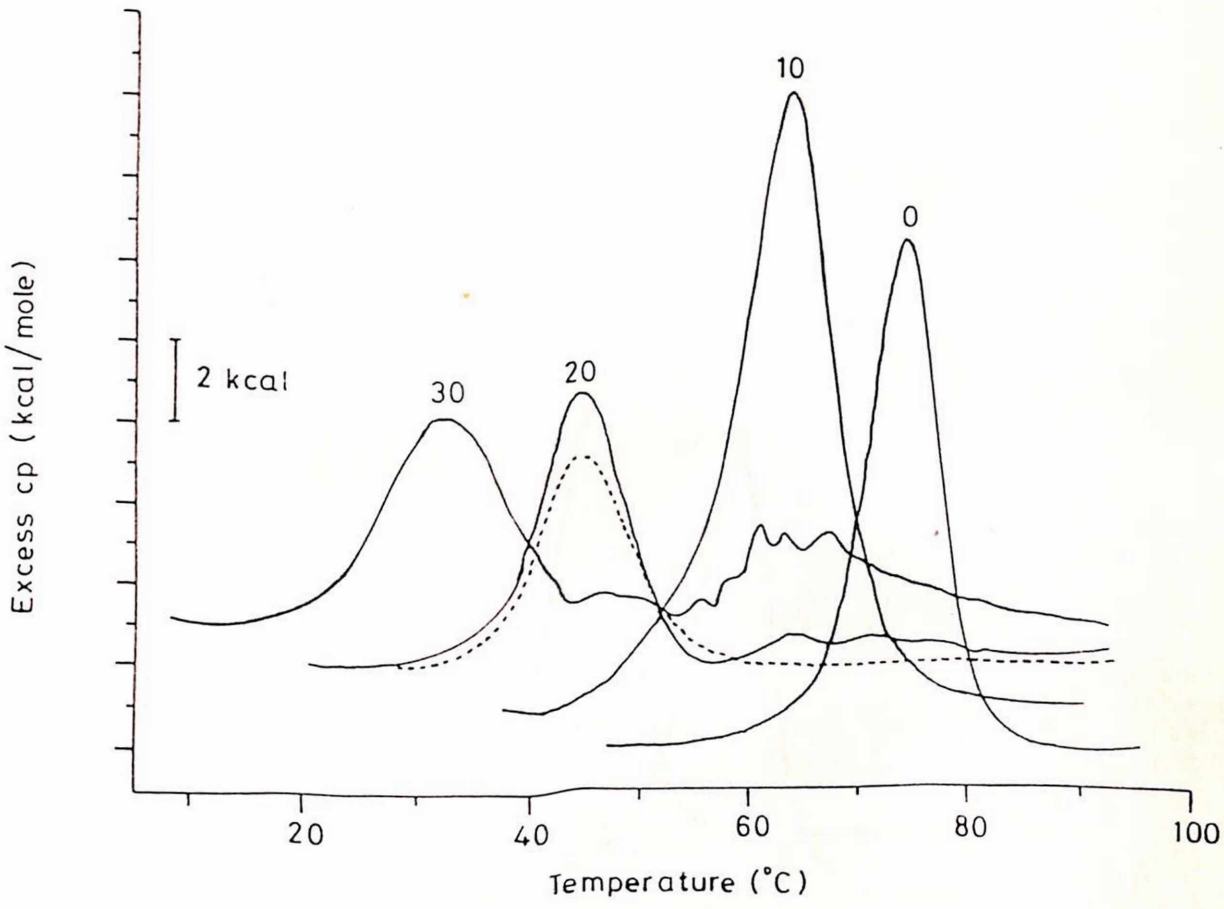


Figure 5.1. Excess heat capacity versus temperature curves for hen egg white lysozyme at pH 7.0 as a function of TFE concentration. The numbers refer to TFE concentration (% by volume). The dashed line represents the rescan for protein in presence of 20% TFE (by volume).

