CONFORMATIONAL STUDIES OF HYALURON BINDING PROTEIN

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CERTIFICATE

The research work embodied in this dissertation titled "CONFORMATIONAL STUDIES OF HYALURONAN BINDING PROTEIN" has been carried out in the School of Environmental Sciences, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted in part or full for any other degree or diploma of any university.

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ABBREVIATIONS

Amp	Amperes
ANS	1-anilinonaphtalene-8-sulfonate
APS	Ammonium persulphate
BSA	Bovine serum albumin
CBB	Commassie Brilliant Blue R-250
D	Denatured state
ECM	Extracellular matrix
EDC	N-ethyl-N'-(3-dimethylaminopropyl) Carbodiimide.
EDTA	Ethylene diamine tetra-acetic acid
GAGs	Glycosaminoglycan
HA	Hyaluronic acid or hyaluronan
HABP	Hyaluronic acid binding protin or Hyaluronectin
IP_3	Inositol-1,4,5-triphosphate
kDa	Kilodalton
α-LA	α-Lactalbumin
M	Molar
mM	Millimolar
ml	Milliliter

ml	Milliliter
µg	Microgram
µl	Microliter
MG	Molten globule
Mr	Molecular Weight
N	Native (folded state)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PMSF	Phenylmethylsulfonyl fluoride
rpm	Revolution per minute
SDS	Sodium dodecyl Sulphate
TEMED	N,N,N',N'-tetra methylene diamine
Tris	Tris-(hydroxy methyl) amino methane
U	Unfolded state
V	Volts.

INTRODUCTION

The knowhow about the transfer of information from one dimension to three dimension (i.e. protein conformation) remained as a major unsolved problem in modern molecular biology. Contrasting the progress in understanding of inside-out signaling, there is still much to learn about outside-in signaling. In the process of cell-surface receptor-mediated signal transductions, ligand induced conformational change takes place, that propogate from the extracellular to cytoplasmic domains. In order to know the nature of these conformation change, first objective of the study would be to characterize the conformation of these receptor molecule.

Because the biological properties of proteins arise primarily from their native conformations, considerably more attention during the past 60 years has been focused on the native conformations than on the non-native conformations. In the past few years, however, there has been increasing interest in non-native and denatured states of proteins since these less ordered states play important roles in at least three important phenomena:

1. Protein folding and stability. The denatured states of proteins are equal in importance to the native states in determining the stability of a protein, since stability is defined as the difference in free energies between native and denatured states. In cells and tissues, the native states are in dynamic equilibrium with the denatured conformations (Tanford et al., 1968, 1970, Pace et al., 1975).

2. Transport across membranes. To cross a lipid bilayer after translation and folding, some proteins must first be converted into a partially folded or denatured conformation either spontaneously or with the help of unfoldase (Rothman et al., 1986; Verner et al., 1987; Deshaies et al., 1988)

3. Proteolysis and protein turnover. Numerous studies have implicated the denatured state as the primary target for degradative enzymes, both in state of stress such as heat shock, and under physiological conditions (Mclendon et al., 1978, Holzer et al., 1980, Parsell et al., 1989).

Why Denatured State ?

The denatured state of protein under physiological condition (pH, ionic concentration, temp. etc) is most relevant to an understanding of protein stability. Unfortunately, since most proteins under native conditions are stable by +5 to +20 kcal/mole, then only one out of 10^4 to 10^{15} protein chains is in denatured state. Consequently, experimental studies have concentrated on denatured states under conditions quite different than aqueous buffers at neutral pH and 20°C to 37°C. By analyzing how the properties of denatured states vary under these more extreme conditions, estimates of the properties of denatured state can often be made by extrapolation back to physiological conditions (Tanford et al., 1968, 1970).

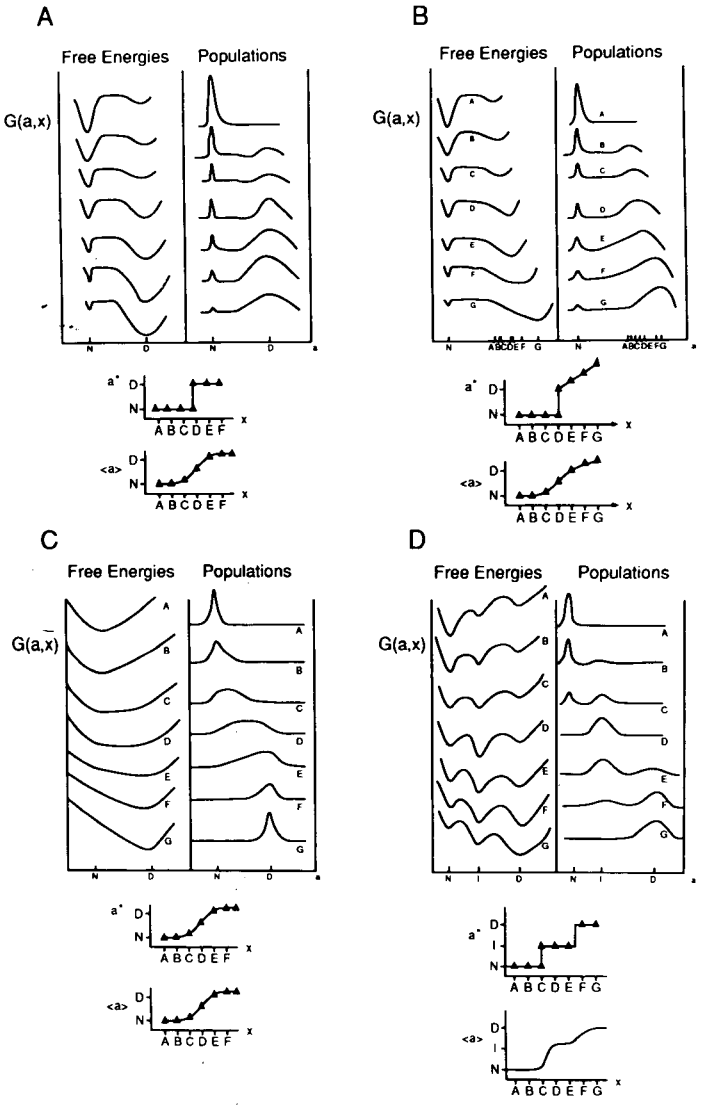

Fig. 1 **Thermodynamic models for transitions such as protein denaturation**. The axis labelled a on the upper figures represents a property, such as the radius of the molecules, that varies from the native to denatured states. The Y-axis shows the free energy. The deepest minimum corresponds to the stable state. The populations are given by the Boltzmann distribution law [proportional to exp (-ΔG/RT)]. The series of curves, $\vec{X}=x_A, x_B, \ldots$ represent increasing quantities of some denaturing agent. The set of curves in each of Figures A-D is summarized by two figures below each set that describe the experimental observables: a* vs x or <a> vs x: for example, molecular radius vs amount of denaturant. Two types of property are shown. I. a* is a type of property (state-distinguishable) in which both states (N and D) are simultaneously observable: the * indicates whichever state has the highest population. 2. Another type of property (state-averaged), such as intrinsic visosity, only detects an average, <a>. The latter will show sigmoidal behavior vs x. Four different transition models are shown: (A) fixed Two-State (first order): free energy barrier separates two fixed states, (B) Variable Two-State: two-state but the denatured state varies with denaturant, (C) Higher Order: no barrier between the states, (D) Fixed Three-State: two barriers separate three stable states (Ref. Ken A. Dill, Annu. Rev. Biochem. 1991, 60, 795-825).

The most widely used strong denaturants are GuHCl and urea, which lower protein stability in proportion to their concentrations. Although, the chemistry of interaction between protein and aqueous solutions of urea and guanidium is not fully understood, most available evidence indicates that these compounds cause water to become a better solvent for the non polar amino acid (Lee et al., 1974; Schellmman et al., 1978) i.e. they weaken their hydrophobic interactions. In contrast of classical experiments by Tanford, there is now evidence that even in 9 M urea or 6 M GuHCl, proteins can have significant amounts of internal structure. Under the much broader range of conditions that are typically used to denature proteins, recent theory and experimental data suggests that denatured proteins are often very compact, with persistent hydrophobic clustering and considerable residual secondary structure. (Ken et al., 1991). In contrast to traditional views, the primary sequence is also a major determinant of denatured conformations.

Thermodynamic Model For Protein Denaturation

Whereas the native state of a protein has a relatively well defined set of atomic coordinates, the denatured states do not, and must be described within a different framework, involving conformational ensembles. Denaturation is referred to a variety of poorly understood irreversible alterations in protein structure that include aggregation, disulfide bond rearrangement, protein isomerisation and backbone cleavage. Unfolded state is referred to a specific subset of denatured states, namely conformations that are highly open and solvent-exposed, with little or no residual structure; such states are generally obtained only under strong denaturating conditions. A second subset of denatured states is "compact denatured" states obtained under weaker denaturing conditions. Whereas "denatured" and "unfolded" have often been used interchangeably, the "state" has its usual thermodynamic meaning; it refers to an ensemble of the many different configurations of individual molecule, the average of which corresponds to a given macroscopically observable quantity or "stable equilibrium state" i.e. an ensemble of configurations at a minimum of free energy, as shown in fig. 1.

(a) "Fixed" two-state model. The free energy behaviour shown in fig. 1 A is referred to as a "first order" or "two state" transition (Lumry et al., 1966); there is a free energy barrier separating two stable states, N and D, which are "fixed" in so far as they do not change with x. Because thefree energy is high between the states N and D, the populations of the intermediate states will be small.

