Instituto Juan March
de Estudios e Investigaciones

89 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Protein Folding

Organized by

A. R. Fersht, M. Rico and L. Serrano

R. L. Baldwin
B. Bukau
F. E. Cohen
W. F. DeGrado
C. M. Dobson
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S. M. V. Freund
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Introduction

A. R. Fersht, M. Rico and L. Serrano

The information encoded in the genome of any living system has to be translated into protein sequences in order to generate biological activity. Protein molecules are synthesized in the ribosomes as linear polypeptide chains, which must fold into their well-defined three dimensional structure in order to be functionally active. Understanding how this functional three-dimensional structure is obtained is one of the fundamental problems still to be solved in modern Biochemistry. Much remains to be known about the forces involved in determining protein folding and stability. Yet the problem is important not just from an academic perspective but from a practical point of view. There is for instance a growing pressure to solve it in order to fill the gap between the number of known sequences provided by the different genome projects, and the number of known structures determined by NMR and X-ray crystallography. Rational proposals for the obtention of, *i.e.*, novel proteins with improved or new biological activities can only be made on the basis of the three-dimensional structure, which for heterologous proteins cannot be predicted from the aminoacid sequence up to the present time. The same can be said for proteins which can be considered as drug receptors, so that folding is also a central issue in drug discovery for use with pharmaceutical and medical purposes. More recently, there is a growing evidence that folding is coupled intimately with the localization and regulation of biological activity. Misfolding events therefore lead to malfunctioning of living systems, and an increasing range of diseases from cystic fibrosis to Creutzfold-Jakob disease and Alzheimer's is now associated with such problems.

During the past few years, recent advances in both experimental and theoretical aspects have offered new insights into the basis of the problem. The aim of the Workshop was to provide a forum for discussion on recent advances and methodological developments in protein folding on the part of leading experts in the field. The Workshop was directed to a wide range of scientists, more or less connected to this important biological problem, and the main intention was to enrich their views on those aspects less familiar to them.

The developing of very simple theoretical models that can reproduce some of the experimental results obtained with real proteins, the so-called lattice models, have captured the imagination of many people in the protein field. These approximations provide some microscopic insight into the process of protein folding and could help in the interpretation and design of future experiments. A long term goal in protein folding is to predict the pathway of folding of proteins by computer simulation. Recent Molecular Dynamics simulations from several laboratories are showing that the goal can be accomplished for the unfolding of small proteins as demonstrated by the good agreement obtained between the predicted pathways with the experimental results coming out from protein engineering studies.

Simple experimental protein systems have shown to be invaluable in obtaining meaningful data about the main factors involved in the folding process. We are approaching a moment in which due to the number of experimental models analyzed, there is enough information to start proposing unified models explaining the folding reaction of small proteins. In particular the development of methods that allow the monitoring of folding stages in the μs range, have provided for the first time insight into the early folding events. Similarly recent advances in NMR and Mass Spectroscopy have allowed us to monitor specific conformations in the denatured state, as well as of the progression of the conformational ensemble to the folded state.

While still we are far from understanding the folding process and from predicting correctly the 3-D structure of a particular sequence, several groups have started to use the information already available to design new proteins. The *de novo* design of proteins implies the possibility of rationally modifying existing proteins, or the design of sequences that should adopt a given target fold. During the past five years this field is coming into a mature state as demonstrated by the results presented in the Workshop, according to which many different successful rational designs have been accomplished. The possibility of increasing the stability, selectivity or catalytic efficiency of a protein has then proven to be possible.

In the last few years a large number of proteins that assist protein folding *in vivo* (chaperones) have been discovered. Some of them have been shown to be highly versatile chaperones since their assistance range from folding of nascent proteins to proteolytic degradation of unstable proteins. While most proteins appear to fold upon release from those chaperones, certain slow-folding and aggregation-sensitive polypeptides seem to be subsequently transferred to a cylindrical protein complex (chaperonin) for folding in the sequestrated environment of its central cavity. Questions related to this important and new field of research were actively discussed at the Workshop.

Finally, two important human diseases associated to protein aggregation due to misfolding were addressed at the Workshop: amyloid related diseases (including Alzheimer's disease) and transmissible encephalopathies caused by a proteinaceous infections agent or prion. Not only invited papers but also the communications presented as posters in this Workshop were important contributions to grasp the current state and the future work to do on this fascinating biological problem. While many of the questions on protein folding are still to be solved, the Workshop did certainly succeed in enriching our view of the problem and setting the scenario for future developments on the field.

A. R. Fersht, M. Rico and L. Serrano

Session 1: Theoretical aspects and simulation

Chair: Robert L. Baldwin

EXPERIMENT AND SIMULATION OF PROTEIN FOLDING

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A long term goal in protein folding is to predict the pathway of folding of proteins by computer simulation. Such simulations require experimental data at atomic resolution to benchmark their validity. Such information is being provided by protein engineering experiments - F-value analysis, which can chart the progress of each side chain as a protein folds up. Molecular dynamics simulations from several laboratories of the unfolding of chymotrypsin inhibitor 2 (CI2) and barnase are in good agreement with the protein engineering studies. The simulations are subject to several assumptions, especially the validity of extrapolating data at 500 K to room temperature. Thus, they require experimental validation. We have now tested predictions on the structure of the transition state of CI2 and verified them by designing faster folding mutants. By bench marking the simulation of the unfolding of barnase using F values and NMR studies on the denatured state, we are able to describe the complete folding pathway of barnase at atomic resolution.

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Protein Folding: Insights from Simulations

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To understand the thermodynamics and dynamics of proteins, simulations are needed to supplement the experimental data. In most cases, simplified models have to be introduced to be able to explore the accessible conformational space. Examples taken from lattice and off-lattice simulations of protein folding and unfolding will be presented.

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Peptide Folding: When Simulation Meets Experiment

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It has been commonly assumed that it is not possible to simulate the folding of a peptide in solution in atomic detail under reversible conditions using molecular dynamics simulation techniques. First, the time scale on which peptides fold is considered beyond that which can be currently simulated using realistic models. Second, the available models are thought to be of insufficient accuracy to reproduce conformational preferences of peptides under realistic conditions. In this report we provide clear evidence, exemplified by studies on β-peptides, of the ability of atomic level dynamics simulations to accurately reproduce the mechanism of peptide folding in solution and to reproduce conformational preferences as a function of amino acid sequence.

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Folding-unfolding thermodynamics of a β-heptapeptide from equilibrium simulations
Proteins (1998) in press

- X. Daura, A.E. Mark and W.F. van Gunsteren
Peptide folding simulations: No solvent required?
Comp. Phys. Comm. (1998) in press

PROTEIN HYDRATION, STABILITY AND UNFOLDING

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Following on our studies on hydration of proteins, we have focused on an analysis of cavities within proteins and specifically on the properties of cavities in native proteins which are found to contain solvent molecules (1). From this approach, we have been able to estimate whether any cavity within a protein is likely to be hydrated. This estimate of the probability of hydration depends on its size and the extent of its polar surface2-3. Such calculations can be carried out at realistic temperatures for unfolding (80C) and within an iterative protocol using a total of 100 ps molecular dynamics. The original algorithm was based on a modelling procedure in which we only had a shell of solvent molecules and as this shell was broken on unfolding it was repaired. More recently, we have improved the algorithm so that we have a more usual insulation in a NVT or NPT ensemble. Thus there is a normal box of solvent and water molecules needed for insertion into cavities are chosen from those near the periphery. We will present data on the use of both the original 4) and modified insertion protocols as well as comparisons with high temperature unfolding simulations. We are currently exploring these approaches to study the unfolding of a number of proteins including thermophilic and mesophilic ribonuclease H, p53 and the eye lens crystallins.

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Protein Folding: from Lattice Models to real Proteins. Physical Insights into Protein Evolution.

E.I Shakhnovich

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Theory, simulations and experiment converged on nucleation mechanism of folding as likely scenario for small single-domain proteins whereby passing the transition state requires formation of a small number of obligatory contacts (specific nucleus). The focus of the present talk is on implication of nucleation mechanism of protein folding kinetics for evolution of protein sequences. In particular we will present evidence that for a number of proteins evolution indeed controlled their folding rates (sometimes negatively, i.e. preserving slow folding). Simulations and analytical theory of protein evolution allows to develop a unified model of protein evolution that can be directly applied to the analysis of protein sequences. The theory introduces a new concept of "Conservatism of Conservatism" that accounts for physical degeneracy of protein code and points out to a more consistent way to do evolutionary analysis of protein sequences. We show how analysis of several protein superfamilies along these lines helps to discover evolutionary signals that are responsible for protein stability and folding rate.

In the second part of the talk we will present our most recent findings that shed light on the unusual behavior of prions.

Session 2: The denatured state and kinetics in the early stages

Chair: Alan R. Fersht

Kinetics And Dynamics Of Elementary Processes In Protein Folding.

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Knowing the time scales and mechanisms of elementary processes in protein folding is essential for understanding how proteins fold. These processes include the formation of secondary structure (alpha helices and beta sheets), loops, and long-range contacts, as well as global collapse of the unfolded polypeptide chain. We are investigating their kinetics using nanosecond optical methods for both triggering and observation. The formation of helices and hairpins in isolation, as well as global collapse, are being studied by nanosecond laser T-jump with fluorescence monitoring, while loop formation is being investigated by the quenching rate of excited triplet states. The results are interpreted in terms of simple statistical mechanical models. One of the surprises from this work is that much of the basic physics of protein folding is contained in the dynamics of a structure as small as a beta hairpin forming peptide.

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*These studies are being carried out in collaboration with Stephen J. Hagen, Eric R. Henry, James Hofrichter, Gouri Jas, Lisa Lapidus, Victor Muñoz, and Peggy A. Thompson.

DIRECT OBSERVATION OF CONFORMATIONAL EVENTS IN PROTEIN FOLDING ON THE MICROSECOND TIME SCALE

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While small globular proteins typically require milliseconds to seconds to complete the process of folding, there is growing evidence that important conformational changes, including secondary structure formation and chain collapse, occur on a much shorter time scale. These early events are crucial for understanding how protein folding is initiated and directed along productive channels (1). However, the limited time resolution of conventional kinetic methods has precluded direct observation of the initial collapse of the polypeptide chain, and the mechanism of this critical event remains poorly understood.

In previous kinetic studies on cytochrome c (a 104-residue protein with primarily α-helical structure), a major fraction of the heme-induced quenching of Trp59 fluorescence indicative of chain collapse was found to occur within the first millisecond of refolding (2-5). Using a newly developed continuous-flow capillary mixing method (6), we were able to quantitatively account for the entire fluorescence change associated with refolding of cytochrome c over the time window from tens of microseconds to minutes (7). The kinetics of folding under various conditions exhibits a major exponential process with a time constant of about 50 μs, indicating that a distinct free energy barrier is encountered during the collapse of the polypeptide chain. This major conformational transition occurs long before the rate-limiting formation of specific tertiary interactions, indicating that folding occurs in at least two stages. Continuous-flow experiments on another small protein, the 57-residue B1 domain of protein G, revealed a major fluorescence phase with a time constant of ~600 μs, which accounts for the initial changes in fluorescence that remained unresolved in earlier stopped-flow measurements (8).