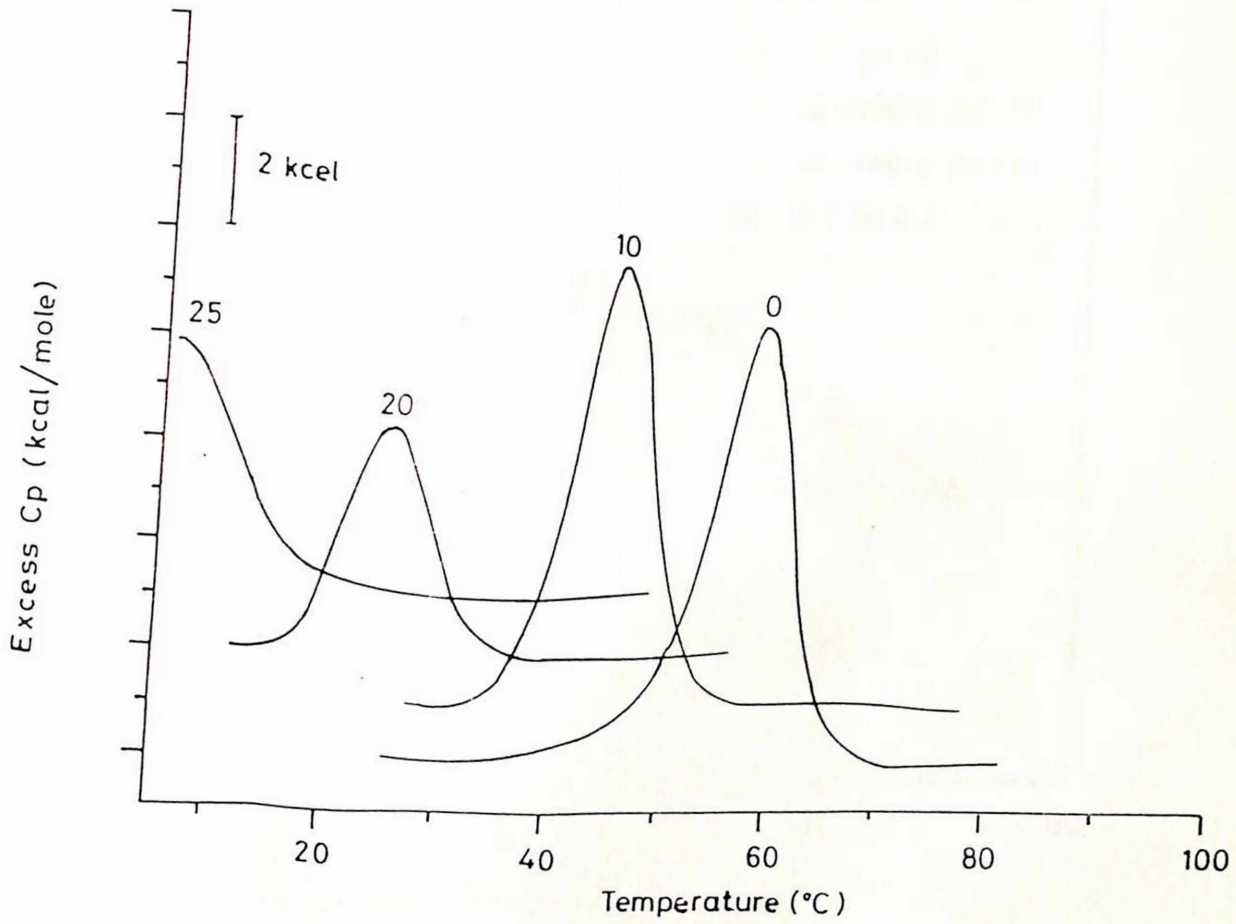


Figure 5.2. Excess heat capacity versus temperature curves for hen egg white lysozyme at pH 2.0 as a function of TFE concentration. The numbers refer to TFE concentration (% by volume).

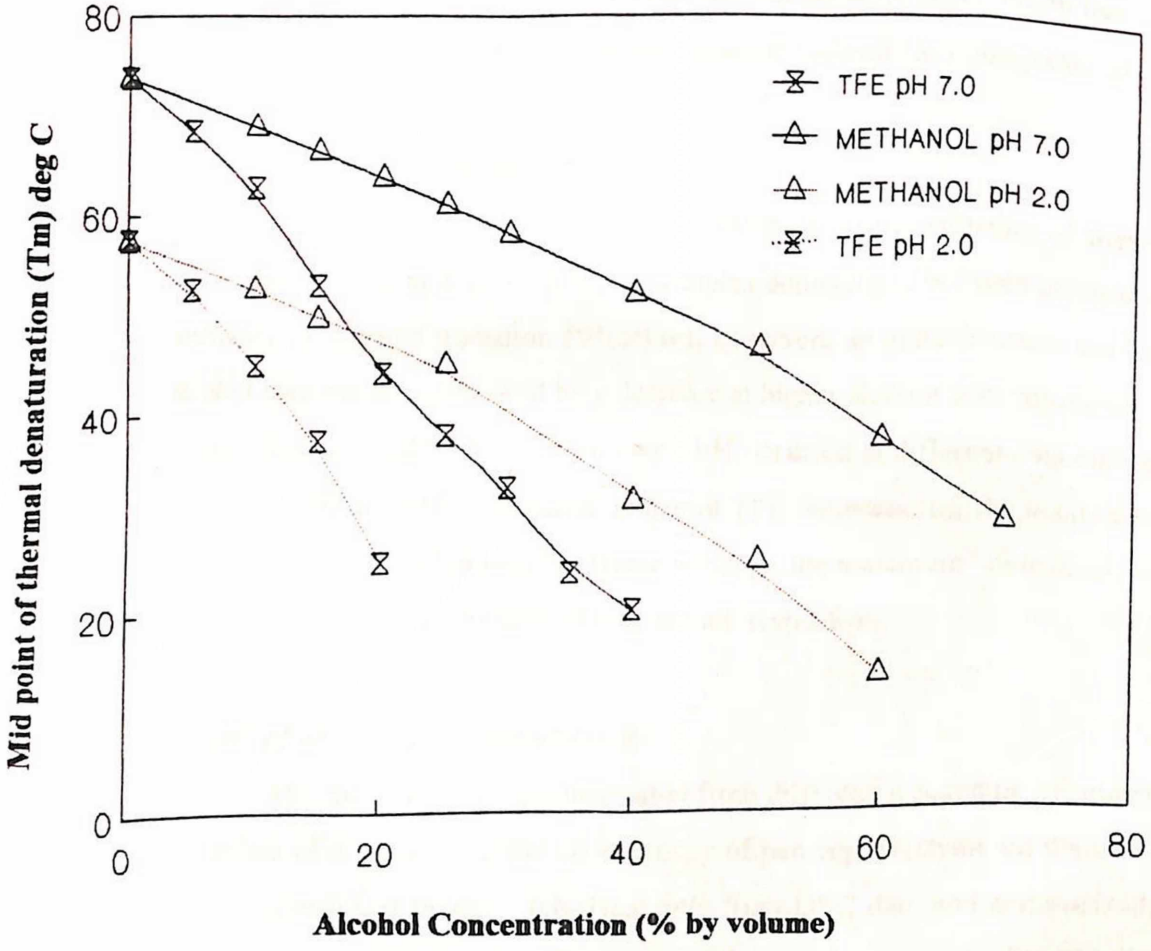


Figure 5.3. Dependence of mid point of thermal denaturation temperature (Tm) on the alcohol concentration (% by volume).

denaturation temperature was dependents on the alcohol concentration and alkyl chain length and follows the order propanol>ethanol>methanol (17, 18). The results presented here showed that TFE in fact lowers the denaturation temperature much more effectively than propanol or methanol, thus suggesting that it is not only the alkyl chain length and alcohol concentration but also the stearic hinderence factor which is responsible for alcohol dependent lowering of Tm because ethanol and TFE have similar alkyl chain length but TFE has a bulkier fluorine molecules resulting into a much bulkier molecule as comopared to ethanol.