(b). Variable two-state model. In this case too, there is a free energy barrier separating two states, but one state (D) changes with x (Fig. 1 B). (Shortle et al., 1987, Dill et al., 1985).

(c) High order transition. In this type of transition, there would be no free energy barrier between states N and D. Therefore, at the transition, significant population of intermediate states would be observed.An example of a higher order transition is the critical point of the liquidgas

phase change (Fig. 1 C).

(d) Classical three state model. In this model, there are three stable states N,D, and intermediate I separated by twofree energy barriers (Fig. 1D).

What forces determine the conformations of denatured proteins?

The balance of forces that contributes to polymer collapse and expansion was first described by Flory et al., 1953, subsequent theoretical developments have recently been reviewed (Chan et al., 1991). The energy of a polymer chain in solution can be divided into three components.

1. Chain elasticity-A characteristic feature of polymer conformations in solution is their statistical nature . For long chains composed of bonds that have relatively free rotations (i.e. small energy differences among rotational isomers), the number of conformations accessible to a chain molecule is large and grows exponentially with chain length. The properties of such chain molecules are governed by the statistical distribution of these conformations. The entropic force is the basis for rubber elasticity and acts to oppose the collapse of a polymer chain to a compact state, as when a protein folds to its native state. This component of the conformational free energy of a chain molecule is due to "local" forces; i.e. this form of "ordering" is of each bond in the chain relative to its immediately neighbouring bonds in the sequence. For peptides, this ordering involves changes in , and side chain angles.

2. Excluded volume entropy- The "excluded volume" entropy is due to "nonlocal" factors, where nonlocal refers to the effect of one monomer on another, that is far apart in the sequence. The steric constraints are nonlocal in the sense that the volume occupied within any region of space can be due to monomers quite distant in the sequence. Excluded volume contributions to the entropy are large for a chain molecule that becomes as extraordinarily compact as the native state of a globular protein. So this chain configurational entropy also opposes the collapse of a polymer molecule to compact states i.e. protein folding.

3. Polymer-solvent contact interactions-Even though entropic effects 1 and 2 oppose the collapse of polymers and proteins to their compact states, polymer can be driven to compactness if they are dissolved in an unfavourable solvent. In a solvent, in which the monomer-monomer attractions are stronger than monomer - solvent attractions, a chain will contract because contraction increases the number of monomer-monomer contacts and this free energy can be sufficient to overcome the effects of chain elasticity and excluded volume.

Folding Intermediates

In 1982, those scientists who were studying folding intermediates were convinced of their reality, but the wider community was skeptical. The two state model of folding process U N, without observable intermediates) was deeply ingrained and

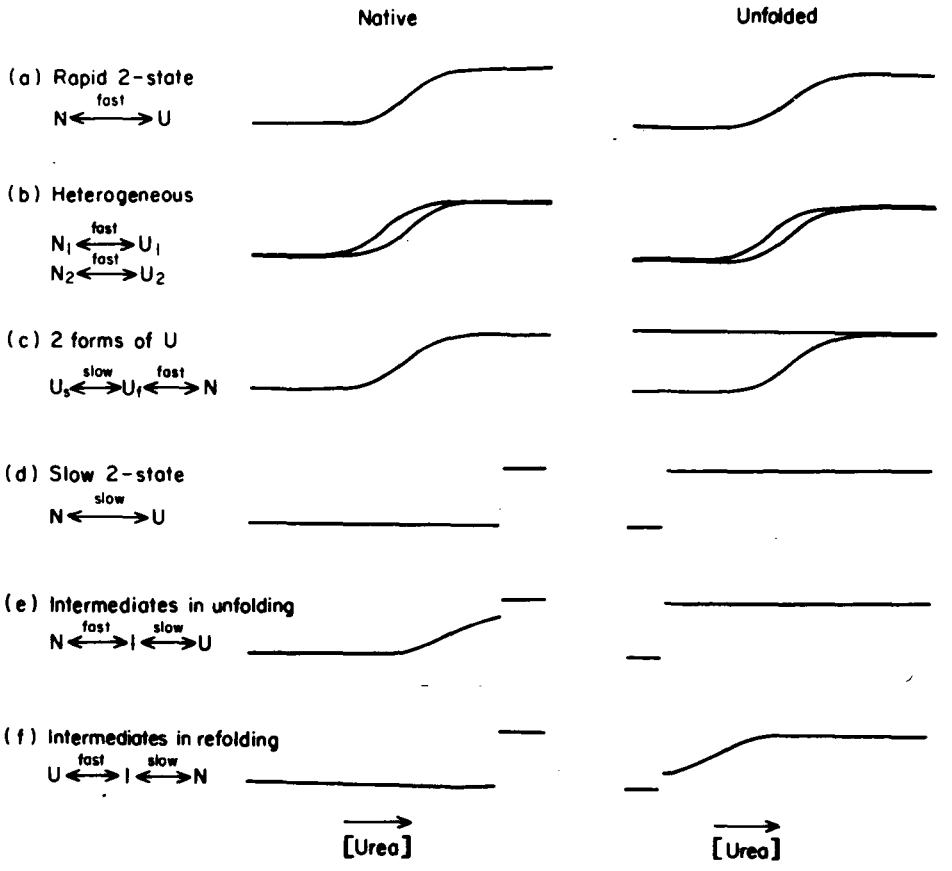

Fig 2. **Schematic illustration of urea gradient electrophoresis patterns expected for some simple protein folding transitions.** The initial protein applied to the gel was either fully folded (left) or fully unfolded (right). All folding transitions occur in the gel, during the electrophoresis separation. For each of the patterns, the urea concentration increases from left to right, and electrophoretic migration is from top to bottom, with the unfolded protein migrating more slowly. The rates described as "fast" or "slow" refer to the apparent rate constant for each transition, which is the sum of the individual rate constants for the forward and reverse directions. The term fast or slow are relative to the duration of the electrophoretic separation, the respective half times of fast and slow transitions should be atleast an order of magnitude smaller or greater than the time of electrophoresis. For intermediate rates the protein is smeared out due to molecules changing their electrophoretic mobility once at varying times during the separation. (a) A rapid transition produces the same pattern with both folded and unfolded protein applied to the gel: the pattern depends upon the equilibrium between U and N, which varies from N predominating at low urea concentrations to U predominating at high urea concentrations. With a 2-state transition, a smooth curve with a single inflection point

is expected. If any third conformational state, with an electrophoretic mobility different from those of U and N, were to be populated significantly, the curve would be more complex, with an additional inflection point. (b) If the protein population is heterogeneous, with a fraction of the molecules unfolding at a different urea concentration, but all having rapid 2-state transitions and the same electrophoretic mobility when folded and unfolded, the band of protein will split in the transition region.The heterogeneity need not be covalent, but it is required that the 2 forms not be interconverted during the electrophoretic separation when both folded and unfolded. Heterogeneity expressed in different mobilities of U or N, or both, will produce separate bands of protein over the corresponding areas of the urea gradient. (c) Two slowly interconverted populations of the initial unfolded protein, one which refold rapidly (U_F) and the other slowly (U_S), as expected where incorrect *cis-trans* isomers of proline peptide bonds in (U_S) blocks refolding, will produce a band of protein due to a rapid 2-state transition between N and the U_F fraction of unfolded molecules, but the U_S fraction will stay unfolded and produce another band with the mobility of U across the gel at low urea concentrations. When starting with folded protein, only the rapid transition between N and U_F will be observed, since U_S will not be formed to a significant extent during the electrophoretic separation. (d) Slow rates of unfolding and refolding will produce patterns in which the original form of the protein persists. However, the rates of both unfolding refolding are often observed to be markedly dependent on the urea concentration, so the diagrams show unfolding to become rapid at very high urea concentration and refolding to become rapid at a very low urea concentration. At intermediate concentration, where the rates are neither rapid nor slow, the protein band will be smeared out by molecules unfolding or refolding, thereby changing mobility, at various times during the electrophoretic separation. (e) Rapid and reversible partial unfolding of the native protein to less compact conformations (I), prior to complete unfolding, will result in the band of folded protein curving upwards. The degree of curvature will depend upon the average mobility of the state I, which could be one or very many interconvertible conformations, and upon the equilibrium between N and I. For a folding transition at equilibrium, such intermediates can only be stable at urea concentrations above that of the equilibrium unfolding transition. (f) Intermediates in refolding will produce patterns complementary to those of (e), where the band of unfolded protein will migrate more rapidly at urea concentrations below the equilibrium transition.
Ref. Creighton, T.E. 1980 J. Mol. Biol. 137, 61-80.

implied that folding intermediates were difficult perhaps impossible to detect. The following developments were influential in convincing others of the reality of folding intermediates.

First, compact kinetic intermediates can be seen directly on gels when the folding transition is examined by urea-gradient electro phoresis near 0°C, conditions that allow the kinetics of folding to be analyzed for some proteins (Fig. 2) (Creighton et al., 1980).

Second, probes of secondary structure (i.e. amide hydrogen exchange, ^3H labeling) easily detect structural intermediates in kinetic folding experiments, especially when folding is studied in strong native conditions (Schmid et al., 1979, Kim et al., 1980). Later, fast CD measurements revealed the presence of early intermediates in kinetic studies (Kuwajima et al., 1977).

Third, an equilibrium form of some proteins has been found that is obviously different from both the native and unfolded form and may be an intermediate in folding. A molten globule model has been proposed to describe this collapsed form (Ptitsyn et al., 1987)

Fourth, fragments of small proteins provide models for folding intermediates, by forming weak but folded structures, especially at low temperature (0°C). The structures in these fragments are native like and the results suggest that the entire polypeptide chain is not needed for folding to occur. (Dyson et al., 1988; Oas et al., 1988).