Complementary structural information on early events in folding can be obtained by combining hydrogen exchange labeling, rapid mixing and 2D NMR methods. By observing the competition between H/D exchange and refolding during the 2-ms dead time of a quenched-flow experiment, we were able to measure protection factors for 40 individual amide protons in horse cytochrome c (9). At low denaturant concentration, we found weak but significant protection for amide protons in three helices with little or no protection elsewhere, indicating that the early intermediate contains a loosely packed α-helical core. Thus, the collapse of the chain on the submillisecond time scale is a barrier-limited event resulting in a dynamic ensemble of partially folded states with non-random structural preferences, which appears to facilitate folding by limiting the conformational search during the initial stages.

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Stefan Freund

Abstract : Structure and Dynamics of denatured β-Proteins

The energy landscape of the denatured state of a protein provides a key to understanding early folding events. In studies of the denatured states of barnase and barstar, some of the embryonic initiation sites, regions that possess loacl residual structure, as determined by NMR methods, are found to be productive for protein folding. These regions contribute to tertiary interactions involved in the transition state of protein folding as derived from extensive protein engineering studies.

We have attempted to map the energy landscape for the third fibronectin Type III domain from human Tenascin (Tnfn3), a compact 9.5 KD β-sandwich protein, through measurements of 15N backbone dynamics on the milli- to picosecond timescale and a number of structural parameters. Tnfn3 was fully denatured with 5M urea, no structural differences were observed for the 6M urea denatured state. Secondary chemical shifts, 3JHNHα coupling constants, amide proton temperature coefficients, interresidue NOE intensities, 15N relaxation rates and {1H-15N} steady state NOE enhancements were analyzed. Off-resonance T1ρ experiments reveal a lack of mobility on the milli- to microsecond time scale, indicating that no element of residual structure in the denatured state is persistent. Reduced mobility correlates with regions of extreme hydrophobicity or polarity. In these regions, several other measures of random coil behavior are perturbed. Evidence for two nascent turn-like structures corresponding to loop regions in the native fold is reported.

The results are compared to denatured states of an immunoglobulin superfamily 9.5 kD domain (IgSF), Ig18' sharing the same β-sandwich fold. Analysis of structural and dynamic data of the 4.2M urea and the 6M urea denatured state reveal that they are both highly disordered with some local turn-like residual structure. The 6M urea denatured state has a similar dynamic range like the 5M urea denatured state of Tnfn3, with nascent turn like structure in region 32-36 corresponding to the BC loop in the native protein. In the 4.2M urea denatured state, additional turn like structure and substantial chemical exchange on the mill- to microsecond timescale were found in the C-terminal region 80-93. This indicates that the structural and dynamic properties of denatured Ig18' can be characteristically altered when the protein is subject to different denaturant concentrations.

Both studies indicate that residual structure and local compactness correlates more strongly to characteristics of individual residues than to structural elements of the native state. The explicit measurement of chemical exchange provides new ways to characterise conformational restrictions that may play a role in the early stages of folding, but will also enable to probe oligomerisation or folded-unfolded equilibria.

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A closeup view of a protein folding :

a residue-specific study from the denatured to the folded state in a chemotactic protein from Escherichia Coli, *CheY*

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So far, proteins denatured states, as well as early folding stages, are poorly understood. Even though structural evidence is lacking, it is now admitted that denatured states do no necessarily correspond to the random coil model. It is also important to understand the physical properties governing unfolded proteins, as i) they are determinant of protein stability, ii) seem to play a crucial role in protein folding initiation and iii) have been shown to be implicated in pathological processes.

In this poster, we describe a new technique to investigate the folding process of a protein at the residue level, from the denatured state to the fully folded protein, based on multidimensional NMR. The unfolded state of CheY V83T/F14N mutant has been shown to be more compact than what should be expected for a fully denatured protein and the packing of the N-terminal extremity of the molecule would correspond to a folding nucleus upon which the rest of the architecture condenses.

This protein has then been studied in presence of 5 M urea by heteronuclear multidimensional NMR using 3D ^{15}N-separated TOCSY-HSQC and 3D ^{15}N-separated NOESY-HSQC experiments. In the TOCSY-HSQC spectra, all side chains protons are correlated to their intra-residue amide protons. This, and the fact that almost all α-protons of a residue are correlated with the previous residue's amide proton in the NOESY-HSQC spectra, allowed the assignment of 112 residues, out of 129.

The observation of the "conformational shift" then obtained, as well as the backbone ^{15}N relaxation times, suggests that two sequence regions present less dynamic properties in the unfolded state. One region, from residue 8 to 22, corresponds to the regions proposed previously as a folding nucleus. The other region, from residues 70 to 97, corresponds to the mutation region, suggesting that this substitution could also alter the denatured state. Eventually, a study of peptides encompassing these specific regions has allowed us to determine whether the stabilisation of this compact state arises from local or non-local interactions.

The assignment of the unfolded state then allowed us to follow the changes in environment for each backbone amide proton. The cross-peak intensity of each proton in a serie of 20 HSQC spectra, recorded with different urea concentrations, has been used as a conformational probe. As CheY denaturation is completely reversible, it is then feasible to monitor its folding process residue by residue. 75 residues, distributed all along the protein sequence, have then been studied. This work clearly shows the existence of an intermediate which is not formed cooperatively. This intermediates forms according to two folding sub-domains, consisting in residues 1 to 70 and 70 to 129. Moreover, a region remains collapsed under very high denaturating conditions, even though the majority of the protein is completely unfolded. This region corresponds to the one shown in the kinetics studies as the folding nucleus, supporting a model for protein folding in which the core provides a template for a further compaction of the rest of the polypeptide chain.

This amino-acid scale information also allows a thermodynamic analysis and the evaluation of the stability of each residue in the native protein as well as in the denatured one. In conclusion, this technique allows us to describe the protein folding process as never seen before, probed from the unfolded state to the native one at the residue level.

PROTEIN FOLDING AND CANCER

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Many diseases, including cancer, result from mutations in proteins that affect their folding and stability. For example, some 50% of human cancers are associated with mutations in the tumour suppressor p53. There have been spectacular advances in recent years in our understanding of the pathway and stability of folding of small proteins derived from experiments on engineered mutants. Fundamental principles derived from the model studies are now being applied to understanding the behaviour of the mutant proteins that affect health. This will be illustrated in this lecture by experiments on p53 protein that illustrate the power of the protein engineering approach. Methods are described for measuring the reversible stability of the core domain, in which most of the carcinogenic mutations occur. This has allowed the identification of the nature of the mutations. The carcinogenic mutations tend to lower the stability of the core domain. The folding assay has allowed the screening of mutations that could stabilise wild-type protein and be used to prepare mutants that are more stable than wild-type. A semi-rational approach for the design of such stable mutants will be described. p53 is a tetramer which exhibits the phenomenon of negative dominance: an inactive mutant can hybridize with wild-type protein to produce an inactive tetramer. The mechanism of assembly of the tetramerization domain of p53 has been rigorously established by kinetic and thermodynamic analysis of the folding of rationally designed mutants at almost every position in the sequence. We have been able to redesign the subunit interfaces of the tetramerization domain to overcome negative dominance. Studies such as these may aid in the designing of drugs for therapy and designing proteins for gene therapy.

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ADVANCES IN PROTEIN DESIGN

Luis Serrano

The *de Novo* design of proteins implies the possibility of rationally modifying existing proteins, or the design of sequences that should adopt a given target fold.. Until very recently, the majority of the protein design projects resulted in general in proteins that adopted the expected secondary structure, but not a defined structure. Similarly, rational modification of proteins relied more in the intuition of the researcher and require a good doses of serendipity. However, during the past five years the field seems to be coming into a mature state in which many different successful rational designs have been published (1). Starting with the simple design of isolated secondary structure elements: a-helices (2) and b-hairpins (3), followed by supersecondary structure elements (i.e.three-stranded b-sheets (4) and full proteins (5-7). Similarly, the possibility of increasing the properties of proteins (i.e thermostability (8)), or improving the affinity of a protein for its ligand (9), has been proven to be possible. For many of these designed molecules, the methodology used was based partly on the statistical analysis of the protein database and previous experimental information on particular interactions. However, in the past year a new approach that relies solely on known energy force fields and intensive exploration of the sequence and conformational space using mathematical tricks, has proven to effective (5). This technology in combination with the possibility of allowing backbone flexibility (6), could in principle in the near future allow to do fully automatic De Novo design of proteins. Thus, de Novo protein design is going to be a very powerful approach to obtain new catalysts, drugs and materials.

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Session 3: Protein design and folding pathways

Chair: Martin Karplus

Folding and Design of Coiled Coils

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Coiled coils are protein structures found in many naturally occurring proteins including medically relevant proteins such as those involved in HIV entry (1). Coiled coils consist of bundles of two or more helices that are supercoiled around each other. The factors that determine specificity in such a seemingly simple system are numerous. For example, coiled coils are known to exist in four different oligomerization states, as homo- or hetero-oligomers, and in parallel or antiparallel orientations.

A peptide denoted GCN4-p1, corresponding to the "leucine zipper" region of the yeast transcription factor GCN4, serves as a model system for many studies (2, 3). A striking periodicity, extending over six helical turns, is observed in the hydrogen-deuterium exchange rates for amide protons in a GCN4 peptide (4). Amino acid substitutions in the GCN4-p1 homodimer, at the predominantly hydrophobic helix-interface positions, result in an oligomerization switch to trimeric or tetrameric coiled coils (5, 6).

Peptides corresponding to the isolated coiled coils of the nuclear oncoproteins Fos and Jun preferentially form heterodimers over homodimers by at least 1000-fold (7). Characterization of these coiled coils indicates that electrostatic interactions are critical components of specificity (8). Remarkably, destabilization of the Fos homodimer provides a major driving force for preferential heterodimer formation.

Protein design serves as a useful test of our knowledge base about coiled coils. A simple heterodimeric coiled coil, denoted "peptide velcro", designed based on principles derived from studies of the Fos/Jun heterodimer, folds in physiological buffer with a dissociation constant of ~30nM, and at least a 10^5-fold preference for heterodimer formation over the homodimers (9). Peptide velcro forms a stable and unique structure, exhibiting a cooperative thermal unfolding transition, significant NMR chemical-shift dispersion, and amide-protection factors in excess of 10^4.

Replacing the single buried-polar residue (Asn-14) in the peptide velcro peptides with leucine leads to formation of a heterotetramer (instead of a heterodimer) that does not fold into a unique structure: in particular the helices lack a unique orientation (10). Moreover, in the context of the same peptide velcro system, if the positions of the Asn residues are altered such that a buried interstrand hydrogen bond can only occur when the helices are in an antiparallel orientation, heterodimers are formed with a pronounced antiparallel preference (11). These results indicate that a single buried-polar interaction in the interface between the helices of a coiled coil can determine oligomerization order, impart structural uniqueness, and determine the relative orientation of the constituent helices.

