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Minireview

How does protein synthesis give rise to the 3D-structure?

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The recent experimental data on stages and kinetic intermediates in protein folding are reviewed. It is emphasized that these data are consistent with the 'framework model' proposed by the author in 1973. The model implies that protein folds by stage mechanism (secondary structure – molten globule state – native state) in such a way that the results of previous stages are not reconsidered in subsequent ones. Arguments are presented that both these hypotheses and available experimental data do not contradict the assumption that native structures of at least small proteins are nevertheless under thermodynamic rather than kinetic control, i.e. correspond to global minima of free energy.

Protein folding; Molten globule; Kinetic intermediate; NMR of protein; Tertiary fold

1. INTRODUCTION

The biosynthesis of protein chains on ribosomes is only the first step in the creation of a functioning protein. The next step is *protein folding*, i.e. the formation of a native 3D-structure from a linear polypeptide. Nobody knows how the protein folds in vivo, simultaneously or just after biosynthesis or secretion. However, we know that the protein chain can spontaneously refold in vitro, i.e. it can restore its native 3D-structure and its native function from a completely unfolded state after the removal of a denaturing agent. After this fundamental fact had been first established for ribonuclease A [1] it was confirmed for so many proteins that there is almost no doubt that *in principle* the protein sequence contains all the information which is necessary for protein folding. This does not mean that there are no factors which can facilitate protein folding in vivo, However, this implies that these factors can accelerate a proper folding or prevent proteins from 'non-productive' folding pathways (like misfolding or aggregation) rather than determine the final structure of the molecule. In fact, enzymes which are or can be involved in protein folding (protein disulphide isomerase and peptidyl-prolyl *cis-trans* isomerase [2]) do not dictate the final protein structure as the same structure is also achieved in their absence. The same is true for chaperones of the HSP-60 type which are believed to prevent proteins from misfolding and non-specific aggregation [2–4] and also do not dictate their final 3D-structure.

It seems therefore that the central dogma of molecular biology can be extended from protein biosynthesis to protein folding [4]. In other words, all information which is required for the specific 3D-structure of the product of a given gene is contained in the nucleotide sequence of this gene.

However, there is a fundamental difficulty in the *realization* of this dogma at the stage of protein folding. Two main enemies of the creation of a desired protein are *miscoding*, i.e. mistakes in protein synthesis, and *misfolding*, i.e. mistakes in protein folding. We know that proteins are strongly protected against miscoding, but how are they protected against mistakes in folding? This problem is very serious for protein molecules as the number of possible conformations increases exponentially with the increase of chain length. In fact, if each residue has ~10 different conformations, the total number of conformations for a chain of 100 residues will be $\sim 10^{100}$ which is obviously too large to be searched in any reasonable time. This difficulty is called 'paradox of Levinthal' as Levinthal was the first to describe this situation [5].

The aim of this paper is to review the current ideas and experiments related to this paradox.

2. STAGE MECHANISM OF PROTEIN FOLDING

The only logically possible explanation of 'Levinthal's paradox' is that the protein chain *does not* make a complete search among all its possible structures. This is possible only if protein folding is a stepwise process

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and the results of each step are not substantially reconstructed at subsequent steps. This hypothesis proposed by the author in 1973 (see [6]) and called later [7] 'the framework model' does not necessarily imply that the protein structure is under kinetic rather than thermodynamic control (as has been suggested in [5]). It is also consistent with the assumption that the native protein structure corresponds to the global minimum of free energy but that intermediate structures achieved at different folding steps are consistent with this minimum (or are not very far from it). I shall come back to this point at the end of this paper.

The specific mechanism of protein folding proposed in [6] (see also [8]) consists of 3 main steps: (i) formation of 'fluctuating embryos' of regions with secondary structure in an otherwise unfolded chain; (ii) collapse of these regions into an 'intermediate compact structure'; and (iii) adjustment of this intermediate structure to the unique native structure. The important point of the hypothesis is the suggestion that location of the secondary structure in an unfolded chain must be close to its location in the final state and that the crude mutual positions of α- and β-regions ('tertiary fold') in the intermediate compact state must be close to those in the final state.