Fifth, a combined method, stopped flow pulse labeling with subsequent 2D-NMR analysis after folding is complete, and the first result indicate that native like secondary structures are present in early folding intermediate. The method employs exchange with solvent (H_2O or D_2O) to label and trap exposed peptide NH protons in kinetic folding intermediates.

Finally, the linkage relations between folding and disulfide bond formations have been analyzed (Creighton et al., 1984, Creighton et al., 1983), thus clarifying the nature of disulfide intermediates as folding intermediate. Because the process of disulfide reoxidation is coupled to the reduced Bovine pancreatic trypsin inhibitor (BPTI), these disulfide intermediates are also necessarily refolding intermediates.

Nature Of Folding Intermediates

The practical problem in studying folding intermediates is that the folding reactions of most single-domain proteins are highly co-operative, so that intermediates are not well populated at equilibrium. To circumvent this problem, the first is to carry out kinetic refolding experiments, in which activation barriers lead to the transient accumulation of intermediates and low temperature can be used to stabilize intermediates. The second is to find special conditions where partially folded forms of the protein are populated at equilibrium. The third is to identify protein fragments or peptide models that fold autonomously. In addition, consideration of residual structure in the unfolded state has become an important issue in understanding early events

protein folding.

Collapsed forms (Molten globules)

Several years ago it was observed by Kuwajima, Sugai and Coworkers 1976, that α-LA forms an equilibrium intermediate at low pH (originally called the A state). The CD spectrum of A state was essentially identical to that of the native protein in the far UV region, but the aromatic region of the CD spectrum was similar to that of the completely unfolded protein. Thus, the A state approved to be an equilibrium intermediate containing native like secondary structure but lacking tertiary structure (Kuwajima, et al., 1977).

The surprise came when it was found that the A state of α-LA was compact as measured by intrinsic viscosity or dynamic light scattering measurements. These measurements indicated that the A state was much more compact than the unfolded protein (e.g. in GuHCl) but only slightly less compact than the native protein. This lead to Ptitsyn's interpretation of the A state as a "molten globule" a compact folding intermediate with a high content of secondary structure and fluctuating tertiary structure. The name "molten globule" was given by Ohgushi and Wada (Ptitsyn et al., 1987 Ohgushi et al., 1983). A collapsed form has been detected in other proteins (Goto et al., 1989, Brems et al., 1989), and it now seem likely that it occurs commonly but in restricted conditions (e.g. in acid or alkaline pH, at moderate or high salt conditions). Collapsed form have a strong tendency to aggregate making so it is difficult to characterize them.

In kinetic refolding experiments, fast CD measurements indicate that rapidly formed intermediates have at least one property resembling the collapsed form. This suggest that the equilibrium between collapsed form and the unfolded state is fast ($T<1$m sec) wheres the equilibrium between the collapsed form and the native protein is slow ($T>1$ sec). The lack of a large activation barrier between the collapsed form and the unfolded state suggests that the collapsed form may corresponds to the structure of the unfolded protein in refolding conditions. (Kuwajima et al., 1989).

Calorimetric studies of α-LA suggest that the collapsed form has nearly the same heat capacity as the unfolded state. Since the increase in heat capacity that accompanies in folding is thought to result from the exposure of previously burried non polar side chains to water, this result leads to the surprising conclusion that non polar side chains in the collapsed form are solvated. Collapsed forms bind hydrophobic dyes (i.e. ANS) strongly at concentrations where the native or unfolded form of the protein do not bind, suggesting that collapsed forms contain accessible hydrophobic surfaces. These findings, together with the observation that same collapsed forms aggregate readily, raise the possibility that they may be produced by a collapse involving hydrophobic interactions between solvated side chains (Pfeil et al., 1986)

Recent NMR studies of α-LA indicate that some fixed structure is in fact present in this collapsed form, in contrast to the hypothesis that structure in the molten globule is rapidly fluctuating.

Hydrophobic collapse

The notion of a hydrophobic collapse is on the idea that when an unfolded polypeptide with exposed hydrophobic side chains is placed in an aqueous solution, it will collapse to a state that shields hydrophobic groups from solvent. It seems likely that a hydrophobic collapse is at least partially involved in collapsed forms, although this remains to be established firmly. Dill and coworkers (1985), have used lattice models to argue that a large fraction of compact states contain substantial amount of secondary structure. Bowie et al., 1989 give evidence that the hydrophobic nature of allowable amino acid substitutions in protein provides substantial predictive information about secondary structure. Model compound studies suggest that pairwise hydrophobic interactions can occur even when nonpolar side chains are separated by a layer of water molecules (Wood et al., 1990).

Local unfolding reactions

Intermediates in unfolding reactions are difficult to populate even in kinetic experiments, most likely because the intermediates are not stable in unfolding conditions. Transient fluctuations in the structure of a folded protein can be detected using amide proton exchange measurements or by fragment complementation studies, and it is likely that in some cases these fluctuations correspond to local unfolding reactions (Taniuchi et al., 1986).

How Does Protein Fold?

The old mystery of protein folding i.e. to understand how a protein can achieve its unique 3-D structure or conformations despite the large number of alternatives, can be answered by new experiments. At present, we know of three main stage of protein folding-the formation of secondary structure, the folding pattern and the detailed tertiary structure. The first stage, i.e. the formation of secondary structure, is not connected with high potential barriers and can occur more or less independently in different parts of a molecule. This can lead very quickly to the equilibrium for corresponding degrees of freedom. The main problem is connected with the second and the third stages. It is unclear how the protein finds its native-like folding pattern at the stage of molten globule formation and why this folding pattern which is outlined by relatively weak which does not change upon the formation of the rigid structure which can be stabilized by stronger interactions. An important contribution to the answer of the first question has been made by Shakhnovich and Gutin (1990, 1993). They showed that alternative (not native) relatively stable 3 D structures of each given protein chain are not small variations of the native structure but are quite different from it. Therefore, the biological evolution can stabilize the native structure (which corresponds to the global minimum of energy) without altering the stability of alternative structures.

The equilibrium folding passes through partly folded and molten globule intermediates similar to the intermediates accumulated in the kinetic folding pathway.

The equilibrium molten globule is separated from both the native and unfolded (or partly folded) states by "all-or-none" (Phase) transitions and, therefore, is a third thermodynamic state of a protein molecule. The role of the molten globule in the physiological processes predicted by Bychkova et al., 1988 and later confirmed by several other studies. In fact, the molten globule which may exist in the nascent protein chain, is recognized by chaperones (Martin et al., 1991) and is involved in the insertion of proteins into membranes (Van der Goot et al., 1991, 1992), the target release of hydrophobic ligands (Bychkova et al., 1992) cell fusion and probably also protein degradation (Bychkova et al., 1993). Moreover, there are suggestions that some proteins, e.g. insulin (Hua et al., 1993) function in the molten globule state.

Hyaluronic Acid Binding Protein: A Model For Conformational Studies.

The aforesaid roles played by non native and denatured states of protein are quite relevant to the HA-binding protein.

Hyaluronic acid (HA), is an anionic polysaccharide, made up of repeating disaccharide structure of 1-and 4-linked ß-D glucuronate and 1-3 linked 2-acetamido-2-deoxy-ß D glucose. This primary structure is homogeneous and had been conserved throughout evolution from bacteria to mammals. The molecular weight of HA most commonly observed to be in the range of approximately $2\text{-}6\text{x}10^6$, but low molecular weight HA (approximately $0.1\text{-}1\text{x}10^6$) has also been found (Laurent et al., 1983; Laurent et al., 1960; Holmes et al., 1988).

From our laboratory, we have reported a 68 kDa hyaluronic acid binding protein from normal wistar rat heart tissue using HA-Sepharose affinity chromatography. It was shown to be heat susceptible, protease sensitive and further characterized as sialic acid containing glycoprotein. Babu et al., 1990, demonstrated that 68 kDa HA-binding protein is a homodimer of 34 kDa subunits. Amino acid analysis has shown that, it is rich in glycine and glutamic acid content and has 209 residues in the monomer subunit including nine proline residues (Deb et al., 1995).

Protein folding and stability for a protein is an obligatory requirement for ligand-receptor interactions. In our lab, we examined the enhanced phosphorylation of HA-binding protein in lymphocytes, when treated with hyaluronan, Con A and PMA, without changing its protein level. Interestingly, signal propagation through the phosphorylation of hyaluronic acid binding protein is further supported by increased formation of IP_3 in HA and Con A treated lymphocytes, which is inhibited by about 60% when pretreated with anti-HA-binding protein antibodies. Consequently, the role of 68 kDa hyaluronan binding protein as a signal transducing molecule in lymphocytes in response to mitogens is obvious and ligand HA: HA-binding protein interaction is the first and prime event to lead all these subsequent biochemical events (Rao et al., 1985).

An obligatory requirement for a protein to attain denatured or non-native state for transport across membrane is also applicable to the 68 kDa HA-binding protein. Because, HA-binding protein existence as an extracellular matrix protein is much prominent, although HA-binding protein existence inside the nucleus is also reported.