The simple, repeating units of structure in coiled coils make then particularly amenable to computer-based recognition methods. In 1982, Parry (Biosci. Rep. 2, 1017) used a template-based method to identify coiled coils from primary amino acid sequence. An extension of this strategy to include pairwise residue correlations in coiled-coil sequences, implemented in the computer program PAIRCOIL, shows improved discrimination between dimeric coiled-coil and non-coiled coil sequences (12). A multidimensional scoring program (MULTICOIL), that scores sequences using dimeric and trimeric databases, classifies the oligomerization state of coiled-coil sequences (www.wi.mit.edu/kim/computing.html). Additionally, using MULTICOIL and a maximum likelihood approach, it is estimated that approximately 3% of protein residues take part in coiled-coil structures (13).

The repeating nature of coiled-coil structures also makes them well suited for computational enumeration of detailed core-packing interactions. An algebraic parameterization of coiled coils given by Crick in 1953 (Acta Cryst. 6, 689) allows these types of calculations to be performed while allowing backbone freedom, and the results show an unprecedented agreement between calculation and experiment (14). The most significant test of the parametric backbone approach to packing calculations, however, is provided by its successful application in the design of coiled coils with an unnatural, right-handed superhelical twist (15).

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Structure, stability and folding of small model proteins

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I will present data from our studies using the four-helix bundle protein, Rop, as a model system. In particular I will discuss our work in which we manipulate the length and nature of helix-helix connecting loops and the details of packing in the hydrophobic core of the molecule. I will discuss the effect of these changes on protein structure, stability, activity and folding. I will also discuss recent studies in which affinity and specificity of RNA binding have been manipulated. In addition to the studies on Rop, I will also discuss our work on the B1 domain of IgG-binding protein G, a model system in which to investigate the energetics of beta sheet stability and folding.

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INTERACTION BETWEEN WATER AND POLAR GROUPS OF THE HELIX BACKBONE: A DETERMINANT OF HELIX PROPENSITIES

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Helix propensities determined in peptide helices are context dependent. The values found in alanine – based peptides follow the same rank order as in natural sequence peptides but the propensity ratios, relative to a reference amino acid, are substantially larger in alanine-based peptides. To find the reason, we study a set of five 13-residues alanine-based peptides which differ only by a single test amino acid: alanine, leucine, isoleucine, valine or glycine. The rank order of helix propensities is given by the relative helix contents of the five peptides. Surprisingly, the rank order changes as either temperature or solvent composition (trifluoroethanol concentration) is varied. The enthalpy of helix formation varies sharply among the five peptides and accounts for the change in rank order with temperature.

Model compound data in the literature show that the free energy of interaction of the peptide group with water is enthalpic. Model building shows that nonpolar side-chains shield the interaction between water and peptide polar groups in a helix in a sidechain–specific and rotamer–specific manner. A straightforward explanation for our results is that water still interacts with peptide polar groups after the helix is formed, as suggested earlier by Ben Naim and by Honig and coworkers, and that nonpolar sidechains shield this interaction. This rationale helps to explain why alanine is the only amino acid with a favorable helix propensity in water and to reconcile the discrepancy between the unfolding enthalpies of the alanine helix and helical proteins, as well as to explain the context dependence of helix propensities in peptide helices.

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OBLIGATORY STEPS IN PROTEIN FOLDING AND THE CONFORMATIONAL DIVERSITY OF THE TRANSITION STATE.

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There is substantial experimental evidence that in mesophilic isoforms the stability and folding rate of proteins are not optimal. Modulation of protein stability does not seem to be a difficult task (Muñoz et al.,1996; Villegas et al., 1996). In many examples where a designed or casual mutation stabilize the folded structure there is also an increase in the refolding rate (Viguera et al., 1996; Kim et al,1997; Martinez et al., 1998) suggesting that as well as the stability of the native state the refolding rate can easily be increased. However, some destabilized mutants are also able to refold faster than the wild type (Mila et. al. 1995, Viguera et al., 1996). These later examples are most probably revealing impeding points in the refolding of the parent polypeptide. The protein engineering method (folding kinetics analysis of individual side-chain mutations, Fersht, 1996) provides a mean to have a representation of the energy contributions of given protein groups in the transition state. Residues that loss a high fraction of the destabilization energy in the refolding semireaction (high $\phi_{t\text{-}u}$ value) are said to be in a folded conformation in the transition state. The interactions established by these groups can also be said to be promoting folding (part of the folding nucleus), while the ones that have no contribution to the energy of the transition state are probably formed as a consequence in the following down-hill steps. However, it has been proposed that among residues showing high $\phi_{t\text{-}u}$ values there can also be some that are not part of the folding nucleus, but that rather represent early kinetic barriers in the refolding process (obligatory steps, Martinez *et al.*, 1998). In parallel, theoretical analysis using lattice simulations has also postulated the existence of these obligatory steps that are due partly due to topological constraints (Nymeyer *et al.*, 1998). Distinguishing between residues that are part of the folding nucleus and those which are folded early in the folding reaction because they are part of obligatory steps in the folding reaction, is critical to understand the folding reaction in small two-state proteins. To address these questions, we have done two different types of experiments:

I) On one hand we have introduced in the type II' turn of SH3 an exogenous secondary structure element (β-hairpin) that folds in the absence of tertiary contacts, to facilitate the folding of an unstable hairpin which is part of the folding nucleus.

II) On the other, we have analyzed the folding reaction of several mutants of the spectrin SH3 domain at three different pHs, to identify regions of the protein that fold in a similar manner indepently of the destabilization of the protein.

Our results, together with previous analysis of other Spectrin and Src SH3 mutants, confirm that in the folding reaction there could be obligatory steps that are not necessarily part of the folding nucleus. It seems that the transition state ensembles in β-sheet proteins could be quite defined and conformationally restricted ('mechanic folding nucleus' and in these cases the structure of the transition state constraints could be evolutionarily conserved. More interestingly, we have found that an excessive stabilization of a folding nucleus could accelerates folding but also result in the appearance of folding intermediates in otherwise two-state folding proteins.

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The Folding Mechanism of Larger Model Proteins

Aaron R. Dinner and Martin Karplus

We investigated the folding mechanism of a 125-bead heteropolymer model for proteins subject to Monte Carlo dynamics on a simple cubic lattice. The behavior of the model was found to be more complex than that of smaller systems. Study of a few sequences showed that the mechanism involves two slow steps: a random search restricted by kinetically accessible, cooperative secondary structure for a core that drives folding to an intermediate and rearrangement from that intermediate to the native state. The generality of these results was confirmed by statistical analysis of a 200 sequence database by a method that employs a genetic algorithm to pick the sequence attributes that are important for folding and a neural network to derive the corresponding functional dependence of folding ability on the chosen attributes. The kinetic and thermodynamic behavior of a representative sequence is explored in greater detail by mapping the reaction to a space of two coordinates: one monitors the formation of the core and the other monitors whether the chain is trapped in a long-lived intermediate. In this space, the trajectories can be classified into a relatively small number of average pathways: a "fast track" in which the chain forms a stable core that folds directly to the native conformation and several "slow tracks" in which particular contacts form before the core is complete and cause the chain to misfold. From multicanonical Monte Carlo simulations, we obtain an estimate for the density of states and calculate equilibrium averages, including the free energy, energy, and entropy, as functions of the reaction coordinates at two temperatures. The equilibrium averages are found to be in good agreement with the observed kinetics; the transition states correspond to barriers or plateaus in the free energy while the intermediates are energetically stabilized local free energy minima. The folding mechanism bears striking similarity to that of lysozyme, a well-studied protein which is comparable in length.

Session 4: Experimental aspects and folding mechanisms

Chair: Fred E. Cohen

STABILITY AND FOLDING OF CRO PROTEIN FROM PHAGE 434

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The bacteriophage 434 Cro is a monomeric, 71-residue, site-specific DNA-binding protein of known X-ray structure and whose NMR structure we determined. Its single domain consists of five α-helices and shows striking structural similarity to the N-terminal DNA-binding domains of phage 434 repressor (R1-69) and phage λ repressor ($\lambda_{6\text{-}85}$). These three proteins thus serve as excellent models for comparative studies on folding and DNA-binding. Spectroscopic and calorimetric studies indicate that the equilibrium unfolding behaviour of 434 Cro is two-state, and that the protein has modest stability (ΔG (293K) _ 3 kcalmol^{-1}). The kinetics of 434 Cro folding monitored by stop-flow fluorescence indicates that it folds in the millisecond time-scale (k_f = 33 s^{-1}, k_u = 0.04 s^{-1} at 293 K, pH 6.0). The kinetic data reveal the presence of an intermediate which is populated at pH 6.0 but is absent at pH 4.0, and the transition state for folding appears to be native-like. By contrast, $\lambda_{6\text{-}85}$, the structural homologue of 434 Cro, has been shown to have folding times in the microsecond range (k_f _ 5000 s^{-1}, k_u= 30 s^{-1}), and its variants containing helix-stabilizing mutations fold even faster (k_f up to 61000 s^{-1}, k_u = 2s^{-1}). Examination of the folding propensities of synthetic peptides which together span the entire sequence of 434 Cro indicates that the individual helical propensities in 434 Cro appear to be significantly lower than in $\lambda_{6\text{-}85}$. In both proteins, the highest intrinsic helix propensity is observed for helix 1, but even this helix in 434 Cro is less stable than in $\lambda_{6\text{-}85}$. The native structure of 434 Cro contains a salt-bridge between a buried Arg (Arg 12) and a buried Glu (Glu 37), but is absent in $\lambda_{6\text{-}85}$. The observed folding behaviour of 434 Cro could thus be a reflection of the presence of the buried salt-bridge in the native structure of the protein, as well as of the low intrinsic folding propensities of its five helices. The latter explanation would also conform, at least qualitatively, to the diffusion-collision model which incorporates intrinsic helix propensities, recently described by Oas and colleagues.

STABILITY AND FOLDING OF APOFLAVODOXIN

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Flavodoxins are redox proteins that transfer electrons in the photosynthetic reactions that lead to $NADP^+$ reduction. Flavodoxin is in fact a tight non-covalent complex between the apoprotein (displaying an α/β fold) and one molecule of flavin mononucleotide (FMN). As the FMN can be reversibly removed from the complex, the apoprotein can be easily prepared. We use the apoflavodoxin from the cyanobacteria *Anabæna* PCC 7119 as a model for stability, folding and binding studies. The flavodoxin gene is cloned, the X-ray structure is known[1], the solution structure is in progress, and the stability of the wild type form has been characterised[2].

Wild type apoflavodoxin is well folded as judged from the near UV CD and ^{1}H-NMR spectra and the X-ray structure. The equilibrium unfolding conforms to a two-state model, the stability at pH 7 and low ionic strength being at 4 kcal mol^{-1}.