Effect of alcohols on the enthalpy.

Figure 5.4 displays the effect of alcohols on the enthalpy of thermal transition of lysozyme at native and acidic pHs. A complex dependence between alcohol composition and enthalpy of thermal transition (ΔHcal) was observed: an initial increase in ΔHcal upto a certain alcohol concentration followed by a decrease at higher alcohol concentrations. The maximum in the value of ΔHcal for lysozyme at native pH occurred at different concentrations of alcohols which was in the order methanol. propanol TFE whereas, for the maximum increase in the value of Hcal observed showed a reverse trend. i.e the maximum value of ΔHcal was observed for TFE followed by propanol and methanol. respectively.

Effect of alcohols on cooperativity.

The most interesting observation from differential scanning calorimetric studies was the effect of alcohols on the cooperativity of hen egg lysozyme on thermal denaturation. This is determined by the ΔHvh/ΔHcal ratio from DSC data and summarized in Figure 5.5. Unity or near-unity values for this ratio provide a necessary and sufficient criterion for a two-state process [19]. Lysozyme in presence of methanol at native or acidic pH and TFE at acidic pH the ratio of van't Hoff to calorimetric enthalpy was found to be unity irrespective of alcohol concentration. Whereas for native lysozyme in presence of propanol or TFE a significant decrease (upto 0.5) in van't Hoff to calorimetric enthalpy ratio was observed at low alcohol concentrations (10 and 15% by volume for TFE and propanol, respectively). These observations suggest that for lysozyme in presence of methanol at native or acidic pH and TFE

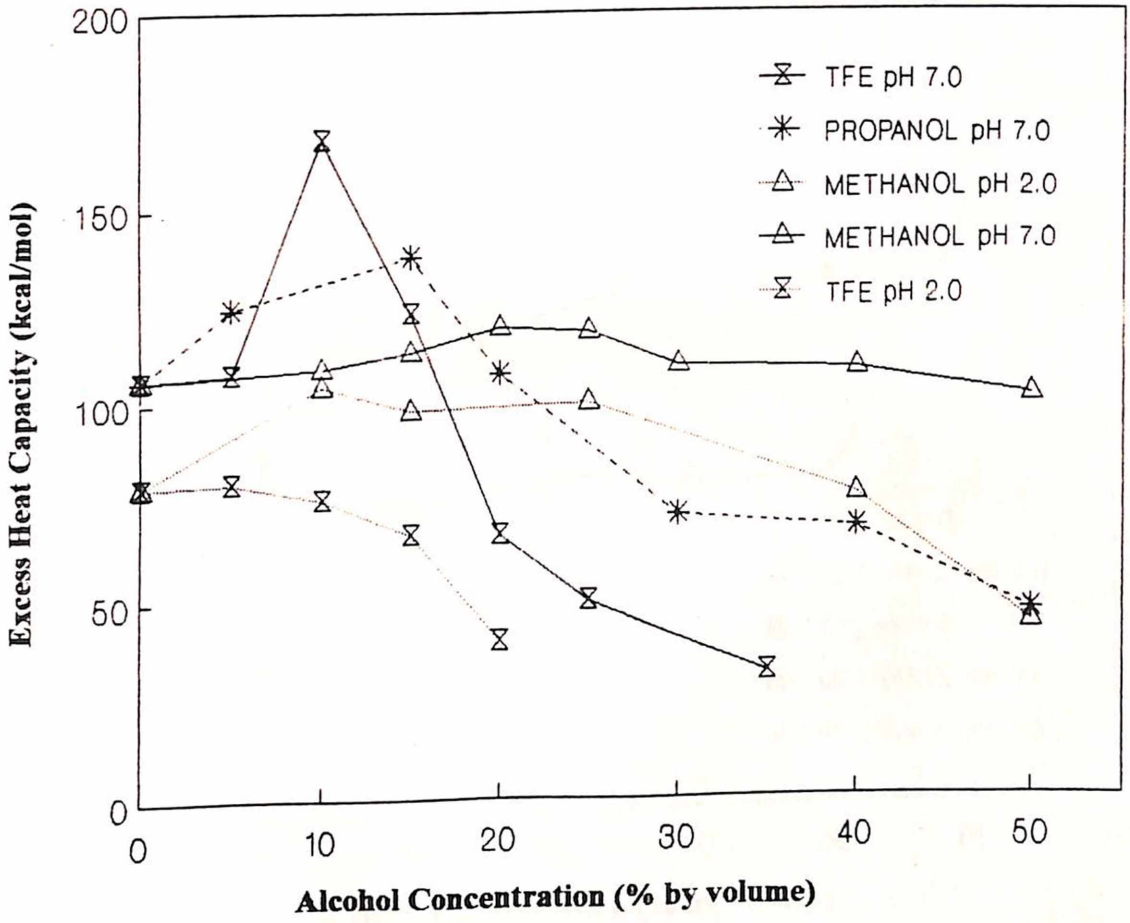


Figure 5.4. Dependence of calorimetric enthalpy on the alcohol concentration (% by volume).