In our lab 68 kDa HA-binding protein is isolated from tissue by merely treating with 0.2 M glycine-HCl pH 2.2, without using any membrane solublizing ionic or nonionic detergent. Gupta et al., 1993 showed that HA-binding protein is attached to extracellular membrane with specific binding proteins having approximate molecular mass of 37 kDa and 40 kDa in brain, liver and fibrosarcoma. During stress and physiological condition, change in conformation of protein governs the level of proteolysis and protein turnover. In arthritis, a degenerative disorder of cartilage, the HA turnover is proportional to the proteolysis of HA-binding protein which remains in complex form with HA (Bignami et al., 1991). Besides matrix HA-binding protein, the turnover of cell surface HA-binding protein (e.g. CD44) is also reported to be expressed more in case of lymphocyte homing, neoplastic phenomena and inflammation (Brown et al., 1991; Penneys et al., 1993).

AIM AND OBJECTIVES

Cellular responses to contact with the extracellular matrix (ECM) are varied and include adhesion, spreading, differentiation, migration, invasion and remodelling. The multiple role of ECM constituents are reflected in the responses of cells if they express the appropriate receptor repertoire. In most of the cases, cell-ECM contact via these receptors, promotes rapid organization of the cytoskeleton and shape change indicating that the contact signal is transmitted across the plasma membrane to effector molecules associated with the cytoskeleton. It is the ligand-receptor interactions which communicates a variety of signals from the ECM to the cell interior that can influence gene expression.

Till date, several classes of the ECM receptors have been characterized. Amongst the newly described cell adhesion receptors "integrins" are best characterized and functionally implicated in each of the above mentioned biological processes which involve cell adhesion and migration. The integrins serve as a transmembrane linker between its extracellular ligands and its intracellular components and in their capacity, facilitate the transmission of signals.

Hyaluronan (HA), is a polymeric anionic glycosaminoglycan (GAG) that exhibits a wide variety of biological effects mediated by specific binding to cell surface and extracellular matrix proteins. Recently, "Hyaladherin" (HA-binding protein), a new member of the ECM glycoproteins has been reported from many laboratories including ours and their roles have also been demonstrated in few cases. In our laboratory we have purified a 68 kDa HA-binding protein belongs to "integrins" from rat tissues. It plays an important role in cell attachment, tumorigenesis, solid tumor formation, lymphocyte aggregation and many other biological processes. We have further demonstrated that hyaluronic acid (HA) binding to cell surface receptor –

– HA-binding protein induces phosphorylation of HA-binding protein and increased formation of IP_3, which are intermediate steps in signal transduction for biological functioning. All our experimental evidences suggest an important role of the cell surface HA-binding protein in cellular signalling.

Being a cell surface protein, after its synthesis in cytoplasmic compartment, it may be transported across the cell membrane in order to facilitate its biological activity. A protein to cross the lipid bilayer, is expected to convert into a partially folded or denatured conformation before it gets its actual folded structure on cell surface. Thus, in order to understand the structure-function relationship of HA-binding protein in HA induced cell signalling phenomenon, as an initial step, a perturbation technique is applied. In this programme our aim is to study

(a) unfolding of native HA-binding protein in terms of pathway and its kinetics by transverse urea gradient gel.

(b) refolding of unfolded HA-binding protein in terms of pathway and its kinetics by transverse urea gradient gel.

(c) Comparative hydrodynamic volume of HA-binding protein at different urea concentrations by size exclusion chromatography.

(d) Secondary structure of HA-binding protein at different urea concentrations through proteolytic susceptibility.

MATERIALS AND METHODS

Electrophoresis Materials:

Acrylamide, N-N' methylene bis-acrylamide, Bromophenol blue, Sodium dodecyl sulphate, ß-mercaptoethanol, Coomassie Brilliant Blue R-250, TEMED were obtained from Sigma chemicals, St. Louis. USA. Marker kit for SDS-PAGE i.e., Bovine serum albumin (67,000), Ovalbumin (43,000), Carbonic anhydrase (30,000), Trypsin inhibitor (20,100), Lactalbumin (14,400) were purchased from Pharmacia Fine Chemicals, Sweden.

CHEMICALS

Unless and otherwise mentioned chemicals were purchased from Sigma Chemicals, St. Louis, U.S.A. EAH-Sepharose-4B was purchased from Pharmacia Fine Chemicals, Sweden. Hyaluronic acid (Grade I) from human umbilical cord, Agarose, EDTA, BSA, PMSF, Trypsin were purchased from Sigma Chemicals, St. Louis, USA. Sodium azide (LOBA-CHEMIE Indo-Austranal Co.,Bombay), Folin's reagent (Bio-Rad Laboratories), Sucrose (Analar) were used for different analysis. Sepharose CL-4B and Blue dextran 2000 were purchased from Pharmacia Fine Chemicals, Sweden. Urea was obtained from United States Biochemical Corporation.

Processing of dialysis tubings:

Dialysis tubings (Sigma) were boiled for 10 minutes in EDTA (10mM) solution and then washed with double distilled water.

METHODS

Preparation of hyaluronic acid-Sepharose-4B-affinity column:

Hyaluronic acid from human umbilical cord (Grade I) was coupled to EAH-Sepharose 4B beads by Carbodiimide coupling using N-ethyl-N'-(3- dimethylaminopropyl) carbodimide (EDC) as coupling reagent. Here, EDC acts as homobifunctional reactant which conjugates the ligand (e.g. HA) to amino group of the gel beads.

Reaction was performed in distilled water adjusted to pH 4.5-6.0 for 12 hours. For 20 ml of EAH-Sepharose 4B (7-10 µmole-NH_2/ml), 310 mg of EDC and 50 mg of hyaluronic acid were added and pH was checked intermittently and kept in between 4.5 - 6.0 by adding 1N NaoH. The slurry was washed thoroughly with water and alternately with 0.2 M glycine-HCl pH 2.2 and 0.1 N $NaHCO_3$ containing 0.5 M Nacl.

The activated Sepharose was blocked by 1 M acetic acid, pH 4.0 for 4 hours at 4°C under constant stirring condition and again washed as before. The amount of hyaluronic acid bound per ml of gel was estimated by carbazole test (Bitter. et al., 1962) using glucuronic acid as standard and found to be 1.75 mg. Column was packed with HA-Sepharose matrix and equilibrated with 0.01 M PBS, pH 7.2.

Purification of HA-binding protein from rat heart tissue:

Purification of HA-binding protein was carried out according to the method of Gupta et al., (1991). The heart tissue was minced, weighed and washed with 0.01 M PBS, pH 7.2. Homogenate was centrifuged at 12,000 rpm for 30 minutes. The pellet was resuspended in double the volume of 0.2 M glycine-HCl, pH 2.2, homogenized and centrifuged at 17,000 rpm for 30 minutes.

The supernatant was neutralized, and the precipitate was removed by centrifugation at 10,000 rpm for 20 minutes. The supernatant was dialyzed against PBS at 4°C. The precipitated proteins were pelleted down by centrifugation at 10,000 rpm for 30 minutes. The clear supernatant was extensively dialyzed against PBS, loaded on to a hyaluronate-Sepharose 4B affinity column and incubated for 45 minutes. After, the column was washed with PBS and then eluted with 0.2 M glycine-HCl, pH 2.2. The peak fractions were pooled, concentrated in sucrose, followed by extensive dialysis against distilled water and 0.01 M PBS, pH 7.2.

Estimation of protein:

Protein content in the eluted sample was estimated by Lowry's method (Lowry et al., 1951), using BSA solution as standard.

Electrophoretic procedure:

Sodium dodecyl sulphate-polyacrylamide slab gel electrophoresis (SDS-PAGE) was carried out by method of Laemmli (1970). The separating gel was made of 12.5% acrylamide, 0.2% N-N' methylene bis-acrylamide, 0.1% SDS and 0.375 M Tris-HCl, pH 8.8. The solutions were poured and polymerization was initiated by adding freshly prepared 0.05% ammonium persulfate (APS) and 0.05% TEMED. The stacking gel contained 4% acrylamide, 0.106% N-N' methylene bis-acrylamide, 0.01% SDS, 0.125 M Tris-HCl, pH 6.8, 0.05% APS and 0.05% TEMED.

The running buffer was composed of Tris 0.025 M, glycine 0.192 M buffer pH 8.3 and 0.01% SDS. The sample buffer for SDS-PAGE was prepared as follows:

Tris-HCl	0.0625 M, pH 6.8
SDS	2 %
Glycerol	10%
ß-mercaptoethanol	5% (V/V)
Bromophenol blue	0.001%

Low molecular weight standards and protein samples were applied to the gel and electrophoresed. The protein bands were visualized by Coomassie Brilliant Blue

(Merril et al., 1981).

Polyacrylamide gradient gel electrophoresis (Gradient PAGE):

For analysis of the trypsin digested product of HA-binding protein, discontinuous polyacrylamide gel electrophoresis (Davis et al., 1964) in a vertical slab gel was performed under denaturing conditions (i.e. in the presence of 0.1% SDS) at pH 8.9 for 2000 volt-hours, i.e. at 150 volt (constant voltage). The running gel had 5 to 20% acrylamide, 0.106 to 0.8% N,N'-methylene bis acrylamide and 18% sucrose in 0.375 M Tris-HCl, pH 8.8. The stacking gel had 4% acrylamide and 0.106% N,N'-methylene bis-acrylamide in 0.062 M Tris-HCl, pH 6.8. The electrophoresis buffer contained 0.05 M Tris-HCl and 0.38 M glycine, pH 8.3. The molecular weight markers were also run in the same gel. The protein bands were visualized by staining the gel with Coomassie Brilliant Blue.