Apoflavodoxin is a good model to analyse the contribution of side chain interactions to protein stability. So far we have used this protein to quantify the strength of cation/π interactions and that of a hydrogen bond. Apoflavodoxin contains a single histidine (H34) that is hydrogen bonded to the side chain oxygen of Y47 and close to the phenyl ring of F7. We have determined, by double mutant cycle analysis and by pK_a measurements, that the strength of the cation/π interaction formed between protonated histidine and phenylalanine is of around 0.5 kcal mol^{-1}. This value is close to the one found for a similar interaction in barnase (0.3 kcal mol^{-1}) and demonstrates that cation/π interactions can effectively stabilise proteins. Using the same methodology (double mutant cycle analysis and pK_a measurements) we have determined that the contribution of the neutral histidine/tyrosine hydrogen bond to apoflavodoxin stability is of 1.3 kcal mol^{-1}. Since this value is obtained from a double mutant cycle, and not from simpler deletion studies, it provides direct evidence of a stabilising effect of hydrogen bonds on protein structure. Unexpectedly the hydrogen bond becomes less stable when the histidine protonates, its contribution to protein stability becoming of only 0.7 kcal mol^{-1}. Since this is a rather surprising result we wanted to test it by extending our studies to other hydrogen bonds in the protein using the following strategy. Surface exposed hydrogen bonds involving either an aspartate or a glutamate have been made neutral by replacement of the acidic residues by the corresponding amides (asparragine or glutamine), and the stabilities of the mutants have been compared with that of the wild type protein. In the three cases analysed, neutralisation of the hydrogen bond made the protein more stable. Parallel studies with control mutations, where non

hydrogen bonded acidic residues have been made neutral, indicates that the stabilising effect observed upon neutralisation of the hydrogen bonds is not related to the lower net charge of the protein, supporting the idea that neutral hydrogen bonds in folded proteins can be more stable that charged ones.

Despite the simple two-state equilibrium unfolding, the folding and unfolding kinetics of apoflavodoxin are more complex, with two refolding phases (of comparable amplitude) and two unfolding phases (one major and one minor). None of these phases are related to proline isomerisation nor to protein oligomerisation, and the overall amplitude of the unfolding phases equals that of the refolding ones within error. We thus detect the accumulation of an intermediate that can be directly observed in our stopped-flow time scale and not a burst phase or a curvature of a single observable kinetic refolding phase. A global analysis of the urea dependence of the observed rate constants and amplitudes is consistent with a triangular mechanism where most of the molecules fold via the unfolded state: the intermediate is thus mainly off-pathway. A mutational analysis of the structure of the intermediate and of the transition states is currently in progress.

The fact that an intermediate accumulates during the folding of apoflavodoxin makes possible, in principle, to find conditions to stabilise intermediate conformations at equilibrium. To search for such stable intermediates we destabilise the native structure by truncation at engineered methionines. We have found that removal of the C-terminal helix (fragment 1-149) produces a monomeric molten globule fragment[3]. The stability of this molten globule has been studied by urea denaturation (monitoring triptophan fluorescence, secondary structure content, and molecule size) and by thermal denaturation (monitoring triptophane fluorescence and absorption, and secondary structure content). Our results indicate that both the urea and the thermal unfolding are cooperative despite the low stability of the fragment (1 kcal mol^{-1}). The 1-149 fragment displays cold denaturation and this has allowed us to measure the ΔC_p of unfolding which is significantly lower than that of the entire protein. We have also truncated apoflavodoxin at the N-terminus by removing 38 amino acids and the resulting fragment (39-169) is also cooperatively stabilised. Removal of a long loop (20 amino acids) splitting the β5 strand weakens the protein but the shortened apoflavodoxin is in this case well folded. Our studies of several shortened apoflavodoxins thus indicate that substantial cooperativity remains after deletion of several parts of the protein (that add up to 50 %) what suggest that during the folding reaction of proteins different intermediates resulting from the condensation of different parts of the protein may all be cooperatively stabilised.

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Folding mechanisms of proteins with simple folds and complex topologies

Our understanding of how a protein folds has increased enormously in recent years, principally through the combination of novel protein engineering experiments, ingenious experimental methods and rapid developments in theoretical approaches. Views of folding ranging from rapid simple two-state transitions to more complex mechanisms involving parallel pathways and partially folded states have emerged (see for a recent review). Thus far our insights into folding mechanisms has arisen from very detailed studies of a handful of proteins. An important question that remains is whether current views of folding are representative of the wide variety of native protein structures found in the structure database. Important new clues about folding might therefore emerge from investigations of the folding of different protein folds. One set of proteins which has been under-represented in folding studies thus far are β-sheet proteins. These proteins are particularly important, not only in that they are a major structural class of globular proteins, but their complex topologies raise particularly intriguing questions about the role of hydrophobic collapse and hydrogen bond formation in folding . In addition, how correct strand pairing is achieved and alternative arrangements avoided pose intriguing questions, that have medical relevance in terms of the role of misfolding of β-sheet structures in amyloid diseases. To address some of these issues we have initiated studies of the folding the Greek key protein, pseudoazurin, which has a complex double wound Greek key fold . Our recent results on the folding of this protein and the insights learned will be described.

In contrast with our studies on the folding of the complex β-sheet proteins, we have also recently initiated a study of the family the bacterial immunity proteins, which have a very simple four helix fold . These proteins inactivate bacterial colicins by binding to them with fmol affinity. The proteins are small (~86 amino acids), have no disulphides, cis proline residues or prosthetic groups and hence are ideal model proteins for folding studies. We have analysed the folding of two of the family members, Im7 and Im9. Our data show that these proteins fold very rapidly, but despite being 60% identical, the proteins appear to fold with mechanisms of different complexity. These data will be reviewed and the implications of the results obtained thus far for the folding of helical proteins discussed.

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Structural Transitions in Protein Folding and Misfolding

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We have been investigating the structural transitions involved in protein folding by means of the simultaneous application of a wide range of complementary biophysical techniques[1]. NMR spectroscopy has played a prominent part in this process, which is directed at understanding the fundamental mechanisms by which proteins fold[2]. The combination of theoretical and experimental methodologies is essential for success in this enterprise, and we have been particularly concerned with strategies which bring together these approaches[3].

As part of this research we have been exploring the links between protein folding and misfolding and various types of disease. In particular we have investigated the nature of the formation of protein fibrils of the type associated with tamyloidogenic diseases. One system of particular interest to us has been c-type lysozyme. This has been one of our model systems for studying fundamental spects of folding for some time[4], and the discovery that clinical cases of amyloidosis are connected with single point mutations in the lysozyme gene has enabled us to explore the molecular basis of this disease in a well defined model system[5].

This work has recently been extended by the discovery that many proteins not associated with clinical manifestations of disease can form amyloid fibrils in the laboratory under specific conditions[6,7]. This has enabled us to explore the nature of the structure and means of formation of these fibrils in some detail[8]. This talk will report recent results from our laboratory and the significance of these for understanding the thermodynamics and kinetics of the interconversion of different states of proteins.

The work discussed involves collaborations with many colleagues, some of whose names are included in the reference. I should like to acknowledge all of these, along with generous funding from the UK Research Councils, The Wellcome Trust, the Howard Hughes Medical Institute, The European Commission and many individual charities.

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De Novo design of Helical Proteins

William F. DeGrado (Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA 19104-6059).

Our group has recently adopted a synthetic approach to understanding the structural basis for protein function. In order to test some of the rules and concepts that are believed to be important for protein folding and stability we have designed several simple proteins that fold into predetermined three-dimensional structures. A number of three- and four-helix bundle motifs have been designed, and structurally characterized to determine the features that are important for folding into a native-like structures versus mis-folded dynamic states. Of particular interest are sites along the solvent-exposed surface of the protein, which -- although unimportant for stabilizing the native structure of the protein -- nevertheless contribute to conformational specificity by destabilizing alternatively folded structures. Also, the sequences of the designed helical bundles have been elaborated to introduce binding sites for small ligands and metal ions.

Session 5: Folding *"in vivo"* and misfolding in disease

Chair: Christopher M. Dobson

Mad Cows And Englishmen: The Prion Folding Problem

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The transmissible encephalopathies including Kuru, Creutzfeldt-Jakob Disease and Bovine Spongiform Encephalopathy appear to be caused by a proteinaceous infectious agent or prion. I will describe theoretical and experimental studies of the prion protein that help to explain how one disease can present in infectious, sporadic and inherited modes and how a protein can "replicate" *in vivo*. Current work suggests that the transmissible encephalopathies are diseases of protein folding where the tertiary structures of the normal cellular isoform, PrP^{C}, and the disease causing isoform PrP^{Sc}, are distinct while their covalent structures are identical. The molecular basis of the species barrier and the strain phenomena will be discussed.

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The pathogenesis and consequences of amyloidosis

Dr P N Hawkins

Amyloidosis is a generic term for a heterogeneous group of disorders associated with the extracellular accumulation of normally soluble proteins in a distinctive, highly abnormal fibrillar form. Amyloid can be hereditary or acquired, localised or systemic, and can be potentially lethal or merely an incidental finding. It is a model example of a protein folding disease and is associated with a diverse spectrum of clinical manifestations. Amyloid related diseases include multiple organ failure in systemic amyloidosis, Alzheimer's disease, type II diabetes and the transmissible spongiform encephalopathies.

Amyloid fibril formation provides clear evidence that amino acid sequence is not the only determinant of a protein's tertiary form. Proteins that can form amyloid fibrils are evidently able to exist as two radically different stable structures, i.e. a normal soluble form and the highly abnormal fibrillar form.

The pathogenesis of amyloid thus centres around off-pathway folding of the various fibril precursor proteins into an alternative conformation that is rich in β-sheet structure and can auto-aggregate in a highly ordered fashion to create the characteristic fibrils. Fibril diffraction studies have confirmed that the β–strands within the β–sheets are arranged specifically, and that all amyloid fibrils share an essentially similar core structure. This underlies their distinctive common physicochemical properties including their ability to bind Congo red in a spatially organised manner, their relative resistance to proteolysis, and their capacity to bind the normal plasma protein serum amyloid P component (SAP) in a specific calcium dependent manner. The specific binding interaction between SAP and all amyloid fibrils is the basis for our development of radiolabelled SAP as a diagnostic nuclear medicine tracer. Most fibril precursor proteins can form amyloid fibrils *in vitro* and in some instances the fibrils *in vivo* are composed of intact whole precursor molecules, for example genetic variants of transthyretin and lysozyme in hereditary amyloidosis and β_2-microglobulin in dialysis related amyloidosis (DRA). In other situations the precursor proteins undergo partial cleavage, although it is not known exactly when during fibril formation that this occurs.

Although amino acid sequence determines the potential for a protein to form amyloid, and an essential prerequisite for developing amyloidosis is a sustained supply of the respective fibril precursor, little is yet known about the genetic or environmental factors that control individual susceptibility to amyloid, or those which govern its anatomical distribution and clinical effects. For example, only a small proportion of patients with chronic inflammatory disease develop AA (secondary) amyloidosis despite the fact that most such individuals have sustained very high circulating levels of the AA fibril precursor, serum amyloid A protein (SAA). However, parenteral injection of a minute extract of amyloidotic tissue primes mice for explosively rapid AA amyloid deposition within days (rather than weeks) of an inflammatory stimulus, and although the significance of this so called 'amyloid enhancing factor' (AEF) has long been appreciated, it remains surprisingly poorly characterised. AEF presumably promotes amyloidogenic off-pathway folding of susceptible proteins, possibly by capturing them on to an 'amyloid template' when they transiently populate unstable partly unfolded forms. Since all amyloid is massively rich in AEF, the conditions necessary for amyloid formation *in vivo* are probably self-perpetuating once an initial nucleus of amyloid material has been laid down, depending only on continued supply of the fibril precursor protein. It is of interest that some normal tissues contain low levels of AEF, raising the possibility that proteins may quite commonly be processed transiently in an amyloid-like manner.