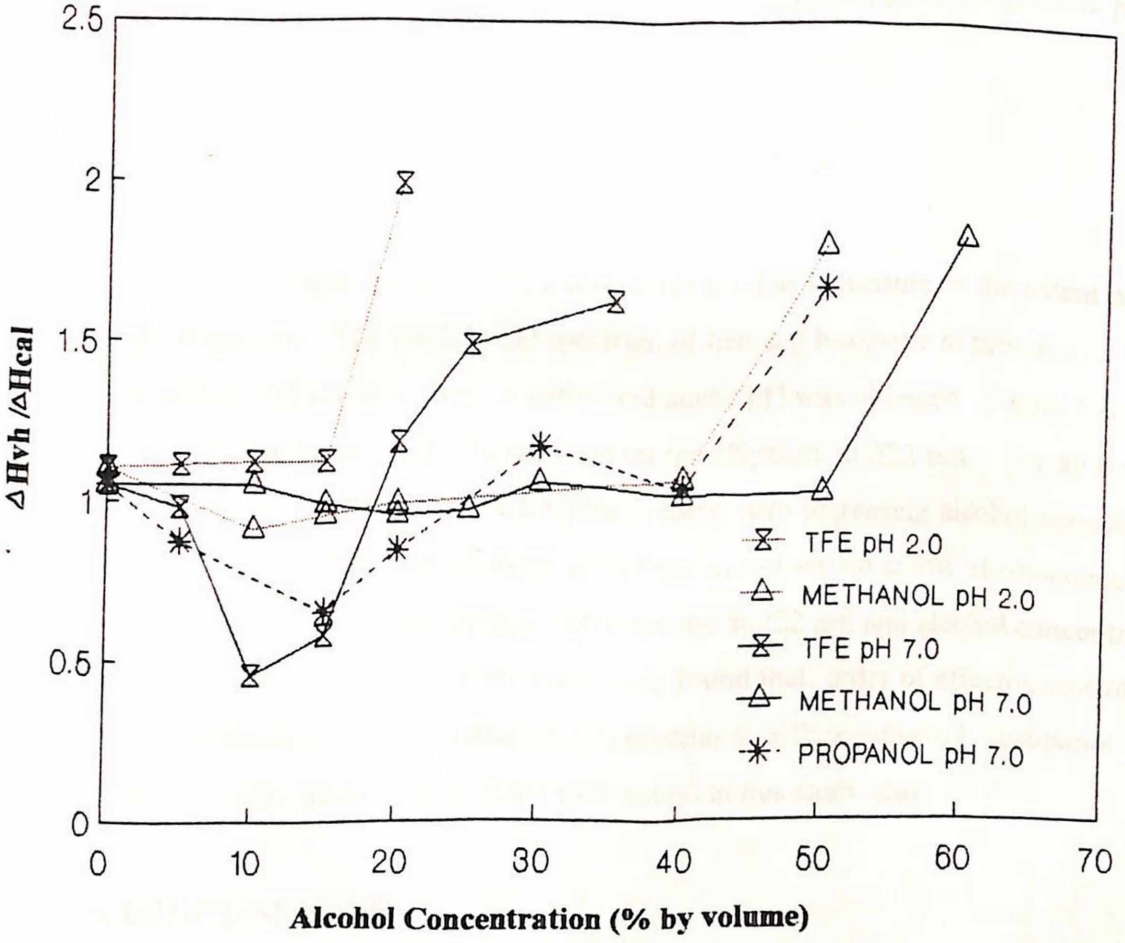


Figure 5.5. Dependence of ratio of van't Hoff to calorimetric enthalpy on the alcohol concentration (% by volume).

at acidic pH. follows a a two state mechanism of thermal denaturation, whereas, for low propanol and TFE concentrations , the temperature dependent denaturation of hen egg lysozyme showed a shift from two-state-process to a non-two state process, but at higher propanol concentration the melting process was again found to be following a two state process, thus indicating that addition of lower concentrations of propanol brings about non-cooperativity in the native protein which otherwise is a prototype of cooperative protein [20, 21].

Effect of alcohol on the structural features of lysozyme.

Secondary structure.

The CD ellipticities at 222 nm is commonly used as a measure of the extent of α-helical content in proteins. The far-UV CD spectrum of hen egg lysozyme in presence of increasing concentrations of alcohols both at native and acidic pH was obtained. Figure 5.6 shows the effect of increasing alcohol concentration on the ellipticity at 222 nm. For all the alcohols studied, native lysozyme showed a biphasic curve with increasing alcohol concentration in which no significant change in ellipticity at 222 nm was observed at low alcohol concentration. At pH 2.0 a sigmoidal relationship between ellipticity at 222 nm and alcohol concentration was observed. Based on several studies it has been found that order of effectiveness of alcohols towards stabilization of helical structures in proteins is trifluoroethanol > propanol > ethanol > methanol (25). Similar observation were found in this study also.

5.4 DISCUSSIONS

It is well known that various substances cause changes in the native conformation of proteins when added to the aqueous protein solution. Various additives, such as guanidine hydrochloride, urea and alcohols, affect proteins in different ways. Addition of alcohols to aqueous solution of proteins have been shown to stabilize helical structure (6,11,12,22,23), but destabilize tertiary structure of a protein (16, 24, 25). Due to the presence of these mixed properties, alcohols are helpful in inducing partially folded intermediates. In fact addition of alcohols decreases cooperativity of transition and induces partially folded states in a number