Some of the main features of this hypothesis have been confirmed by a set of experiments which were done by my own and other groups during the last 10 years. First, in 1981 a novel equilibrium state of protein was discovered [9] (see also [8,10–12] which later [13] was called the 'molten globule' state. A number of proteins can be transformed into this state at mild denaturing conditions [12]. The molten globule state is rather compact, has a pronounced secondary structure but has lost rigid tight packing of side chains typical for native proteins. Almost nothing is known about the tertiary fold of a protein in this state though there is indirect evidence [14–17] that it can preserve some features of the native tertiary fold. Second, the study of kinetics of protein folding (see reviews [2,7,8,11,12,18–21]) revealed a number of kinetic intermediates. These intermediates include the compact state with a pronounced secondary structure but without rigid tertiary structure, i.e. the state which is very similar to the equilibrium molten globule state [22–28] (see also [8,11,12,20]).

The following 3 main stages of protein folding have been revealed:

(1) Very fast (within 0.01 s) formation of a pronounced secondary structure monitored by a large increase of circular dichroism in the far ultraviolet region ([23,26,29,30] and unpublished data of Semisotnov and Kuwazima reviewed in [20]). Though circular dichroism even in the far ultraviolet region can be influenced by the contribution of aromatic groups [31–33], a very fast change of this dichroism (long before it begins to change in the aromatic region [8,11,20,22,23,26,29,30]) can be due only to the formation of secondary structure [12].

(2) Fast (within ~1 s) formation of the molten globule state, i.e. the collapse of a protein chain with pronounced secondary structure in a compact globule without rigid tertiary structure and without enzymatic activity. This collapse has been monitored by an increase of energy transfer from tryptophan indole rings to fluorescent dansyl labels [20,24,34]. If a protein molecule has several tryptophan residues and several dansyl labels, the energy transfer is averaged over many tryptophan-dansyl pairs and therefore reflects the overall compactness of a molecule. This has been checked by a parallel study of the changes of energy transfer and intrinsic viscosity upon equilibrium protein unfolding [20,34]. The important fact that the collapse occurs *after* the formation of a pronounced secondary structure has been established by direct comparison (at identical conditions) of the times of energy transfer (~1 s) and far ultraviolet circular dichroism (<0.01 s) increases for carbonic anhydrase [20,24] and β-lactoglobulin [20,29]. Similar information has been obtained for carbonic anhydrase using electron spin resonance labels [20,24,34,35].

The collapse of the protein molecule is accompanied by the formation of a solvent-accessible core from non-polar groups monitored by an increase of protein affinity for hydrophobic probes such as 1-anilinonaphtalene-8-sulfonate (ANS) [20,24–26,36]. The increase of affinity to ANS is typical also for the equilibrium molten globule state [36,37].

(3) Formation of the native protein structure monitored by the restoration of NMR [20,25], of circular dichroism in aromatic (near ultraviolet) region [20,22–24,29,30,38,39] and of enzymatic activity [20,22,39–41] as well as by desorption of ANS [24–26,36]. In a number of proteins all these processes occur simultaneously [20,26] suggesting that there is a *single* process of transformation of the loosely packed molten globule into the tightly packed native protein. However, there are proteins (e.g. carbonic anhydrase [24]) in which ANS desorption and the appearance of high field NMR signals are observed *before* the restoration of aromatic circular dichroism and enzymatic activity, thus suggesting that a non-polar core becomes tightly packed and solvent-inaccessible before the full restoration of the native protein structure. The time of this full restoration can be as large as 500–2500 s (at 25°C) which is often [2,7,18,21] (but not always [42,43]) connected with proline *trans-cis* isomerization.

These 3 main stages of protein folding are consistent with the hypothesis proposed by the author as early as 1973 [6].

3. STRUCTURE OF KINETIC INTERMEDIATES

The experiments briefly reviewed above give very

useful information on the *overall* state of the protein molecule (like its secondary structure content and compactness). However, they tell us nothing on the *specificity* of protein structure (like the location of α- and β-regions in the chain and their mutual positions in 3D-space). Some powerful methods have been developed recently which can provide an answer to this important question.

Hydrogen exchange has been used to label kinetic structural intermediates at different folding stages with the subsequent analysis of proton-labelling patterns by 2D-NMR spectra [44,45]. As a strong protection of NH-groups from hydrogen exchange needs almost certainly *both* their involvement in hydrogen bonding and their screening from solvent, this method probably reflects mainly the formation of a *screened* secondary structure rather than the formation of fluctuating embryos of secondary structure in an unfolded chain.