Transverse urea gradient gel electrophoresis :

Urea gradient polyacrylamide gels were prepared essentially as described by Creighton (1986) with slight modifications. 0.05 M Tris-acetate, pH 8.0 was used to prepare 92x82mm, 1.0mm thick polyacrylamide gels containing a horizontal linear gradient of 0 M - 8 M urea and a compensatory inverse gradient of 10% - 7.5% acrylamide. The concentration of cross linking N,N-methylene bis-acrylamide was kept constant at 0.0266 (w/w) that of acrylamide. A 5% acrylamide stacking gel, pH 6.8 was used. Transverse urea gradient gel electrophoresis was performed using either native proteins or proteins unfolded for 20 minutes at 25°C in 8 M urea. For the analysis of proteins previously unfolded in 8 M urea, the stacking gel contained the same concentration of denaturing agent. Proteins were prepared for sample loading by the addition of 10% glycerol, 0.002% bromophenol blue and for unfolded samples, 8 M urea was also added. Electrophoresis was performed at 4°C in a 0.05 M Tris glycine buffer, pH 8.0, so that the urea gradient was perpendicular to the direction of protein migration. The electrophoresis was started by applying about 10 mA/gel current; after the marker dye had crossed the stacking gel, the current was increased and kept constant at nearly 50 mA/gel. After completion of the electrophoresis, the gels were stained overnight in 0.1% (W/V) Coomassie Brilliant Blue in 25% methanol with 7% glacial acetic acid. The gels were destained by diffusion against 25% methanol with 10% glacial acetic acid.

Limited proteolysis by trypsin:

HA-binding protein in 0.05 M Tris-HCl, pH 8.0 was equilibrated at various urea concentrations for 20 minutes at 37°C before the addition of trypsin at a final enzyme: substrate ratio of 1: 100 (W/W). The reaction was stopped by adding Phenylmethylsulfonyl fluoride (PMSF) at final concentration of 0.003 M at various time of incubation. Analysis of the digested material was made by discontinuous gradient SDS-PAGE followed by Coomassie Brilliant Blue staining.

Size exclusion charomatography:

The apparent hydrodynamic volume of HA-binding protein in 0 M, 3 M, 5 M and 7 M urea state was determined by size exclusion chromatography on Sepharose CL-4B column (0.7x27 cm) (Ackers et al., 1967, 1970; Corbett et al., 1984). The column was equilibrated with 0.05 M Tris acetate, pH 8.0 at 4°C at a constant flow rate of 0.5 ml/5 minute. After equilibration, the void volume of the column was determined by passing 200 µl of Blue Dextran 2000 (1mg/ml in Tris-acetate buffer). The maximum peak position of the elution at 280 nm was taken as the elution volume of Blue Dextran as well as the void volume (Vo) of the column. Preincubated HA-binding protein samples with various concentrations of urea were run and 0.5 ml/5 minute fractions were collected and monitored at 280 nm. From their elution volumes, hydrodynamic volume of HA-binding protein at various concentrations of urea were compared 0.15 M KCl was also added to Tris-acetate buffer to avoid non specific binding of protein to the column.

RESULTS

Rationale of protein unfolding and refolding by urea gradient electrophoresis:

A uniform sample of protein, either native or unfolded is subjected to electrophoresis through a slab gel of polyacrylamide in which there is a transverse gradient of a nonionic denaturant typically 0 M to 8 M urea (Creighton et al., 1979, 1986). The same protein is migrating differentially in different portion of the gel, depending upon the concentration of urea in gel. Its presence alters the rates and equilibrium of the interconversion between the native and denatured states. Compact, folded conformations migrate more rapidly than do expanded conformations. Different conformations can also differ in their net charge (Hollecker et al., 1982), but this is usually minor compared to the effect on mobility caused by the increase in hydrodynamic volume that occurs upon unfolding.

Urea gradient electrophoresis, like other separation methods, can be used to detect different conformational isomers only if the separation can be at least as rapid as the rate of their interconversion. If one or more conformations are rapidly interconverted with half-time no greater than about 0.1 the duration of the electrophoretic separation, a single band will result with a mobility that is the average of the conformations present (Creighton et al., 1979, 1986). Consequently, unfolded proteins usually migrate as a single apparently homogeneous band with a breadth no greater than that of the folded protein: many unfolded conformational samples are rapidly interconverted and have similar average mobilities. Conversely, different populations of conformations that are interconverted with half-times substantially greater than the duration of the electrophoresis will produce separate bands. Conformational transitions with half-times comparable to the electrophoretic separation time tend to produce diffuse, smeared patterns because the various protein molecules undergo the conformation transitions at different times during the separation. Consequently, the various molecules have overall mobilities that vary widely between the two extremes of the initial and final conformations.

Ribonuclease A and Hen egg white lysozyme can remain unfolded and folded, respectively, within the urea gradient gel and they are used as internal standards to check constant mobility at all urea concentrations (Hollecker et al., 1982).

A rapid and reversible unfolding transition produces a continuous band of protein, which depicts the equilibrium between the conformations present with a co-operative two state transition, there should be a sigmoidal transition between the bands resulting from the two end states, with a single inflection point at about the midpoint of the transition.

A partly folded state of a protein is expected o have an electrophoretic mobility intermediate between those of the native and unfolded states and to unfold in a less abrupt transition than the native conformation.

Not all proteins unfold in co-operative two state transitions, especially larger ones composed of multiple domains (Privalov et al., 1982), which might unfold independently at different urea concentrations. The two stages probably correspond to the independent unfolding of two domains at different urea concentrations (Drabikowski et al., 1982).

Kinetic studies of protein unfolding and refolding are extremely important since the process is highly cooperative. Partially folded intermediate which define the folding pathway are inherently unstable and are not populated to significant extents at equilibrium. However, they might accumulate as transient kinetic intermediate. The advantages of urea gradient gels for studying the thermodynamics of protein folding transitions also apply to kinetic studies (Creighton et al., 1980). The kinetics of unfolding and refolding have been studied by urea gradient gels, starting the electrophoresis with the folded and unfolded forms of protein. The separation of kinetically isolated conformations was optimized by carrying out the electrophoresis at low temperature (2°C) to slow the folding transition, at high voltage to maximize the rate of migration, in thin gels to minimize the heat generated and for as short a period of time as possible. The pattern generated from such an experiment can be quite complex, since the final distribution of protein depends upon the species present initially and upon the rates of their interconversion, which are usually highly sensitive to the urea concentration, and thus are different across the gel. Nevertheless, comparisons of the patterns observed starting with both the unfolded and folded forms of the protein permitted estimation of the rates of unfolding and refolding and the relative hydrodynamic volumes of protein species, at continuously varying urea concentrations. The unfolded states of many proteins have been found to consist of two or more populations which differ in their rates of refolding and are only slowly interconverted. The different unfolded forms of protein have been shown to differ in their *cis-trans* isomerization about the peptide bonds preceding proline residue (Schmid et al., 1978; Lin et al., 1983). Proline *cis-trans* isomerization is inherently independent of denaturant concentration and very slow at low temperatures occuring with a half-time of about 20 minute at 0°C, so different isomers might be resolved by rapid electrophoresis, a pattern that would be expected on the simplest basis that incorrect proline isomers completely block refolding.

Four different species of a slow unfolding and refolding of staphylococcal pencillinase protein have been identified by urea gradient electrophoresis: the Native (N) unfolded (U) and two intermediate conformation (I and H) (Creighton et al., 1980). The observation suggests that the unfolded protein is in rapid equilibrium with more compact conformations under refolding conditions. In contrast, residual folded protein showed no sign of rapid, reversible partial unfolding under denaturing conditions. The slowest step in each direction is the interconversion of the fully folded, native state, with all the others. Starting with N, there is no indication of any partial unfolding preceding complete unfolding, since the band of protein has constant mobility across the gel. Starting with the fully unfolded state, U, which predominates at high urea concentrations, there is one rapidly reversible transition to a some what more compact state, H. The conformational state corresponds to one which had been shown to be the

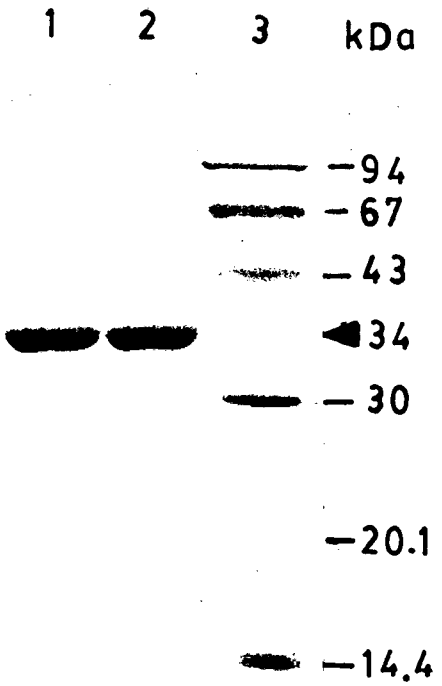

Fig 3. **Homogeneity of purified rat heart HA-binding protein by SDS-PAGE.**

Lanes 1 and 2: 7 μg of pure rat heart HA-binding protein in the absence of ß-mercaptoethanol (non reducing conditions) and in the presence of ß-mercaptoethanol (reducing conditions) respectively; lane 3 : 4 μg of low-molecular-weight marker proteins: phosphorylase b (94 kDa), BSA (67 kDa), Ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and lactalbumin (14.4 kDa). Electrophoresis was carried out in a 12.5% polyacrylamide slab gel in the presence of 0.1% SDS using the Laemmli discontinuous buffer system. The protein bands were visualized by the Coomassie Brilliant Blue staining. The HA-binding protein band migrated to the same position of 34 kDa in both under reducing and nonreducing conditons.