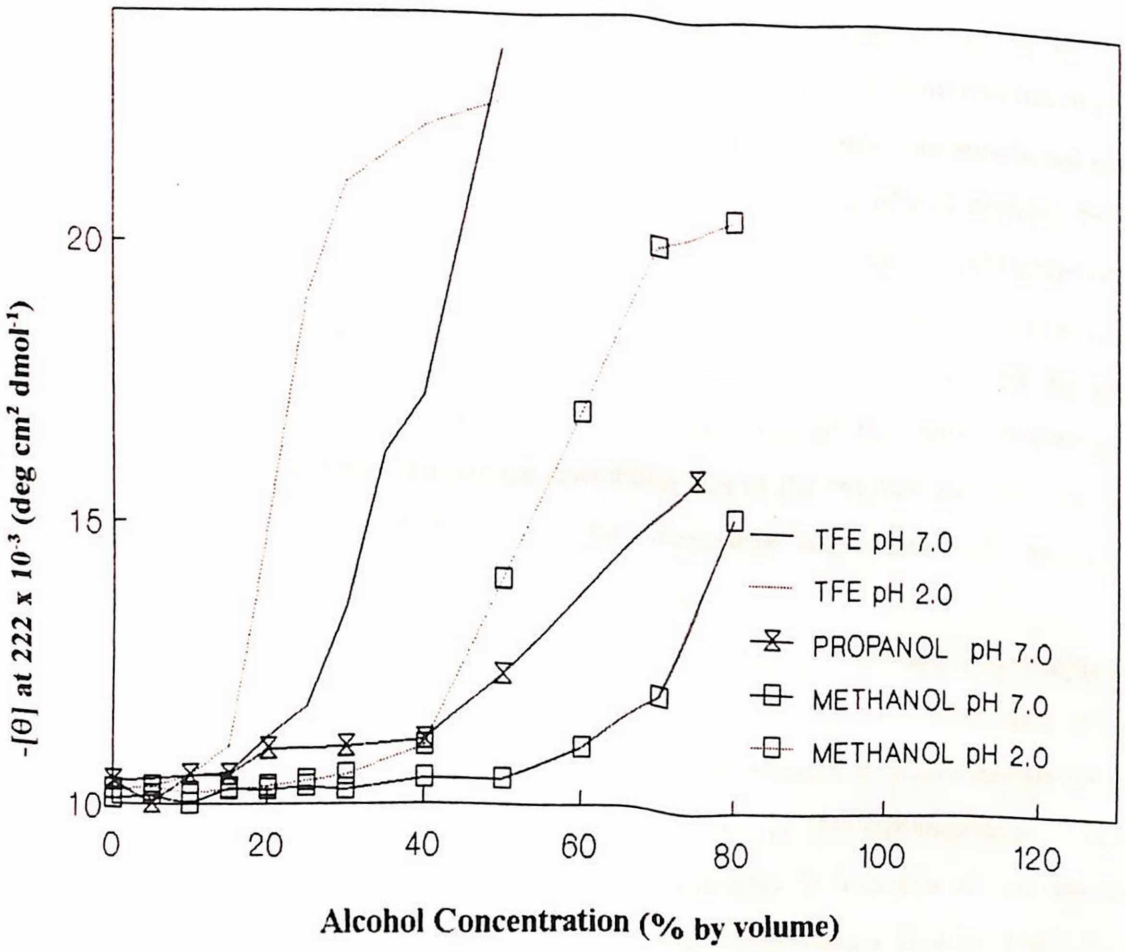


Figure 5.6. Dependence of ellipticity at 222 nm on the alcohol concentration (% by volume).

of globular proteins (17, 18). An emphasis has been put on the remarkable effects of alcohols in increasing the α-helical structure in proteins when added at high concentrations.

In several recent studies, the effect of addition of TFE to an aqueous solution of hen egg-white lysozyme both at acidic and native pH as studied by circular dichroism, NMR and limited proteolysis has been presented (3, 7, 13). It was demonstrated that trifluoroethanol causes a conformational transition of lysozyme into a stable conformational state characterized by a high degree of helical secondary structure and a loss of well defined tertiary structure of the protein under both the conditions. For native lysozyme the mid-point of this transition is near 20% TFE (by volume) and is complete by 50% TFE (by volume) at room temperature. Overall, the data were interpreted as indicating that TFE stabilizes the protein secondary structure, but destabilizes the hydrophobic core of the native lysozyme , leading to a trifluoroethanol state of lysozyme resembling that of the "molten globule", i.e. a more expanded and flexible conformational state characterized by largely native-like secondary structure but lacking specific tertiary interactions (3, 13).

In order to assess that whether the molten globule state observed in lysozyme at high alcohol concentration is due to the effect of alcohol on the native state or on the denatured state, we have carried out temperature dependent structural studies and thermodynamic studies on lysozyme at native and acidic pH in presence of TFE and methanol. The thermodynamic studies on the thermal denaturation of lysozyme in presence of varying concentration of alcohols showed that, presence of increasing concentration of alcohol in lysozyme solution at native and acidic pH resulted in a decrease in denaturation temperature. In fact in presence of 40% and 20% TFE (by volume) the lysozyme solution at pH 7 and 2 respectively had a Tm of about 20 ^{0}C, which suggests that lysozyme under these conditions and also at TFE concentrations higher than these will be in a thermally unfolded state at room temperature i.e. 25 ^{0}C and hence the structural features observed at room temperature as reported earlier (3, 13) will be due to the effect of TFE on the thermally denatured protein and not the native protein.

In order to access the validity of this hypothesis we carried out temperature dependent CD studies with native lysozyme in presence increasing concentration of TFE. The data is summarized in Figure 5.7. Figure 5.8 shows the far-UV CD spectra of native lysozyme in presence of 35, 40 and 50 % TFE (by volume) at 4 and 25 ^{0}C. As seen from the figure, a