The main result obtained by this method is that in all proteins studied so far an early kinetic intermediate has been revealed in which all or at least some of native α- and β-regions are already formed and partly screened from solvent (as their NH-groups are partly protected from hydrogen exchange). NH-groups of the remaining α- and β-regions as well as NH-groups involved in tertiary hydrogen bonds become protected later, at the final folding stage.

In ribonuclease A [46] the NH-protons of all β-strands included in the V-shaped β-sheet, as well as NH-protons of 2 α-helices, are protected rapidly (within 0.4 s) while NH-protons of the N-terminal α-helix are protected in several seconds, simultaneously with the restoration of tertiary structure. In barnase [47] NH-protons of all 3 native α-helices and of the native β-sheet (as well as NH-protons of some native reverse turns) are protected in 2 stages with half-times of <0.003 and ~ 0.1 s while NH-protons involved in long-range hydrogen bonds are protected only in the slow phase. Thus, in both these proteins the secondary structure is completely or almost completely formed in the proper ('native') chain regions *before* the formation of the native tertiary structure. Moreover, it has been shown by site-directed mutagenesis [27] that even some specific interactions near α-helical termini are present in the early kinetic intermediate while other specific interactions near these termini are switched on only in the final folded state. The same method has also shown that the hydrophobic core between α-helices and β-sheets in barnase begins to consolidate in the early kinetic intermediate, becomes more condensed in the transition state (on the kinetic barrier), and finally reaches its tight packing in the completely folded state [27,50].

In cytochrome *c* [45,48] NH-protons of N- and C-terminal α-helices are 60% protected in the first 0.03 s of refolding and this protection is almost certainly connected with the formation of helix-helix contacts similar to those in the native structure. All other NH-protons (including those involved in other native α-helices, as well as in native reverse turns and tertiary hydrogen bonds) become protected much later. It is interesting that fluorescence of a single tryptophan residue (which is in contact with haem in the native protein) is already partly quenched at the early stage of folding while its indole NH-group becomes protected only at the slow stage. This suggests that a protein chain becomes compact simultaneously with the docking of 2 terminal α-helices.

Using a different approach based on competition between exchange and folding, Dobson et al. have shown [28] that the 'helical' subdomain of lysozyme (consisting of 5 N-terminal and C-terminal helices) is formed much faster (with a half-time of about 0.01 s) than the 'β-structural' subdomain (consisting of the central part of a chain). This 'non-homogeneous' structure of the kinetic intermediate in lysozyme folding is similar to the structure of the equilibrium molten globule state of α-lactalbumin [17,28], a protein whose native 3D-structure is almost identical with that of lysozyme [49].

Thus in cytochrome *c* and lysozyme, not only are some α-helices formed and screened from the solvent in early kinetic intermediates, but it is very likely that even the mutual positions of these helices in the intermediates are similar to those in the native protein.

These early kinetic intermediates in barnase [27] and lysozyme [28] have been identified with the molten globule state and it seems that the same is true for ribonuclease A and cytochrome *c*. Therefore, these data can be considered as evidence that in these relatively small and simple proteins, the native location of at least some α-helices and β-strands in a chain and moreover their native mutual positions (tertiary fold) are already present in the early compact kinetic intermediate (the molten globule state).

The situation in a larger protein has been studied for the β_2-subunit of tryptophan synthase [26,52,53]. A substantial part of its secondary structure is formed with a half-time of $\ll 0.01$ s [26], the half-time for the formation of the molten globule state is ~ 0.05 s [26] while the half-time of formation of a native-like antigenic determinant is ~ 10 s [52,53], and the half-time of formation of the native tertiary structure is about 100 s [26]. Therefore, a native-like antigenic determinant is formed *within the molten globule state*. Two other probes of native-like structural organization (approaches of Trp-177 to labelled Lys-87 and Cys-170 monitored by the address energy transfer [51]) appear even later than the formation of antigenic determinants. These approaches need rather a time which is comparable with that for the native tertiary structure.