The universal presence in amyloid of certain glycosaminoglycans, notably heparan and dermatan sulphate, and SAP has long suggested that these moieties might contribute to its

pathogenesis. The role of SAP has lately been confirmed in SAP 'knock-out' mice in which experimentally induced AA amyloidosis was substantially reduced. Similarly, there is indirect evidence that the fibril-associated GAGs enhance amyloidogenesis. Although neither SAP nor GAGs are required for amyloid fibrillogenesis *per se*, they both remain valid therapeutic targets.

Many of the pathological effects of amyloid can be attributed to its physical presence. The deposits accumulate in the extracellular space, progressively disrupting tissue architecture and can impair both organ function and produce space occupying effects. Amyloid fibrils may also be cytotoxic, possibly by enhancing apoptosis, which could account for their damaging consequences in Alzheimer's disease or the prion disorders in which deposits are scanty. The typically progressive nature of amyloidosis has given rise to the notion that amyloid is inert and irreversible, although in reality this more closely reflects the unremitting character of the various conditions which underlie it. Regression of amyloid has been described in numerous case reports and systematic radiolabelled SAP scans in over 1000 patients have lately confirmed that the deposits can regress quite rapidly when the supply of fibril precursors is reduced. Although amyloid deposits are remarkably stable, they do turnover continuously *in vivo*, albeit at a low and variable rate which can readily exceeded by their rate of deposition.

Hereditary amyloidosis is extraordinarily rare but represents an invaluable model for studying the disease. At the molecular level, genetically variant proteins with a strong propensity to form amyloid can be compared with their wild-type counterparts. Variants of transthyretin (TTR) have been studied most widely, but even normal TTR forms fibrils in senile systemic amyloidosis. Our recent discovery of hereditary systemic amyloidosis caused by human lysozyme mutations promised much, since, like TTR, its complete three-dimensional structure was known but wild-type lysozyme is not associated with amyloidosis. The amyloidogenic lysozyme variants, Thr56 and His67, proved to be less stable than wild-type and can form amyloid fibrils *in vitro* either following heating or prolonged standing at 4°C. These variants can unfold partially *in vitro* whilst retaining secondary structure, suggesting they are able to adopt a molten globule intermediate form. It is thought that both mutations destabilise lysozyme sufficiently for this to happen transiently *in vivo* enabling intermediates, which retain major elements of the β-structure of the native fold, to re-stabilise themselves by intermolecular aggregation and thereby producing the cross-β fold typical of amyloid fibrils. Remarkably, this process is completely reversible since fully functional soluble lysozyme His67 can be recovered *in vitro* from isolated *ex vivo* amyloid fibrils. Elucidation of the intermolecular β-sheet association that occurs between pre-fibril intermediates may assist the development of generic fibrillogenesis inhibitors.

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Structure-Function Analysis of Hsp70 Chaperones

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Hsp70 proteins are highly versatile chaperones which assist protein folding processes within the entire lifespan of proteins, ranging from folding of nascent proteins to assistance of proteolytic degradation of unstable proteins (Bukau and Horwich, 1998; Hartl, 1996). This versatility requires Hsp70 to associate non-specifically with misfolded proteins but also highly specifically with selected target proteins such as low abundant regulatory proteins. There are at least two mechanistic principles to account for this versatility (Mayer and Bukau, 1998). First, cells have amplified the number of Hsp70 chaperones, e.g. 3 in *E. coli* and 15 in *S. cerevisiae*, which frequently have distinct functions within the same cellular compartment without apparent cross talk. Second, Hsp70 chaperones have evolved regulatory devices to control their basic functional cycle which allows them to act on a large variety of substrates. This basic cycle consists of ATP driven conformational changes in Hsp70 which control the affinity of the chaperone for substrates. ATP hydrolysis in the ATPase domain of Hsp70 causes the locking-in of substrate in the substrate binding domain. One important mode of regulation of Hsp70 is the control of the key step of the functional cycle, ATP hydrolysis, which is triggered by DnaJ co-chaperones. DnaJ proteins thereby target Hsp70 to specific substrates and the co-existance of various DnaJ homologs with distinct substrate specificities allows binding of Hsp70 to a variety of substrates. Another mode of Hsp70 regulation is the control of nucleotide exchange, and hence of substrate binding, by nucleotide exchange factors. The nucleotide exchange factor for Hsp70 homologs from bacteria, chloroplasts and mitochondria are the members of the GrpE family which catalyze the exchange reaction by 5000-fold (Liberek *et al.*, 1991; Packschies *et al.*, 1997) through binding to the ATPase domain (Harrison *et al.*, 1997; Schönfeld *et al.*, 1995). Some Hsp70 homologs do not require nucleotide exchange factors for chaperone activity. This selective utilisation of nucleotide exchange factors by Hsp70 chaperones is of potential regulatory role. We

set out to analyze the molecular basis for the interaction of DnaJ and GrpE co-chaperones with Hsp70 chaperones using the *E. coli* homologs as model system.

Interaction of DnaJ with DnaK

We show for DnaK that stimulation of ATP hydrolysis by DnaJ requires the linked ATPase and substrate-binding domains of DnaK. Functional interaction with DnaJ is affected by mutations in an exposed channel located in the ATPase domain of DnaK. It is proposed that binding to this channel, possibly involving the conserved J-domain of DnaJ, allows DnaJ to couple substrate binding with ATP hydrolysis by DnaK. Evolutionary conservation of this channel suggests conservation of the mechanism of action of DnaJ proteins.

Interaction of GrpE with DnaK and HscA

Starting point for the analysis was our discovery that the HscA homolog (Hesterkamp and Bukau, 1998; Lelivelt and Kawula, 1995; Vickery, 1997) of DnaK does not functionally and physically interact with GrpE although it co-exists with DnaK in the *E. coli* cytosol. Further inspection of the structures and the structural models of the ATPase domains of various Hsp70 proteins revealed that GrpE contact sites are altered in those Hsp70 homologs that do not require a nucleotide exchange factor. A GrpE signature motif is defined that is shared only by Hsp70 homologs interacting with GrpE homologs. Furthermore, we found that Hsp70 homologs differ with respect to the number of salt bridges connecting the two lobes forming the ATP binding cleft. We provide evidence that these salt bridges are crucial for the control of the off rate of DnaK-bound ADP. We propose that GrpE is needed do open these salt bridges of DnaK, and the lack of these bridges in some Hsp70 homologs may abolish the need for a nucleotide exchange factor. A functional analysis of DnaK salt bridge mutants provides support for this proposal. Our data show a surprisingly high degree of versatility of Hsp70 proteins with respect to the regulation of nucleotide exchange.

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MECHANISMS OF MOLECULAR CHAPERONE ACTION IN PROTEIN FOLDING

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Although the folded structure of a protein is determined by the information contained in its amino acid sequence, efficient realization of this information in vivo may require assistance by molecular chaperones and folding catalysts (1). Folding of many newly-synthesized polypeptides in the cytosol depends on molecular chaperones of the Hsp70 family and on the cylindrical chaperonins. Little is known about the contribution of these chaperone systems to overall protein folding. According to our current model, Hsp70 binds to nascent polypeptides on ribosomes, preventing misfolding until all the information required for productive folding is available. While most proteins appear to fold upon release from Hsp70 (or other nascent chain-binding chaperones), certain slow-folding and aggregation-sensitive polypeptides are subsequently transferred to a chaperonin for folding in the sequestrated environment of its central cavity. Chaperone pathways seem to be tightly coupled in order to avoid the exposure of non-native folding intermediates to the bulk cytosol.

Hsp70 is indeed the major chaperone interacting with nascent chains, at least in eukaryotes. The flux of protein through the GroEL chaperonin of E. coli has recently been analyzed (2), and similar studies are under way for the eukaryotic chaperonin TRiC. These studies show that GroEL assists the folding of a limited subset of cytosolic proteins (~10-20% of total) with a size cut-off at about 55 kD, consistent with the volume of the GroEL folding cage. While the majority of bacterial proteins are smaller than 55 kD, eukaryotes contain a significantly greater number and proportion of large, modular polypeptides. These proteins fold co-translationally in a chaperonin-independent mechanism as their domains emerge from the ribosome (3,4). Co-translational and sequential domain folding appears to be more efficient in eukaryotes than in prokaryotes, and this may have contributed to the explosive evolution of multi-domain polypeptides in eukaryotes.

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STRUCTURAL CONSEQUENCES OF NATURALLY OCCURRING AMINO ACID EXCHANGES IN THE PRION PROTEIN

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Prion proteins in healthy mammalians occur as membrane-anchored monomeric molecules on the cell surface, whereas in individuals affected by transmissible spongiform encephalopathies (TSE) aggregates of prion proteins with increased proteinase resistance are typically observed ("scrapie" form). In humans, TSEs may occur either spontaneously, due to infections or by inheritance. Inherited human diseases have been related with about a dozen mutations that were detected in the protein sequence of affected families. In the healthy form the prion protein adopts a compact globular domain structure for the C-terminal part (residues 124-226), while the N-terminal part remains unstructured in aqueous solution at pH 5. Antibodies that are specific to the scrapie form recognize epitope regions, which are not connected in the 3D-structure of the healthy form of the prion protein.

For studies of structure-function relations and of folding (and "missfolding") considerations a database with experimental structures from NMR and computer simulations is being accumulated. It consists of the NMR structures of mouse and hamster, and presently the structures of various fragments of the human and bovine prion proteins are added. Computer simulations include extensive calculations on mutations related to human inherited diseases. In additional studies amino acid exchanges among different mammalian species are investigated for a better understanding of the occurrence and extent of infection barriers between various species.

The results that will be presented and discussed include structural differences among prion proteins from several species, and the relative thermodynamic stability of various naturally occurring mutations.

POSTERS

STRUCTURAL DEFECTS UNDERLYING PROTEIN DYSFUNCTION IN HUMAN G6PD DEFICIENCY BY VARIANT A^{-}

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Glucose-6-phosphate dehydrogenase (G6PD) A^{-} (V68M and N126D) is the most widely distributed polymorphic deficient G6PD variant in Africa and in people of African ancestry. The loss of intrinsic folding determinants in this double mutant but not in the single mutant G6PD A (N126D) nor in the wild-type G6PD B hinder its *in vitro* refolding capacity preventing the formation of the catalitically active dimer.

In an effort to determining the structural lesions responsible for instability in the deficient molecule G6PD A^{-} producing its low red cell enzymatic activity level we have studied their folding and structural properties by intrinsic and extrinsic fluorescence, circular dichroism, fluorescence energy transfer and differential scanning calorimetry.