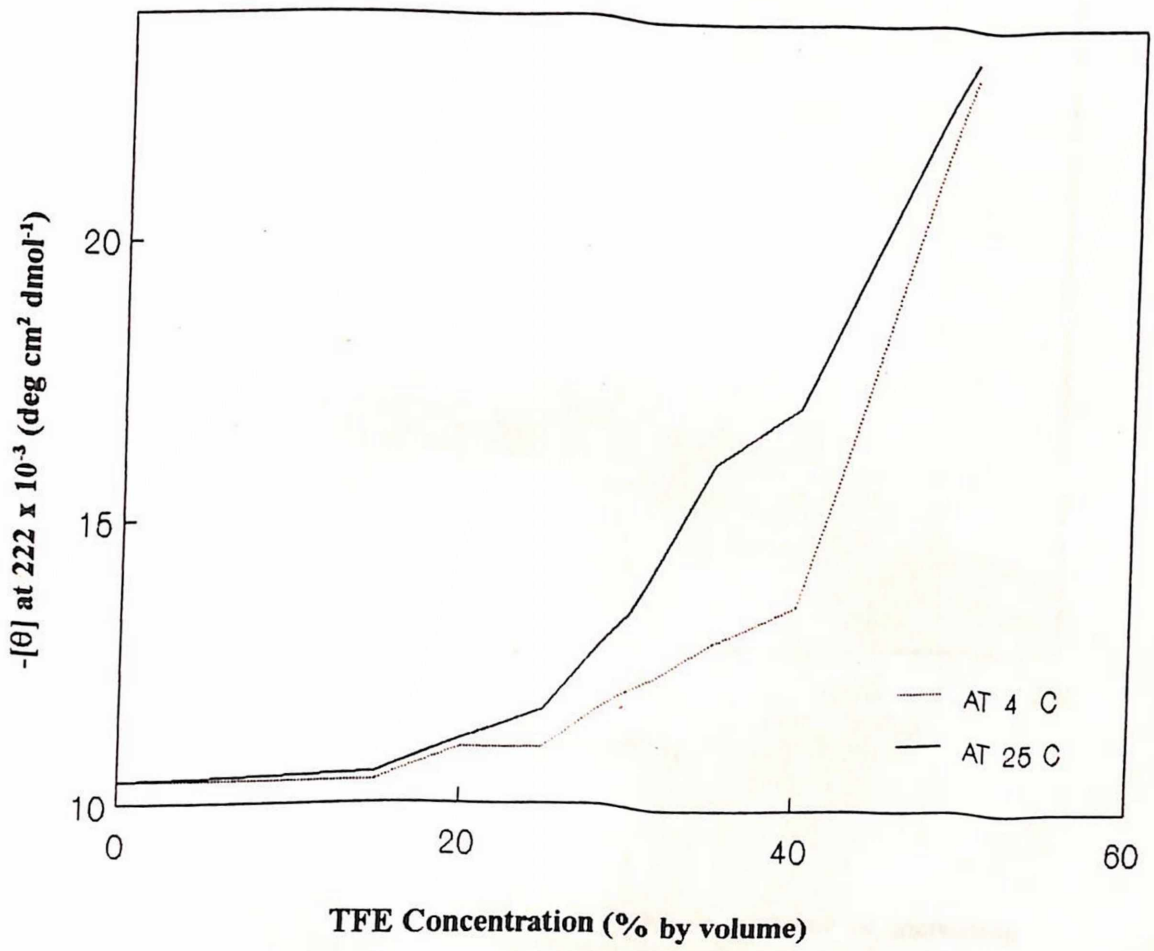


Figure 5.7. Temperature dependent changes in ellipticity at 222 nm of native lysozyme in presence of increasing concentrations of TFE (% by volume).

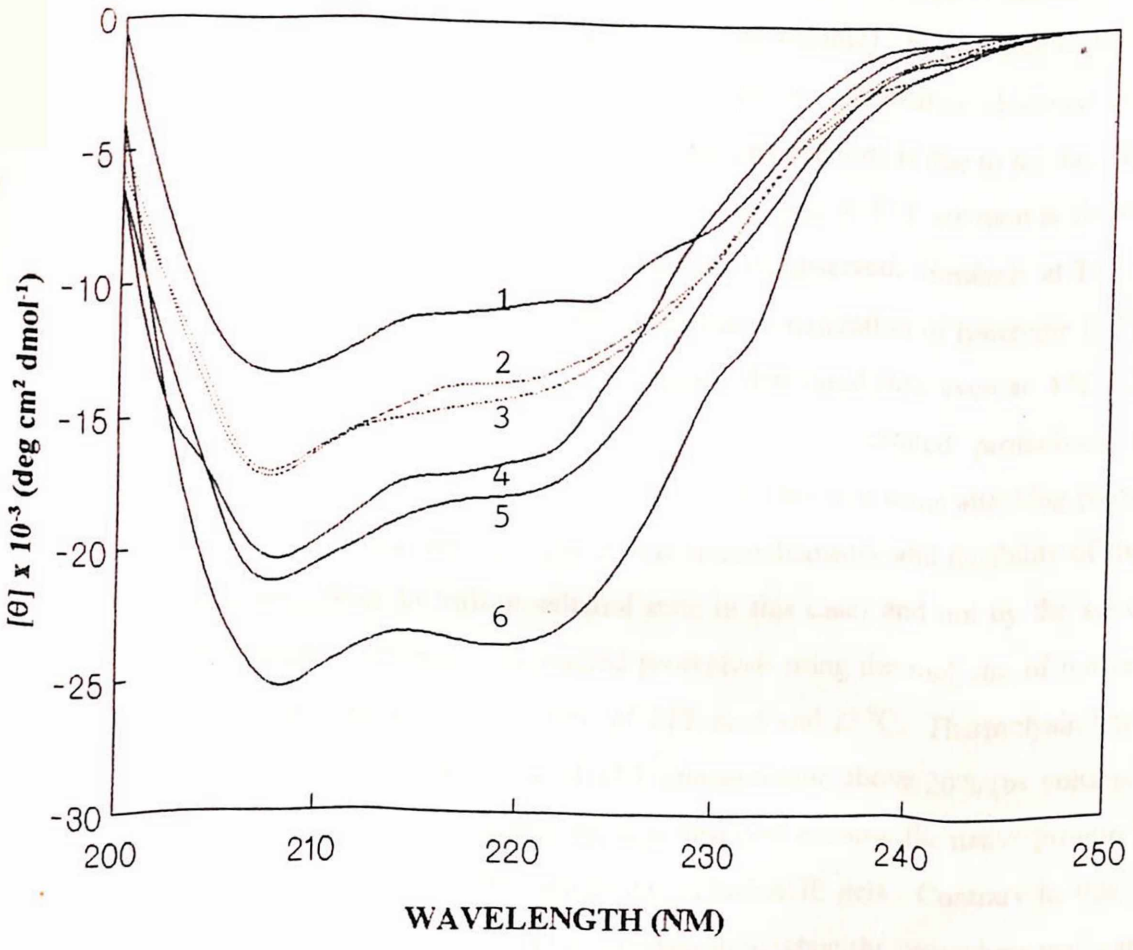


Figure 5.8. Far-UV CD spectra of lysozyme in presence of increasing concentrations of TFE at pH 7.0. The dotted lines represent spectra obtained at 4 ^{0}C and the continuous line represent spectra at 25 ^{0}C. Curve 1 is at 0% TFE, curve 2 and 4 at 35% TFE; curve 3 and 5 at 40% TFE and curve 6 at 50 % TFE.