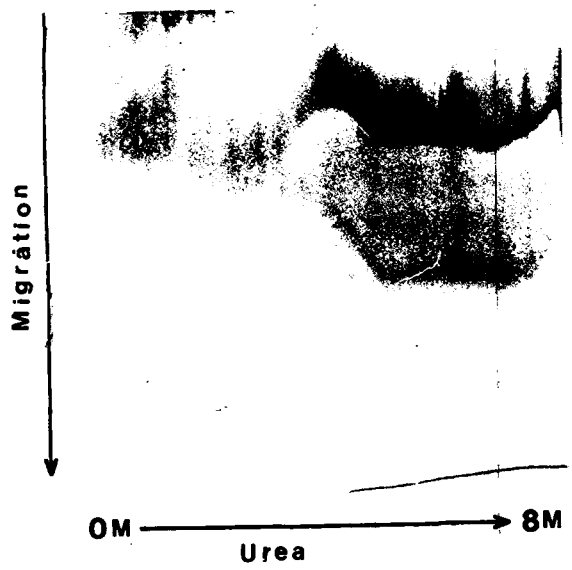

Fig 4. **Transverse urea gradient gel electrophoresis pattern of native HA binding protein**

The linear gradient of 0 M to 8 M urea was superimposed on an inverse linear gradient of 10% to 7.5% acrylamide. Just prior to electrophoresis a 200 µl sample containing 70µg of purified HA-binding protein was layered on the top of the gel. Electrophoresis towards the anods was at 4°C and 50 mA/gel. The gels were stained with 0.1% Coomassie Brilliant Blue in 25% methanol with 7% glacial acetic acid, then destained by diffusion against 10% acetic acid and 25% methanol.

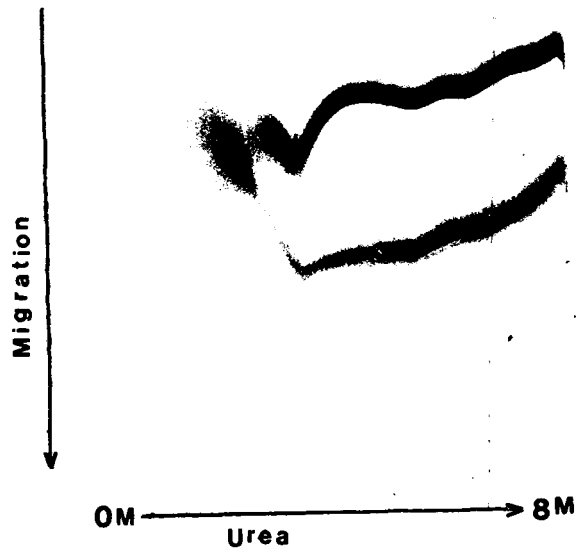

Fig 5. **Transverse urea gradient gel electrophoresis pattern of unfolded HA-binding protein.**

The linear gradient of 0M to 8 M urea was superimposed on an inverse linear gradient of 10% to 7.5% acrylamide. Just prior to electrophoresis a 200 µl sample containing 70 µg of purified HA-binding protein was unfolded in 8M urea for 20 minutes at 25°C and layered on the top of the gel. For the analysis of proteins previously unfolded in 8M urea, the stacking gel contained the same concentration of urea. Electrophoresis towards the anods was at 4°C and 50 mA/gel. The gels were stained with 0.1% Coomassie Brilliant Blue in 25% methanol with 7% glacial acetic acid, then destained by diffusion against 10% acetic acid and 25% methanol.

most thermodynamically stable at intermediate urea concentrations. At very low urea concentrations, there is a second transition to a previously unknown, highly compact species, I, approaching the mobility of N; it is only a transient intermediate because slow refolding to N occurs under these conditions. Keeping this in view, we planned to study the conformational states of a novel 68 kDa HA-binding protein under urea gradient electrophoresis.

We have purified HA-binding protein homogeneously using HA-Sepharose affinity column and confirmed the purity of this protein as a single band of 34 kDa as observed under SDS-PAGE (Fig. 3). This purified HA-binding protein has been used throughout the experiment.

As evident from Fig. 4, complex unfolding transition of native HA-binding protein was seen in urea gradient electrophoresis at pH 8.0 and 4°C. Relatively slow unfolding occurred during electrophoresis at low urea concentrations indicated by smeared band between O M and 3 M urea. Whereas, a continuous and intense band of protein appeared between 3 M and 8 M urea suggesting a rapid unfolding. Protein band between 3M and 8 M urea shows an interesting hypobolic pattern of unfolding. At the high urea concentration particularly between 3 M and 8 M, a continuous faint protein band having higher electrophoretic mobility designated as fast refolding species (U_F) appeared below the slow refolding species (U_S) of slow electrophoretic mobility.

In order to further study the refolding transition of HA-binding protein, it was allowed to unfold by treating with 8 M urea for 20 minutes at 25°C prior to layering on urea gradient electrophoresis. As shown in fig. 5 describes the refolding transition of unfolded HA-binding protein in urea gradient gel electrophoresis at pH 8.0 and 4°C. Relatively slow refolding occured during electrophoresis at low urea concentrations as indicated by smeared protein band between O M and 3 M urea. An abrupt change in refolding transition take place at about 3M urea. A quite intense and continuous protein band appeared between 8 M and 3 M urea indicating a rapid refolding. This protein band between 3 M and 8 M urea shows gradual decrease in electrophoretic mobility at higher urea concentration in perpendicular direction of the migration. Also at the high urea concentration, particularly between 3 M and 8 M, a comparatively faint but continuous protein band having higher electrophoretic mobility, designated as U_F, appeared below the U_S of slow electrophoretic mobility.

Rationale of hydrodynamic volume change detection by size exclusion chromatography:

The use of chromatography to detect conformational differences between proteins is an another area of investigation, the first attempt to use analytical gel filtration for studies of protein denaturation in various solvents done by Tanford and Co-workers (Fish et al., 1970). Conformational changes in several proteins have been detected by size-exclusion chromatography (SEC) in terms of changes in retention time which correlates with the changes of Stokes radii of the protein conformers (Corbett et al., 1993, 1984; Brems et al., 1985). Corbett and Roche, 1984 studied unfolding of those proteins which denatured usually in two state manner (i.e. $N \rightleftharpoons U$ scheme) by size

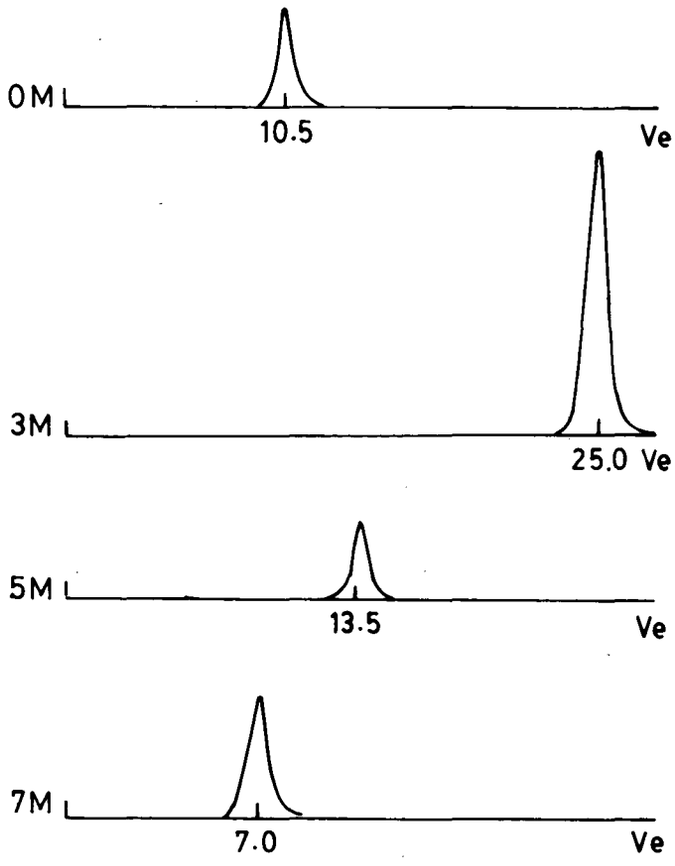

Fig 6. **Elution profile of HA-binding protein at different concentrations by size exclusion chromatography**

Preincubated HA-binding protein (4 μg) was loaded into sepharose CL-4B column, which was pre equilibrated at 4°C in 0.05 M Tris-acetate, 0.15 M kCl, pH 8.0 with respective urea concentration. Elution fraction was collected at 4°C in same buffer at a constant rate of 0.5 ml/5 minutes and monitored at 280 nm.

exclusion chromatography and determined that neither N nor U states interact with the column matrix i.e. a column does not shift the equilibrium between these conformers. Further, Uversky; 1993 suggested that size exclusion chromatography is an "inert" technique, i.e., it does not shift the equilibrium between N, MG and U states and therefore can be used for the studies of the unfolding proteins that denature through the molten globule state, i.e. on the $N \rightleftharpoons MG \rightleftharpoons U$ denaturation scheme.