The results show that the conformational stability of G6PD A (N126D) does not significantly differ respect its parental G6PD B. The presence of the additional mutation in G6PD A^{-} (V68M on top of N126D) renders a conformation with decreased stability of the structure as a consequence of the loss of the interaction of both mutated residues (about 8Å apart) in the coenzyme domain. These determinants which are essential for maintaining secondary structure element(s) in G6PD are also required for its active folding.

The Greek key protein apo-pseudoazurin folds through an obligate and on-pathway intermediate. **A. P. Capaldi and S. E. Radford**

We have studied the folding and unfolding of the 123 amino acid Greek key protein apo-pseudoazurin. Double jump refolding shows that the protein collapses from the unfolded state with all of the prolines in their native conformation to an intermediate within the dead-time of the stopped flow CD experiment. The CD spectra of this intermediate (reconstructed from the folding kinetics at a number of wavelengths) shows that the ensemble contains significant secondary structure. The urea dependence of the folding and unfolding of the protein have also been followed. Surprisingly, despite the clear evidence for a folding intermediate in the burst phase, the natural logarithm of the rate of folding changes linearly with denaturant to the transition midpoint concentration. The data do not fit to a two-state mechanism, however, as the ratio of the folding to unfolding rate is several orders of magnitude too slow to account for the measured equilibrium stability. The data can be rationalised, however, by a model which includes an intermediate that denatures at very high denaturant. Accordingly, kinetic and equilibrium measurements were combined to fit the chevron plot to an on-pathway scheme ($U \Leftrightarrow I \supset N$). The fit shows that the protein folds through an intermediate that is stabilised by 25kJ/mol before refolding to the native state at a rate of $2s^{-1}$. Although the data can also be fit to an off-pathway scheme ($I \Leftrightarrow U \supset N$), the resulting kinetic parameters indicate that the protein must fold to the native state with a time constant of only 12μs ($k=8x10^4 s^{-1}$). This is only 4 fold slower than the rate of formation of a 16 residue β-hairpin (Muñoz et. al., (1997), Nature, **390** 196-199) and vastly exceeds the rate of formation expected for a protein of this size. Similarly, models in which this intermediate is bypassed also lead to unreasonably fast refolding rates. Thus, the intermediate populated during the refolding of apo-pseudoazurin is almost certainly obligate and on the folding pathway. We suggest, based on data from this study and others that, at least for some proteins, intermediates are not kinetic traps as has been argued by others, but instead play a critical role in limiting the search to the native state.

INTERCONVERTING α-HELICES AND β-SHEETS. Seema Dalal, Suganthi Balasubramanian, and Lynne Regan. Yale University, New Haven, CT-06511

In response to the "Paracelsus Challenge" [G. D. Rose and T. P. Creamer, *Proteins Structure Function Genetics* **19**, 1 (1994)] we have successfully transformed a predominantly β-sheet protein (the B1 domain) into a stable α-helical protein while retaining 50% sequence identity to the B1 domain [S. Dalal, S. Balasubramanian, and L. Regan, *Nature Structural Biology* **4**, 548 (1997)]. Using the designed protein, which we have named Janus, as a starting point, we have constructed a series of mutants to evaluate the balance of forces necessary to stabilize an α-helix vs. a β-sheet fold. These mutants include a series in which we have assessed the contributions of the various design considerations to the overall stability and fold of Janus. One goal is to determine the maximum number of B1 domain residues that can be accommodated while maintaining a helical fold, a variant that would be a minimal version of Janus. As a part of this study, we have created a protein that has 61% identity to the B1 domain, but continues to adopt a helical fold. Another aim is to induce a conformational switch from α-helix to β-sheet, perhaps by changing a few residues in minimal Janus or through ligand binding. With these goals in mind, we have created two proteins, one which is 66% identical to the B1 domain and another that incorporates a potential metal binding site.

Our study emphasizes that a subset of a protein's amino acid sequence is required for determining its fold and has important implications for structure prediction and design.

REVERSIBLE PEPTIDE FOLDING IN SOLUTION BY MOLECULAR DYNAMICS SIMULATION

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Understanding the process of peptide folding is a critical first step toward understanding protein folding. The ability to predict peptide folding would also facilitate the de novo design of peptides with predetermined structures and properties for biotechnological applications. Peptides are, however, highly flexible and in solution can adopt a variety of conformations depending on the temperature and solvent conditions. Determining the structure of small peptides in solution experimentally is non–trivial. This makes methodology to predict how peptides fold in solution of fundamental importance in structural biochemistry. In principle, the process of peptide folding could be simulated directly on a computer. It had been commonly assumed, however, that the volume of conformational space accessible, even to a small peptide, meant that simulating the folding of a peptide under realistic conditions was not, and for the foreseeable future would not be, possible. With this work we demonstrate that this is not true by simulating the reversible folding of two β–peptides in solution at different temperatures [1]. The molecular dynamics simulations correctly predict the structures in methanol solution of the two peptides (a β–heptapeptide and a β–hexapeptide), a left–handed helix and a right–handed helix, respectively. The rate of folding and the free energy of folding at different temperatures are estimated. Folding paths and intermediates are detected, and the lifetimes of different populated structures calculated. Although the conformational space potentially accessible to the peptide is extremely large, very few conformers (10^1–10^2) are significantly populated at 20 K above the melting temperature. This implies the search problem in peptide (or even protein) folding is surmountable using dynamics simulations.

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The α-Helix Folds on the Millisecond Time Scale with a Transient Overshoot in Helix Content

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An understanding of the kinetics of secondary structure formation is essential if we are to understand protein folding. Previous work suggested that α-helices fold on the sub-microsecond time scale. Here we measure directly the rate of α-helix formation for the first time in Lysine and Glutamic Acid homopolymers and in two polyAlanine based peptides by stopped flow Synchrotron Circular Dichroism. Folding is initiated by pH jump for poly(Lys) and poly(Glu) or by dilution from 5M GuHCl for the poly(Ala) based peptides. Synchrotron CD is far superior to a conventional UV based instrument and we are therefore able to acquire kinetic data in the 190nm range for the first time. The shorter poly(Ala) based peptide shows first order kinetics with a folding rate of $\approx 20 s^{-1}$ at room temperature and a decrease in rate as the temperature is increased. For the longer peptides, helix folding occurs in two steps on the millisecond time scale, with a transient overshoot of helix content to significantly greater than at equilibrium, similar to that seen in the folding of several proteins. The overshoot may be caused by the formation of a single long helix followed by its breakage into the two or more helices present at equilibrium. We suggest that the rate limiting step in helix formation is the initiation of a new helix and that this occurs at least 10^5 times slower than the previously measured propagation of an existing helix. Helix folding is so slow that it could be the rate limiting step for protein folding in some cases.

Paramagnetic NMR as a Tool to Determine Metal Coordination of Azurin in the Unfolded State

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^{1}H NMR data applied to the paramagnetic cobalt(II) derivative of azurin from *Pseudomonas aeruginosa* has permitted to evidence that the metal ion is bound to the protein in the unfolded state. The relaxation data as well as the low magnetic anisotropy of the metal ion indicate that the cobalt ion is tetrahedral in the unfolded form. The cobalt ligands have been identified as the residues Gly45, His46, Cys112 and His117. Met 121 is not coordinated in the unfolded state. In this state, the metal ion is not constrained to adopt a bipyramidal geometry, as imposed by the protein when it is folded. This is a clear confirmation of the rack induced bonding mechanism previously proposed for the metal ion in azurin.

KINETIC ANALYSIS OF APOFLAVODOXIN FOLDING REVEALS AN OFF-PATHWAY INTERMEDIATE

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Much of current protein folding studies focus on whether intermediates are fundamental species in the folding pathway, or they are trapped states that retard the rate of folding. We have used apoflavodoxin from *Anabaena* PCC 7119 to perform a detailed kinetic analysis of its folding mechanism by stopped-flow methods. Apoflavodoxin is a small globular α/β protein that constitutes a good model for folding studies: its structure is known, it is cloned and expressed with good yields, and a battery of mutations is available to analyse the transient species found in the folding pathway. Unfolding of apoflavodoxin in urea is a monoexponential process with an amplitude that accounts for the total fluorescence change between folded and unfolded species. Refolding of apoflavodoxin shows two exponential phases that account for the total fluorescence change between folded and unfolded states. At low urea concentration, a logarithmic plot of the refolding rate versus urea concentration shows for the slow phase deviation from linearity. A rigorous analytical treatment taking account both the observed rate constants and the amplitudes seems to indicate the presence of a non-productive intermediate. Further mutational characterization of this transient state will hopefully contribute to the general understanding of the mechanism of protein folding.

Folding and Oligomerization of the *phd* Protein of Bacteriophage P1 Plasmid Addiction System Are Coupled to Specific DNA Binding

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The plasmid addiction module of bacteriophage P1 consists of two proteins, *doc* and *phd*. The *doc* protein, a toxin, is a proteolytically stable, whereas *phd*, the toxin's antidote, is unstable. Recently, *phd* was shown to autoregulate its expression by specific DNA binding. We have studied the secondary structure and the thermal stability of *phd* and *doc*, the affinity and stoichiometry of specific DNA binding by *phd*, and the effect of DNA binding on the structure and stability of *phd*. Thermal denaturation experiments monitored by CD suggest that *phd* is predominantly unfolded at 37 °C, having a t_m of approximately 26 °C, while *doc* is fully folded at physiological temperature. The CD spectrum of *phd* at 37 °C revealed that a large fraction of protein is in a random coil conformation. However, when *phd* was titrated with its target DNA sequence, dose dependent increases in both its folding (as determined by the change in ellipticity at 222 nm) and in the thermal stability were observed. When fully DNA-bound, *phd* is stable at physiological temperature, having a t_m of about 48 °C. When non-specific DNA of similar size (P22 Arc repressor operator) was titrated with the protein, no significant change could be observed either in *phd* structure or in its thermal stability. Stoichiometry assays suggest that *phd* binds each of its half DNA recognition sites as a dimer. Taken together, our results suggest that folding and oligomerization of *phd* are coupled to site-specific DNA binding.

Structure of the leader protease of foot-and-mouth disease virus: Implications for self-processing and eIF4G recognition.

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Leader protease of Foot-and-Mouth Disease Virus (FMDV) cleaves itself from the growing polyprotein and inhibits the translation of the host cell RNA capped messengers by the cleavage of the eucaryotic initiation factor (eIF4G). We have recently elucidated the three-dimensional structure of the leader protease. In order to obtain suitable crystals we have worked with two forms of the leader protease: the normal length inactive mutant C51A (residues 29 to 201; Lb^{pro}) and the deletion inactive mutant C51A (residues 29 to 195; sLb^{pro}). Crystal structure of the sLb^{pro} was determined by multiple isomorphous replacement techniques. Subsequently, Lb^{pro} crystal form, with eight molecules in the asymmetric unit, was solved by molecular replacement using sLb^{pro} structure as a model. The leader protease structure has a globular domain (residues 29-183) and a flexible C-terminal extension (CTE, residues 184-201) that is found bound in the substrate binding site of an adjacent molecule. The globular domain reveals the overall fold of papain-like enzymes in spite of the low sequence similarity shared with this family of cysteine-proteases; while the disposition of the CTEs with respect to the globular domain supports the feasibility of a *cis* self-processing mechanism. Specificity in the leader protease appears to be achieved by using differential binding determinants to recognise the two substrates specifically cleaved during viral replication. Thus, the three-dimensional structure allows to establish the molecular basis of substrate binding recognition, and the P' requirements for the eIF4G cleavage.