significantly large enhancement in ellipticity at 25 °C as compared to that at 4 °C was observed for lysozyme in presence of 35 and 40 % TFE (by volume). In presence of lower (upto 30%) and higher (above 45%) concentration of TFE the ellipticities observed at low and high temperatures were similar. The reason for this observations is due to the fact that below 30% TFE the mid point of thermal denaturation of lysozyme in TFE solution is above 40 °C. hence at 25 °C the effect of TFE in the native state is only observed. Similarly at TFE concentration above 45% (by volume) the mid point of thermal denaturation of lysozyme in TFE solution is below 4 °C, hence the protein is in the thermally denatured state even at 4 °C. This finding is further substantiated by the temperature dependent limited proteolysis studies using thermolysin. It has been demonstrated by several studies that using attacking proteases, peptide fission would occur at sites dictated by the stereochemistry and flexibility of the polypeptide substrate (lysozyme in trifluoroethanol state in this case) and not by the specificity of the protease. Figure 5.9 shows the limited proteolysis using thermolysin of native lysozyme in presence of increasing concentrations of TFE at 4 and 25 ^{0}C. Thermolysin has no effect on native lysozyme , but in presence of TFE concentration above 20% (by volume) at 25 ^{0}C a polypeptide bond fission by thermolysin was observed because the native protein breaks down into a smaller size polypeptide seen in the SDS-PAGE gels. Contrary to this, no effect of thermolysin was observed at any TFE concentration when the proteolysis was carried out at 4 ^{0}C. These observations also suggest that the effect of TFE on the structural features of native protein is dependent on the temperature it is studied. Hence it is speculated that the helical protein conformation observed in presence of higher alcohol content is due to the effect of alcohol on the thermally denatured state of protein and not the native state of protein. This is further substantiated by the observation presented in the Figure 5.10, where the ellipticity at 222 nm for lysozyme (as observed from far-UV CD studies and is the measure of α-helical content in the protein) in presence of varying concentrations of alcohols is plotted against the mid point of thermal transition observed from differential scanning calorimetric studies under similar conditions. As shown in figure, no significant difference in ellipticity, irrespective of alcohol or the pH studied, was observed until the mid point of thermal transition reaches 30 ^{0}C. But below this temperature a significant increase in a-helical content was observed. These observations indicate that the alcohol induced α-helical conformation of proteins is

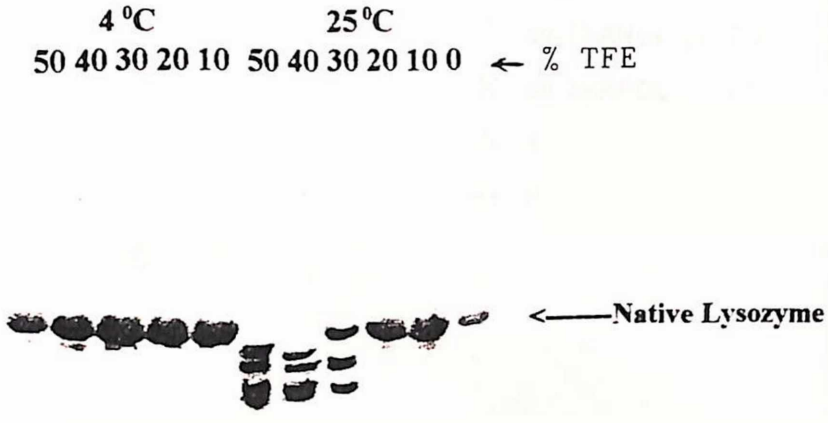


Figure 5.9 SDS/PAGE analysis (reducing conditions) of the proteolysis of hen egg lysozyme by thermolysin in presence of varying concentrations of TFE at 4 °C and 25 °C. The details are give under materials and methods section.