In size exclusion chromatography, order of elution of proteins are inverse of their hydrodynamic diameters. Size exclusion chromatography is performed using porous beads, which have two measurable liquid volumes, the external volume, consisting of the liquid between the beads, and the internal volume, consisting of the liquid within the pores of the beads. Proteins whose hydrodynamic diameter is small relative to the average pore diameter of the beads will access all of the internal volume and are described as being included in the gel matrix. Proteins whose hydrodynamic diameter is comparable to the average pore diameter will access some but not all of the internal volume and are described as being fractionally excluded. Proteins whose hydrodynamic diameter is large relative to the average pore diameter will be unable to access the internal volume and are described as being excluded. Most interestingly, hydrodynamic volume is the operative protein dimension and that an asymmetrical protein will appear to elute with an abnormally high molecular weight compared with globular proteins of similar molecular weight. Thus in order to further confirm the urea gradient gel electrophoresis result, we have studied the changing hydrodynamic volume of HA-binding protein at different urea concentrations by size exclusion chromatography.

Fig. 6. shows the elution profiles for HA-binding protein at different Urea concentrations: O M, 3 M, 5 M and 7 M. In this experiment there is one elution peak which shifts to bigger elution volume with the increase in urea concentration at 3 M, in comparison to O M. But with the increase in urea concentration further at 5 M and 7 M, elution peak get shifted to smaller elution volume. Elution profiles with increase in urea concentration show that the denaturation of HA-binding protein occurs in two stages. At the first stage elution volume increases, this corresponds to decrease in hydrodynamic volume of protein or in Stokes radius. At the second stage elution volume gradually decreases, this corresponds to gradual increase in hydrodynamic volume of protein or Stokes radius.

Rationale of modified proteolytic susceptibility of a protein in the presence of urea:

To be cleaved by a protease, a polypeptide chain must fulfill two requirements:

(i) It must contain the specific sequence that corresponds to the substrate specificity of the protease, and

(ii) this sequence must be sterically accessible to the protease, since native and denatured proteins differ in a characteristic way in their accessibility to protease, most substrate sites are expected to become buried in the process

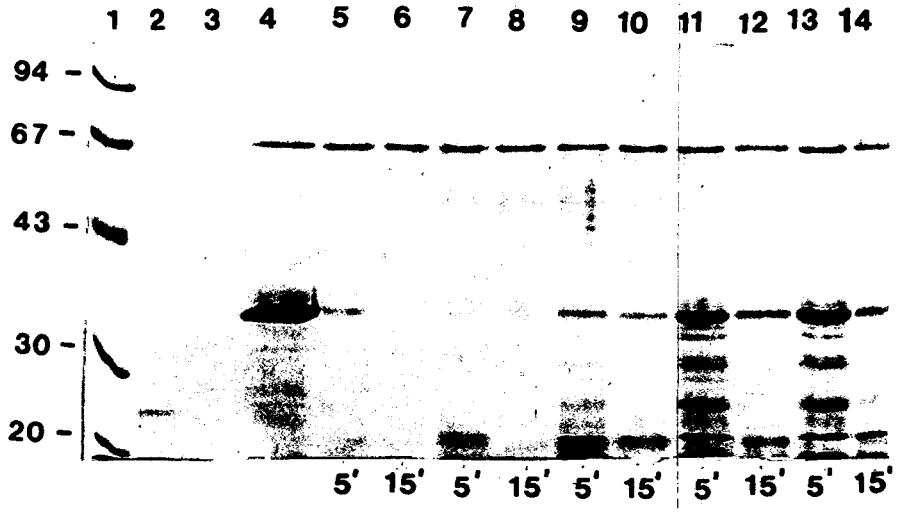

Fig 7. **Modified susceptibility of HA-binding protein to proteolysis by trypsin**

HA-binding protein (10μg) was incubated with various concentrations of urea for 20 minute at 37°C before addition of trypsin to final enzyme: substrate ratio of 1: 100 (W/W). The samples were subjected to gradient SDS-PAGE and Coomassie Blue stained. HA-binding protein in the absence (lanes 4,5 and 6) or presence of urea at the concentration of 2 M (lanes 7 and 8) or 4 M (lanes 9 and 10) or 6 M (lanes 11 and 12) or 8 M (lanes 13 and 14) is shown. Lane 1 corresponds to molecular weight markers in kDa (from top to bottom: Phosphorylase b, 94; Bovine serum albumin, 67; Ovalbumin 43; Carbonic anhydrase 30; Soyabean trypsin inhibitor, 20.1) Lane 2 corresponds to trypsin. Lane 3 corresponds to trypsin with PMSF. Lane 4 corresponds to HA-binding protein in absence of protease and urea. Lane 5,7,9,11 and 13 correspond to protease digestion for 5 minute and 6,8,10,12 and 14 correspond to digestion for 15 minutes.

of protein folding.

If a protein contains a limitednumber of cleavage sites, their disappearance may be directly correlated with the transition of the folding polypeptide chain from the unfolded to the compact, native state. Since guanidinium ion inhibits trypsin activity, urea is used as denaturant (Creighton et al., 1990).Trypsin, a specific proteolytic enzyme, can cleave polypeptide chains on carboxyl side of arginine and lysine residues. In trypsin, the side chains of the amino acids that line the substrate-binding site are negatively charged amino acids, which facilitate the binding of only positively charged (lysine or arginine) residues, instead of the hydrophobic ones.

Thus, upon proteolytic digestion of HA-binding protein in presence of different concentrations of urea, it generates a differential proteolytic fragments.

Fig. 7 shows that HA-binding protein is quite susceptible to digestion by trypsin when equilibrated between 0 M and 2 M urea. The protein becomes increasingly resistant to digestion in a transition between 4 M and 8 M urea. Between 4 M and 8 M urea, digestion produces discrete low molecular weight fragments as opposed to the behaviour at 0 M or below 2 M urea where digestion produces very small peptide chains, which may get eluted out from the resolution limit of gel. From the intensity of 34 kDa band and discrete low molecular weight fragments, it can be inferred that the number of proteolytic attack sites of HA-binding protein are decreasing, in order of increasing urea concentration between 4 M & 8 M. Trypsin is chosen because of its specificity, so that proteolytic patterns reflect exposure of the structure, i.e., hydrophillic sites on the protein surface which have altered degree of accessibility as a function of urea concentration. HA-binding protein has been shown to undergo significant decrease in accessibility to trypsin at high urea concentration.

DISCUSSION

The extracellular matrix protein, HA-binding protein, exhibits a complex unfolding transitions which contrasts with the the usual co-operative two-state transitions of small globular protein in which partially unfolded intermediate conformations are thermodynamically unstable. Two state transitions have only the folded (N) and unfolded (U) states populated substantially, but HA-binding protein has been shown to have an additional third state designated as molten globule (MG), predominant within the transition region at intermediate concentration of urea. Molten globule state (MG) has apparent conformational properties intermediate in many respects between U and N. Further complexities of the folding transition are indicated by biphasic kinetics of refolding, with comparable amplitudes for both phases.

Urea gradient electrophoresis approach to the kinetics of protein unfolding and refolding:

Three distinct conformational states of HA-binding protein have been detected here on the basis of difference in their hydrodynamic volume (1) the native state N, which is dimer of two monomer subunits, each monomer having 209 residues including nine proline residues with covalent modifications (Deb et al., 1995). Conversely, the absence of disulphide cross-linkages, presence of nine proline residues and its more typical size (68 kda) in the native state makes it a favourite subject for denaturant induced unfolding and refolding transition studies than those of the small, stable and usually disulphide cross-linked proteins. (2) the molten globule (MG), which is monomeric subunit of native dimer, which is thermodynamically predominant around 3 M urea and very compact secondary structure having less hydrodynamic volume. (3) partially folded state, U, having still secondary structure at urea concentration between>3M and <8M. All of these states appear to be interconverted in transitions of various degree of co-operation, as judged by smoothness and steepness of the urea induced transitions. The co-operative transitions between them indicates that these are three distinct conformational states.

A smeared band between 0 M and 3 M urea during unfolding transition of native HA-binding protein (Fig. 4) indicates slow equilibrium between native and molten globule state during electrophoresis, this is very obvious with small amounts of the native protein and is probably due to adhesion to the polyacrylamide gel matrix (Labia et al., 1977). However, this band get intense and continuous between around 3 M and 8 M urea indicating that equilibrium between molten globule and partially folded state is rapid, during electrophoresis. The band pattern between around 3 M and 8 M is hypobolic indicating that molten globule state undergo further unfolding at higher urea concentration. This is the first report, were we have demonstrated the appearance of two different species of unfolded HA-binding protein i.e. slow refolding species U_S and fast refolding species U_F. At higher urea concentration, having the same band pattern but different electrophoretic mobility of U_S and U_F indicates relatively slow interconversion of *cis* and *trans* isomers of peptide bonds adjacent to proline residues (Schmid et al., 1978). Our observation is in agreement with the previous findings that the proteins

having the proline residue produces both slow and fast refolding species at higher urea concentration.