Designing amino acid sequences compatible with a given protein fold.

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Understanding the mechanism of protein folding and the factors that govern the conformational stability of proteins and peptides remains a major goal in molecular biology.
'Which sequences are compatible with a given fold ?' is another formulation of the same problem, which may have useful practical application s in *de-novo* protein design.
To try and answer this question we implemented a versatile procedure for selecting sequences that are compatible with a given backbone structure. This procedure consists of a C^{++} interface to the package CHARMM, providing features such as, the construction of side chain atomic models from a library of commonly observed conformations (rotamers), the modification of the amino acid sequence, and conformational energy calculations and minimization. This environment forms the basis for testing a number of sequence/rotamer optimization procedures. The goal is to describe the energy spectrum of the sequences near the global minimum and use it to identify families of sequences that are compatible with a given backbone conformation.
As a first step towards validating our procedure, we tested its ability to re-build the native sidechain conformations for a given backbone structure, searching through all the possible rotamers of each residue. The obtained results were found to compare well with those of several established homology modelling procedures.
These results, together with those obtained from our first attempts to redesign the core of a small protein will be presented.

MAPPING THE INTERACTIONS PRESENT IN THE TRANSITION STATE FOR FOLDING OF FKBP12

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ABSTRACT

FKBP12 is a 107-residue protein with a five-stranded antiparallel β-sheet which packs against a short seven-residue α-helix. The structure of the transition state for folding of the 12 kDa immunophilin FKBP12 has been characterised using a combination of protein engineering techniques and unfolding kinetics. 35 mutants were made at sites throughout the protein. These were used to probe the extent of secondary and tertiary structure in the transition state for folding. The transition state for folding is relatively compact compared to the unfolded state. Approximately 70% of the surface area buried in the native state is inaccessible to solvent in the transition state. All of the interactions in the native state that have been probed so far, are substantially weaker in the transition state. In contrast to other proteins of this size, no element of structure is fully formed in the transition state. Thus, the transition state is similar to that found for smaller proteins such as CI2 and the SH3 domain from α-spectrin. For FKBP12 the central two strands of the β-sheet, β-strand2 and β-strand5, are the most highly structured regions of this protein in the transition state. In particular Val101, which is one of the most highly buried residues and located in the middle of the central β-strand, appears to be in the most highly-structured region of the transition state making approximately 60% of its native interactions. Val101 interacts with Val63, the C-terminal residue in the α-helix, suggesting that some weak tertiary interactions are formed in the transition state. The outer β-strands, and the ends of the central β-strands are formed to a lesser degree. The short α-helix is largely unstructured in the transition state as are the loop regions. The data are consistent with a nucleation-condensation model of folding, the nucleus of which is formed by the side chains of Val24, Val63 and Val101. These residues are distant in the primary sequence demonstrating the importance of tertiary interactions rather than local secondary interactions in stabilising the transition state for folding. A Brønsted analysis suggests that the transition state is an ensemble of states close in structure.

DESIGN AND NMR STUDY OF MONOMERIC β-SHEET FORMING PEPTIDES

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Peptide conformational behavior provides insights into the formation of secondary structure, a key event in the early stages of protein folding. Factors involved in α-helix formation and stability are well-characterised, but this is not so for β-sheet structure. Only recently, a protein fragment[1] and some designed peptides[2-3] were shown to fold into monomeric β-hairpins in aqueous solution. A β-hairpin is the simplest antiparallel β-sheet motif. From the conformational analysis of a series of β-hairpin forming decapeptides[4-6], we concluded that the turn sequence determines the alignment of the β-strands in the β-hairpin, that is, the type of β-hairpin and we identified several favorable cross-strand sidechain interactions for β-hairpin formation.

To check the generality of our conclusions about the role of turn and cross-strand sidechain interactions in the formation of β-hairpins, we have designed and investigated by NMR a series of longer β-hairpin forming peptides. The conformational behavior of these pentadecapeptides evidences the essential role of the turn sequence in determining the kind of β-hairpin formed and that the stabilising effect of cross-strand side chain interactions depends on its closeness to the turn region.

Furthermore, we have designed a 20-residue linear peptide able to fold into a three-stranded antiparallel β-sheet. A ^{1}H and ^{13}C-NMR conformational analysis indicates that the peptide folds into the expected conformation. The exchange kinetics between the three-stranded β-sheet and the unfolded peptide molecules is slow enough on the NMR time scale to estimate a time constant of the order of several microseconds for the coil - β-sheet transition.

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GROEL UNDER HEAT SHOCK: CONFORMATIONAL AND FUNCTIONAL CHANGES

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Chaperonin GroEL from *E. coli*, together with its cochaperonin GroES, are proteins involved in assisting the folding of polypeptides. GroEL is a tetradecamer composed of two heptameric rings which enclose a cavity where folding takes place through multiple cycles of substrate and GroES binding and release. GroEL and GroES are also heat-shock proteins, their synthesis being increased during heat-shock conditions to help the cell coping with the thermal stress. Our results suggest that, as the temperature increases, GroEL decreases its protein folding activity and starts acting as a "protein store". The molecular basis of this behavior is the loss of inter-ring signaling which slows down GroES liberation from GroEL and therefore the release of the unfolded protein from the GroEL cavity. This behavior is reversible and after heat-shock, GroEL reverts to its normal function. This might have a physiological meaning since, under thermal stress conditions, it may be inefficient for the cell to fold thermounstable proteins that are prone to denaturation.

Detection of Folding Nucleus from Analysis of Protein Superfamilies.

LEONID MIRNY
EUGENE SHAKHNOVICH

Recent experimental and theoretical studies suggested a specific mechanism of protein folding where all transition state conformations share a smaller subset of common contacts (folding nucleus). Folding nucleus have already been experimentally identified in several small proteins. Prediction of these residues contributing to the nucleus is a great challenge for theoretical protein folding.

Here we suggest a criterion for detection of folding nucleus in native proteins. The criterion is based on the assumption that location of the nucleus in a protein structure depends primarily on the structure itself, but not on the sequence acquiring this structure. In each superfamily of proteins sharing the same fold and no sequence homology we identify positions which are conserved *within* each family and coincide when non-homologous families are structurally superimposed. Positions identified this way are conserved in all proteins of a particular fold irrespective of their sequences and functions, and hence, are responsible for folding into this structure.

In order to eliminate an obvious explanations of this universal conservatism by low solvent accessibility of selected positions we make a series of statistical tests. For every position in a protein structure we compute conservatism expected according to its solvent accessibility. Comparison of expected and observed conservatism shows that (i) in about 90% of variation in conservatism is explained by solvent accessibility; (ii) universal conservatism used to predict the folding nucleus can not be explained by solvent accessibility. This statistical test is used to identify folding nucleus in the proteins of immunoglobulin, flavodoxin, TIM-barrel and SH3-domain folds. Predictions are compared with experimentally determined folding nuclei.

STRUCTURAL CHARACTERIZATION OF THE NASCENT POLYPEPTIDE CHAIN OF BARNASE

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As a strategy to study the structure of a polypeptide chain as it grows from its N terminus *in vitro*, we have analysed the conformational preferences of barnase nascent fragments. Here we present an extensive conformational characterisation of seven barnase fragments (from B22, (residues 1-22) to B105 (residues 1-105)) by using different biophysical techniques, namely fluorescence, far- and near-UV CD, gel filtration chromatography (SEC), ANS-binding and NMR. Fragments up to B95 appeared to be mainly disordered, although a small amount of secondary structure could be inferred from far-UV CD experiments. Fragment B95 was also essentially disordered, but pH-induced changes in the fluorescence emission spectrum suggested the existence of a native-like tertiary interaction between His18 and Trp94. Fragment B105 showed a native-like structure with all of the probes used. None of the fragments bound ANS, which suggests no accumulation of exposed hydrophobic patches upon chain elongation.

By NMR, fragments B22 to B79 showed a small population of molecules with native-like helical structure, spanning residues Val10 to His18. This residual helical structure increased with the size of the polypeptide chain from fragments B22 to B79. Evidence comes from three NMR parameters: (1) changes in the chemical shifts towards native-like values of residues Asp12 to Tyr17 on going from B22 to B79; (2) a larger number of medium- and short-range NOE contacts in B79 when compared to B22; and, (3) lower temperature coefficients for residues Val10-His18 in B79 compared to B22. Since the first α-helix interacts with the last β-strands in the intact barnase (i. e. residues beyond Ile76), these results suggest that *non-native tertiary interactions stabilised native-like secondary structure in the nascent chain*. We can conclude that long-range interactions, even though they are non-native, are important to stabilise and consolidate the presence of weakly populated conformations of secondary structure in fragments. Thus, in the folding of barnase, the helical structure around α-helix$_1$ is consolidated by long-range non-native interactions, the β-hairpin (residues Ser92-Leu95) region is also consolidated by non-native conformations, and the docking of both regions triggers the acquisition and consolidation of native-like structure in the rest of the protein.

CCH Contains A Novel Peptide Motif Implicated In Protein Oligomerization

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CCH, a 13 kD plant protein from *Arabidopsis thaliana*, is a member of a group of chaperones implicated in intracellular copper trafficking and in oxidative stress responses. The plant protein contains a 48 amino acids C-terminal domain that is not present in CCH homologues from different organisms. This particular domain confers to the protein the capability to dimerize *in vitro* and by itself is able to oligomerize rendering dimers and tetramers as deduced from electrospray mass spectrometry.
We have focused our attention on a novel peptide motif from the amino acid sequence of this plant CCH specific domain, that could play a determinant role in protein oligomerization or in specific molecular recognition events. Secondary structure prediction showed that the motif might fold as a bipolar α-helix with basic residues in one side and acidic residues in the other side of the helix. We have named this motif as "Hello" (Helix with Lateral Opposite charges). In order to define the minimal sequence and structural requirements of Hello as oligomer inducer, we have synthetisized by SPPS the Hello motif and different analogues. Additional Characterisation of these analogues and structure-activity studies of the Hello motif will be discussed.