4. CONCLUSION

At least some important features of the crude native 3D-structure can thus be formed together with the for-

mation of the molten globule state or within this state. It is a very important point as the molten globule state differs from the native one by the absence of tight packing and other specific interactions of protein side chains [8–13,54,55] as confirmed recently by site-directed mutagenesis for barnase [27] and apomyoglobin [56,57]. It may mean that specific van-der-Waals interactions as well as tertiary hydrogen bonds are not of primary importance in the coding of a *crude* 3D-structure. This structure is probably determined mainly by less specific interactions such as backbone hydrogen bonds and 'hydrophobic interactions', i.e. liquid-like interactions of non-polar groups in water environment. This conclusion is consistent with the observations that the code of crude protein 3D-structure is highly degenerate, i.e. quite different sequences can have similar tertiary folds [58,59], thus emphasizing that not all details of sequence (i.e. not all interactions) are important for this coding.

Now we can come back to 'Levinthal's paradox' as formulated at the beginning of this paper. On the one hand, the fact that a protein can find its native 3D-structure among the enormous number of alternatives, suggests that the native structure is just a final step of the programmed folding pathway [5] and does not correspond to the global minimum of free energy. On the other hand, there are some convincing arguments against this point of view. (i) The same protein structure can be achieved in vivo and in vitro, though folding pathways are almost certainly quite different under these conditions. (ii) Permutations in protein sequences which change the folding pathways [60] still do not prevent proteins from correct folding [60,61]. (iii) The set of observed tertiary folds is the most favourable from the thermodynamic point of view [62]. (iv) The first attempts to predict protein tertiary folds from their sequences suggest that each given protein selects from this set a tertiary fold which is thermodynamically the most favourable for its sequence [63–65]. The aim of this last section of the paper is to discuss the possibility that a protein can achieve the global minimum of its free energy without looking for all possible alternatives. To this end it is enough to assume that *a protein achieves its partial equilibrium state at each stage of its folding and that this equilibrium state is consistent with the minimum of free energy at subsequent stages*. In this case the structures which were rejected at preceding stages will not arise again at subsequent stages and do not have to be searched. Therefore, the global search for all possible conformations of this complicated and highly cooperative system can be replaced by a number of subsequent searches in either not very cooperative or not very large systems (cf. [64,65]).

Using as a basis the 'framework model' [6] which has been confirmed now by a number of experiments (see above) we can consider as an example the following set of events in protein folding at native conditions:

(1) First the fluctuating embryos of secondary structure are formed in an otherwise unfolded chain. As an unfolded chain has only marginal ('linear') cooperativity limited by the interaction of each residue with the restricted number of its chain neighbours, the equilibrium state can be achieved quasi-independently in different chain regions and therefore usually by an easy and rapid process.

(2) Embryos of secondary structure collapse into the compact 'molten globule' state. This collapse usually does not change very much the location of α- and β-regions outlined in the unfolded chain. This follows both from comparisons between the theories of secondary structure for unfolded and compact proteins [66] and from kinetic experiments which show that the secondary structure appears much earlier than the collapse and does not change very much simultaneously with the collapse [20,26]. Therefore, the 'new' search at this stage is limited mainly to the probing of a restricted set of allowed tertiary folds which are consistent with the given type of secondary structure [62,63]. As the set of these 'favourable' tertiary folds is very restricted and all possible protein conformations with the framework of a given fold can be probed rather easily and rapidly [64,65] this stage of this search also can be an overcoming task.

(3) The last stage of protein folding is the formation of the native tertiary structure. This stage is the most difficult one as it is connected with a large number of possible side chain conformations in a compact cooperative system. However even this stage seems not to be without hope. Though specific interactions which stabilize side chain packing in the native structure can be larger than interactions stabilizing the molten globule state, one can expect that all really 'good' packings (without overlapping and large holes) have more or less similar energies. Therefore, if each tertiary fold has at least one 'good' packing, the *differences* between their packing energies can be smaller than the differences between the energies which operate at the preceding stages. If this is the case, the search for the most thermodynamically stable tertiary structure can occur mainly within the framework of the previously selected tertiary fold. This search can also be performed rather easily as the highly cooperative molecular core consists of a rather small part of the side chains (see e.g. [67]), while loops and reverse turns, as well as the ends of α- and β-regions forming the molecular shell are not very cooperative.

This scheme does not pretend to provide more than an illustration of how a protein can *in principle* reach the global minimum of its free energy. It should be mentioned that this is not necessarily true for large multidomain proteins. The multidomain structure of large proteins can be expected from the thermodynamic point of view [62] but the fact that each domain usually consists of a *continuous* chain region can be due to the

kinetics of coil-globule transitions in a long chain [62,68].

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