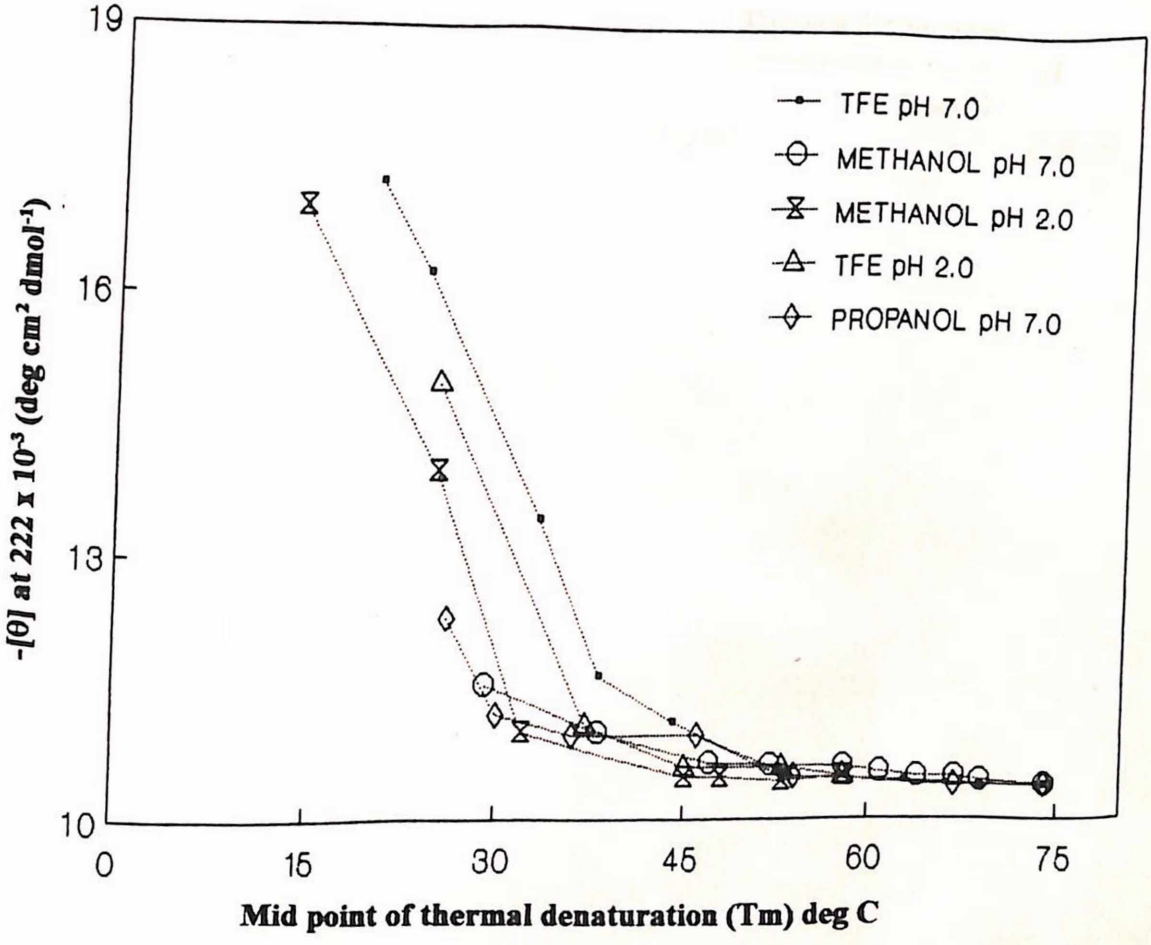
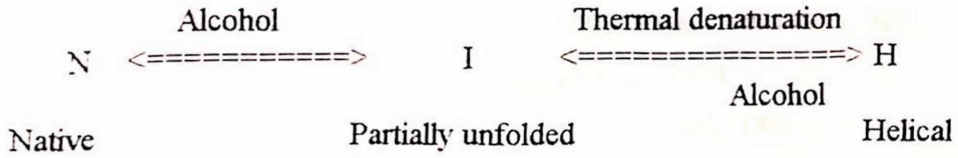


Figure 5. 10. Correlation between mid point of thermal denaturation and ellipticity at 222 nm for native and acid denatured lysozyme in presence of increasing concentration of alcohols.

probably due to strong hydrogen-bond formation between alcohol and polypeptide groups in the unfolded protein. Hence, the helical state observed in presence of high alcohol concentrations is due to the effect of alcohols on the thermally denatured proteins and not the native protein. So the total effect of alcohols on the native proteins can be summarized as follows:



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Papers Communicated:

1) Characterisation of propanol-induced partially folded equilibrium intermediate of Hen Egg-white lysozyme.
K. Ravindra Babu and Vinod Bhakuni

2) An equilibrium partially folded-state of Hen Egg-White lysozyme at low pH: Evidence of its ionic strength dependent transition to a compact conformation and aggregation on thermal denaturation.
K. Ravindra Babu and Vinod Bhakuni

3) Facilitated refolding of a multimeric protein by Alcohol.
Shashi Prajapati. **K. Ravindra Babu** and Vinod Bhakuni.

4) Alcohol-induced partially folded intermediates of small globular proteins: A re-evaluation based on structural and thermodynamic studies.
K. Ravindra Babu and Vinod Bhakuni

Papers Presented/Accepted in Symposia

1. Comparative Structural and thermodynamic features of ovalbumin and the fragment obtained on limited proteolysis by pepsin at acidic pH.
K. Ravindra Babu and Vinod Bhakuni.
National Symposium on Molecular and Cellular Biophysics and 23rd Annual Meeting of Indian Biophysical Society, New Delhi, Feb. 18-21, 1996.

2. Alcohol assisted reconstitution of acid-denatured catalase.
Shashi Prajapati, **K. Ravindra Babu** and Vinod Bhakuni.
National Symposium on Molecular and Cellular Biophysics and 23rd Annual Meeting of Indian Biophysical Society, New Delhi, Feb. 18-21, 1996.

3. A partially folded state of hen egg white lysozyme in propanol: Structural characterisation of the folding intermediate.
Vinod Bhakuni and **K. Ravindra Babu.**
National Symposium on Molecular and Cellular Biophysics and 23rd Annual Meeting of Indian Biophysical Society, New Delhi, Feb. 18-21, 1996.

4. Propanol-Induced two stage transition of Hen Egg-White lysozyme: Characterisation of two partially folded equilibrium Intermediates.
K. Ravindra Babu and Vinod Bhakuni.
65th Annual Meeting of the Society of Biological Chemists (India), Bangalore, Nov. 20-23, 1996.

5. Trifluoroethanol-Induced Partially folded equilibrium Intermediate of Bovine carbonic Anhydrase II at low pH: Structural and thermodynamic characterisation.
Shashi Prajapati, K. **Ravindra Babu** and Vinod Bhakuni
65th Annual Meeting of the Society of Biological Chemists (India), Bangalore, Nov. 20-23, 1996.

6. Propanol-induced three stage transition of cytochrome C: characterization of a Globular and extended conformation of partially folded equilibrium intermediates.
Sonia Varshney, K. **Ravindra Babu**, Shashi Prajapati and Vinod Bhakuni. 65th Annual Meeting of the Society of Biological Chemists (India), Bangalore, Nov. 20-23, 1996.

7. Alcohol-Induced Partially folded equilibrium intermediates of Protein.
K. **Ravindra Babu** and Vinod Bhakuni.
Mahabaleshwar Seminar on Modern Biology (Protein folding and Design) Ootty, Dec. 28, 1996 - Jan. 2nd, 1997.