A smeared band between about 0 M and 3 M urea during refolding transition of unfolded HA-binding protein during refolding transition of unfolded HA-binding protein, (Fig. 5) indicates slow refolding and slow equilibrium between native and molten globule state during electrophoresis. A sharp inclination of migration of protein band about 3 M urea indicates substantial decrease in hydrodynamic volume, which is designated as molten globule state MG. Comparative higher electrophoretic mobility of molten globule state than native state in the same gel support the association of monomeric molten globule state to dimeric native state between 3 M and 0 M urea. This band get intense and continuous between 3 M and 8 M indicates the rapid equilibrium between molten globule state and partially folded state during electrophoresis. This band between 3 M and 8 M shows gradual decrease in electrophoretic mobility in perpendicular direction to migration implies gradual unfolding of molten globule state to partially folded state. The appearance of slow and fast refolding species band during refolding transition, particularly at high urea concentration, support the slow interconversion of *cis* and *trans* isomer of peptide bonds adjacent to proline residues. HA-binding protein (monomer) has nine proline residues but their stereochemistry in the folded state is not known, but at least a substantial fraction of unfolded molecules would be expected, to have one or more incorrect *cis-trans* isomers which refold more slowly than those unfolded molecules with correct isomers (Creighton et al., 1980) especially at low temperature used here. It appears that at least some U_s molecule can refold to native-like state before *cis-trans* isomerization and that the rate of isomerization can be greatly increased by the tendency of U_s molecules to acquire non-random conformations under refolding conditions (Cook et al., 1979)

Size exclusion chromatography approach to the conformational change of protein unfolding:

In size exclusion chromatography (SEC), at least three distinct conformational states of HA-binding protein have been detected on the basis of difference in elution volume, which is inverse of their hydrodynamic diameters, (Fig. 6). These three conformational states of HA-binding protein appeared during two transitions: the first one is interpreted (Ptitsyn et al., 1987) as denaturation of protein (i.e. N→MG transition) and the second one is interpreted as further unfolding of protein (i.e. MG→U transition). Out of three conformational states of HA-binding protein, molten globule MG, state has minimum hydrodynamic volume i.e. compact secondary structure followed by partially unfolded U and native N state.

Modified proteolytic susceptibility approach to the protein structure:

The characteristics of trypsin digested pattern in presence of urea (Fig. 7) shows the transition of HA-binding protein structure as a function of denaturant concentration. This transition takes place in two phases. First, native structure of HA-binding protein which is vulnerable to trypsin attack, and remain vulnerable

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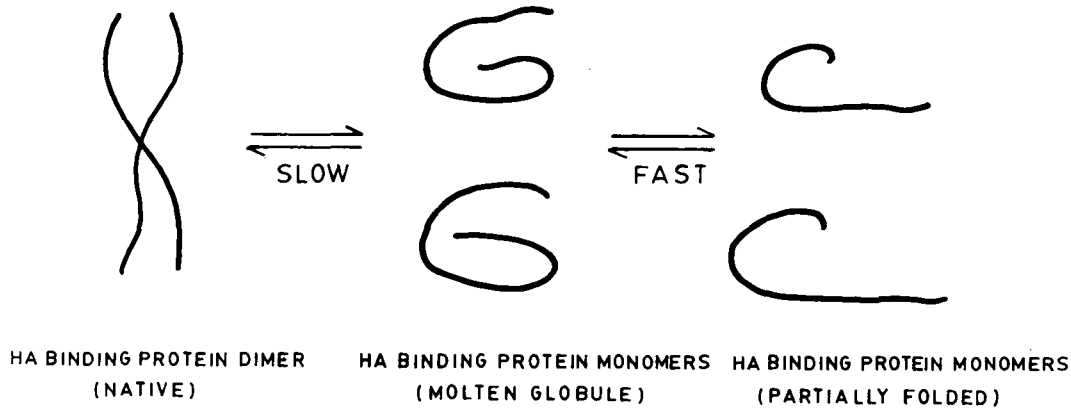

Fig 8. **Hypothetical model for the unfolding and refolding of HA-binding protein-** Urea induced the dissociation from native dimer to molten globule monomer which was slow and non co-operative. This was followed by the highly co-operative and rapid urea induced denaturation from molten globule monomer to partially folded monomer. Partially folded monomer had still some secondary structure at high urea concentration.

substantially up to 2 M urea equilibrated sample. Secondly, between 4 M and 8 M urea equilibration, HA-binding protein structure leads to gradual decrease in accessibility of trypsin, when urea concentration is increased. On the basis of trypsin digestion pattern and intensity of 34 kDa HA-binding protein band at different concentrations of urea, at least three distinct conformational states of HA-binding protein can be assured. (1) Native N; where all proteolytic attack sites are accessible to trypsin means open structure. (2) Partially folded, U, where all proteolytic attack sites are not accessible to trypsin means partially folded structure (3) Molten globule MG, which is most compact structure. The molten globule MG state of protein can be assumed between native and partially unfolded state during unfolding transition and also supported by other results. At urea concentration more than 6 M, the overall digestion pattern look similar to that seen in 6 M, except the the extent of digestion get decreased. To rule out the possibility that some of the cleavage patterns seen at higher urea concentration were the result of digestion by an acid protease contaminant or protein preparation, we examined the specificity of PMSF to the trypsin. The inhibitor completely annealed the potential oftrypsin (lane number 2 and 3 in fig. 7). The specificity of this inhibitor for trypsin thus makes it unlikely that there is any contaminating protease activity.

Hypothetical model for the unfolding and refolding of HA-binding protein

The results demonstrated here, are consistent with hypothetical model (Fig. 8) for unassisted unfolding and folding of HA-binding protein. In this model, native dimer HA-binding protein, when perturbed by increasing urea concentration, initially forms intermediate by the dissociation of native dimer into monomer. The dissociated monomer has characteristic of a molten globule. For example, it is compact with its disordered tertiary structure. As the urea concentration is increased, there is further increase in the denaturation of the molten globule monomer. This denaturation may lead to a partially folded monomer structure, which have still some secondary structure at high urea concentration. Urea gradient electrophoresis suggests that the unfolding transition from native dimer to a molten globule monomer is not very co-operative in comparison to transition from molten globule monomer to a partially folded monomer. The latter transition has rapid equilibrium between molten globule monomer and partially folded monomer than the transition from native dimer to molten globule monomer. The same pathway is followed in the reverse direction during refolding of unfolded HA-binding protein to native dimer in respect of kinetics.

CONCLUSIONS

Hyaluronic acid binding protein is one among the class of cell surface receptor for hyaluronic acid belonging to the family of 'hyaladherins'. This protein after synthesis must be transported across cell membrane in order to facilitate several important adhesion events like cell substrate adhesion, cell-cell adhesion, maintenance of normal cell morphology, embryonic differentiation, cell motility. But, for a protein to cross a lipid bilayer after translation and folding, it may be first converted into a partially folded or denatured conformation, either spontaneously or with the help of unfoldase. To approach the question of how partially folded or denatured conformation of protein acquires its biological three-dimension structure or conformation, a urea-induced perturbation is typically applied to the system.

Hyaluronic acid binding protein (Mr=68 kDa) is a dimeric protein in native state. Each monomer is (Mr=34 kDa) of 209 amino acid residues including 9 proline residues and have no disulphide cross-linkages. Important findings of present study regarding unfolding and refolding mechanisms of HA-binding protein are as follows on the basis of transverse urea gradient gel electrophoresis, size exclusion chromatography and modified proteolytic susceptibility experiments.

(a) Hyaluronic acid binding protein follows $N \rightleftharpoons MG \rightleftharpoons U$ scheme for unfolding and refolding.

(b) HA-binding protein dimer dissociation to molten globule monomer is reversible, slow and non co-operative in kinetics.

(c) Reversible denaturation from molten globule monomer to partially folded monomer is rapid and co-operative in kinetics.

(d) Formation of slow and fast refolding species is due to slow interconversion of *cis* and *trans* isomers of peptide bonds adjacent to proline residues.

Apart from the importance of this report to conformational studies of HA-binding protein for biological functioning, HA-binding protein has become a favourite subject for denaturant induced unfolding and refolding transition due to its more typical size (68 kDa) in native state, presence of nine proline residues and absence of disulphide cross-links than those of small stable and usually disulphide cross linked proteins.

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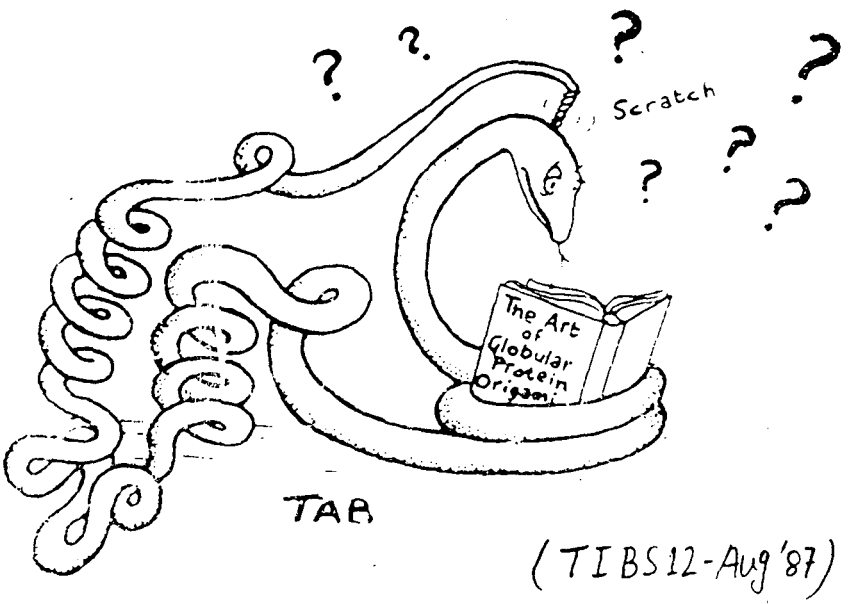
- NOW COLLAPSE DOWN HYDROPHOBIC CORE, AND FOLD OVER HELIX 'A' TO DOTTED LINE, BRINGING CHARGED RESIDUES OF 'A' INTO CLOSE PROXIMITY TO IONIC GROUPS ON OUTER SURFACE OF HELIX 'B' - - -



TAB

(TIBS 12-Aug '87)