KINETICS OF CONFORMATIONAL EQUILIBRIA IN NATIVE PROTEINS FROM NMR AND MS ANALYSIS OF HYDROGEN EXCHANGE

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Our laboratory is investigating the link between thermodynamics and kinetics of folding as monitored by native-state peptide hydrogen (NH) exchange. NH exchange is an increasingly popular tool for the study of protein folding and stability. A number of recent studies have demonstrated that the thermodynamics of protein stability can be measured accurately by monitoring the slowest exchanging NHs in native protein. Some investigators have extended the interpretation of native-state NH exchange to draw conclusions regarding the kinetic pathway of protein folding and unfolding. In previous studies, NH exchange monitored by NMR spectroscopy was used to determine the thermodynamics and kinetics of unfolding and folding at 14 residues in native turkey ovomucoid third domain (OMTKY3) [(1996) *Biochemistry 35*, 171-180; (1997) *Biochemistry 36*, 8686-8691]. Free energies of NH exchange are very similar for all 14 residues, but unfolding rate constants segregate into at least two groups differing by about one order of magnitude: 9 NHs have a mean unfolding rate constant of 0.008 ($\pm$ 0.004) s^{-1} while the other 5 NHs show a mean rate constant of about 0.1 s^{-1}. Our hypothesis was that the slower group of 9 residues were all exchanging at a cooperative global unfolding event. To test this hypothesis, native-state NH/ND exchange at alkaline pH has been monitored by electrospray ionization mass spectrometry (ESI-MS) under the same conditions used in the NMR studies, where the ΔG_u for OMTKY3 is about 7 kcal/mol. The results have been interpreted using a new statistical-mechanical model for simulation of MS peak shapes during NH/ND exchange. The ESI-MS data for OMTKY3 suggest that the hypothesis regarding cooperative exchange at the 9 residues described above is incorrect: in spite of the similarity in their unfolding rates, only about 4 of the 9 residues are exchanging in a completely cooperative manner. The data are most consistent with the other 5 residues exchanging independently of one another. Overall, the 14 NHs with very similar thermodynamics of unfolding are, in fact, involved in a number of different conformational equilibria. Overall, combined NMR and MS analysis of NH exchange provides a rich and complex picture of the ensemble properties of native proteins.

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Molecular dynamics studies of active tertiary folding states in a DNA binding protein.

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Zinc fingers constitute important eukariotic DNA-binding domains in many transcription factors. The protein component, from the Zif268-DNA complex, contains three tandemly repeated zinc finger motifs (Zif) arranged in a semicircular (C-shaped) structure that fits in the major groove of DNA. The question is whether Zif configuration is determined by its interaction with DNA or if the system has enough information in its folding pattern.
Each zinc-finger domain consist of an anti-parallel β sheet and an α-helix with a zinc-ion contributing to hold both secondary structures. Tertiary structure flexibility is basically due to the four residues long "linkers" between the fingers.

Simulation were done to study three issues:

i) Role of zinc in holding the 3D structure;
ii) Role of water solvent ;
iii) Role of charge state of the zinc coordination shell in water and in vacuum.

For all these cases we have examined flexibility issues.

Results:

i) show the importance of zinc as a tertiary structure holder. Although secondary structures are always retained they are perturbed by the absence of zinc. The fluctuation pattern measured for the relative that orientation of the three helices show significant differences when zinc is fully replaced but charge state is conserved (using protonated histidines).
ii) Water surroundings help the tertiary structure to fluctuate around regions compatible with DNA binding. Zinc also contributes to this.
iii) The zinc state of charge was changed. Both +1 and +2 states in water show important fluctuations which are more important for the highest charged form.

The most flexible tertiary structure associated with fluctuations are always related to stable secondary structures. If the fingers loose stiffness, the whole structure collapses with small fluctuations.

The MD simulations underline the importance of water and the presence of zinc to create condition for the folding to be fluctuating in regions where a protein-DNA complex may form.

Electrostatic contributions to protein folding energetics: Relation with guanidine-induced denaturation and protein stabilization by mutation.

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We have characterized the guanidine-induced unfolding of hen-egg-white lysozyme and ubiquitin (both, human and from yeast) on the basis of equilibrium fluorescence measurements, kinetic fluorescence measurements, double-jump unfolding assays and circular dichroism. We have also characterized the thermal unfolding of these proteins by Differential Scanning Calorimetry. The results derived from these studies indicate that the guanidine-concentration dependence of the unfolding Gibbs energy is approximately linear over an extended concentration range but, also, that strong deviations from linearity occur at low guanidine concentrations (below 1M). In addition, these deviations from linearity change with pH, being negative at acidic pH's and positive at pH values near the isoelectric point. This suggests that the deviations are associated to the contribution to the unfolding Gibbs energy that arises from pairwise charge-charge interactions. Thus, this contribution is expected to be stabilizing at pH values near the isoelectric point, since positive and negative charges are distributed on the surface of the protein so as to produce a net electrostatic attraction; on the other hand, it should become destabilizing at acidic pH, due to the neutralization of negative charges and the concomitant repulsion between the remaining positive ones. This hypothesis is supported by theoretical calculations based on the Tanford-Kirkwood sphere model and the Tanford-Roxby mean-field approximation and suggests the possibility of enhancing protein thermal stability by modifying the charge distribution on the protein surface. To this end, we have developed a procedure based on simple electrostatic models that allows us to find potentially stabilizing mutations.

EQUILIBRIUM FOLDING INTERMEDIATES OF AN ALL-BETA PROTEIN: ACIDIC FIBROBLAST GROWTH FACTOR

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We have investigated the existence of equilibrium folding intermediates of acidic fibroblast growth factor (FGF). Moderate low pH (4.0) induces a partly folded state in FGF with a somewhat disrupted tertiary structure and a well conserved secondary structure. This state is very compact, since it undergoes cooperative unfolding transitions induced by urea and heat, and shows an 1-D nuclear magnetic resonance spectrum with well dispersed proton resonance peaks. The low pH-stabilized state is capable of interacting with liposomes, and may correspond to a membrane translocation competent form. On the other hand, we have analysed the effect of 2,2,2-trifluoroethanol(TFE), a known secondary structure stabilizer, on the structure of FGF. TFE induces the formation of alfa-helix in FGF, despite being an all-β protein. Interestingly, a peptide corresponding to the β-strand 8 of FGF acquires an α-helical conformation in aqueous solution at pH 3.0, that is increased upon addition of TFE. This suggests that the folding of FGF is a non-hierarchical process and that non-native intermediates may play an important role in the early steps of FGF folding.

In summary, we present here the description of two partly folded states of FGF that may be relevant in events ocurring in vivo, such as translocation across membranes, and that add information on the folding process of all-β proteins.

Mechanism of folding/unfolding of cytochrome *c*

M. C. Ramachandra Shastry

Although elementary processes, like α-helix or β-turn formation, occur on the nanosecond time scale, the observation that the small proteins typically require ms to s to complete the process of folding stresses the importance of the events on the microsecond time scale. As a general approach to monitor kinetic reactions on the microsecond time scale, we have recently developed a continuous-flow capillary mixing apparatus. In conjunction with the conventional stopped-flow instrument, we have studied the refolding/unfolding of horse cytochrome *c* over six orders of magnitude in time starting from 45 μs, the dead-time of the CF apparatus. Refolding under various initial and final conditions reveals a common exponential process (time constant, 50-75 μs and amplitude, 40-60% of the total fluorescence signal change) accounts for the formation of a compact intermediate (Ic) separated by the unfolded state by a distinct free energy barrier. This initial compaction event which is independent of heme ligation suggests that it reflects an intrinsic conformational event. The barrier limited collapse of the chain into Ic, which occurs long before the formation of the rate limiting process of formation of specific and stable tertiary interactions, is consistent with earlier results that folding occurs in at least two stages. The refolding process has been described based on a kinetic modeling of a scheme involving several intermediates.

Unfolding of cytochrome *c* using GuHCl, on the contrary, is a simple process, which can be described by a single exponential. At higher denaturant concentrations the rollover observed in the chevron plot is attributed to the rate-limiting Met80 deligation of the heme. This was confirmed by initing similar unfolding reactions in the presence of 200 mM imidazole, an extrinsic ligand that eliminates all ligation events, for which a similar plot lacked the rollover.

Proteins must fold in short secondary structure regions

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The secondary structure regions (α helices/β sheets) in proteins have an average length of about 20Å. In large proteins (more than 500 amino acids) this average size does not change (Trends Pol. Sci. 5, 321, 1997). This feature of proteins has been explained as due to the organization of proteins in domains. However it is obvious that no selective pressure towards large secondary structure regions is present when proteins increase in size.

On the other hand we have simulated polymer crystallization by a Monte Carlo method and find the same behaviour: polymer chains fold in regions much shorter than what would be expected from energetic reasons. This is due to the presence of entropy barriers in folding. We conclude that the same process occurs in proteins: if they had long secondary structure regions, the folding process would be very slow. Furthermore, long secondary structure regions would favour random aggregation if they eventually formed.

Simulation of protein models on the cubo-octahedral lattice with the CI algorithm

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Computer simulations of protein models on lattices have been widely used as an aid in the study of protein folding process. Following the suggestion of Raghunathan and Jernigan[1] that the cubo-octahedral lattice can allow a more realistic representation of proteins than other lattices, we have devised the use of a new set of internal coordinates θ for the description of a protein on this lattice. The model, though being a 3D model, presents a complexity typical of a 2D model as the position of a residue can be defined only with respect to the two preceding ones and needs only the internal coordinate θ instead of two internal coordinates usually necessary for 3D models. Moreover, conversion of θ to *xyz* coordinates is very easy as it needs only the vectorial product of unit vectors or the sum of unit vectors, thus having very low time requirements in a computer simulation.

When the Contact Interaction algorithm, already proposed by us[2] for simulations on square or cubic lattices, was applied to the cubo-octahedral lattice, the system obeys the correct thermodynamics derived from the definition of energy. Thus, lattice simulations of protein models, in which secondary structure elements such as α-helices or β-strands can be easily identifiable, can be performed.

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A molecular switch for glutamate-glycine cooperativity in the NMDA receptor

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The NMDA receptor plays a central role in learning and memory, neuronal development, and pathological neuronal death. This receptor constitutes an ionic channel that is highly calcium permeable, and has the unique property of requiring the simultaneous binding of two different agonists, glycine and glutamate, for its opening. At the structural level, the receptor is composed of two pairs of subunits (NR1 and NR2) forming a tetramer containing four ligand binding sites, two for glycine and two for glutamate. Although the binding sites for glycine and glutamate are segregated into the NR1 and NR2 subunits, respectively, there is a reciprocal influence on the apparent binding affinity of one ligand by the other agonist, indicating that both sites interact functionally. Moreover, the extent of this allosteric interaction is dependent on the subtype of the NR2 subunit present in the tetramer.

A deep understanding of the basis of NMDA receptor allosteric heterotropic cooperativity requires the identification of the pertinent intersubunit interacting residues, followed by the structural resolution of the rearrangements that accompany such allosteric interaction. By using chimeras made of different glutamate binding NR2 subunits we have identified a molecular determinant for this intersubunit interaction. This region interacts functionally with the glycine binding site, and funnels the influence of other regions of the NR2 subunit to the NR1 subunit. A three dimensional model based on the structure of homologous bacterial periplasmic amino acid binding proteins place this segment in a loop protruding out of the binding pocket but not involved in ligand binding. Thus, this loop is strategically placed to transmit the conformational changes induced by glutamate in the NR2 subunit to the NR1 subunit.

LIST OF INVITED SPEAKERS

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