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Kinetic and Equilibrium Folding Intermediates

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Kinetic and equilibrium folding intermediates

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SUMMARY

Our recent experiments on the molten globule state and other protein folding intermediates lead to following conclusions: (i) the molten globule is separated by intramolecular first-order phase transitions from the native and unfolded states and therefore is a specific thermodynamic state of protein molecules; (ii) the novel equilibrium folding intermediate (the 'pre-molten globule' state) exists which can be similar to the 'burst' kinetic intermediate of protein folding; (iii) proteins denature and release their non-polar ligands at moderately low pH and moderately low dielectric constant, i.e. under conditions which may be related to those near membranes.

1. INTRODUCTION

The main difficulty of protein folding is to avoid 'traps' (local energy minima) in which protein can fall down. The 'framework' model of protein folding (Ptitsyn 1973, 1987) assumes that protein avoids this difficulty by folding step-by-step in such a way that the results of the each step are not reconsidered but just fastened at subsequent steps. The framework model has predicted two main kinetic intermediates of protein folding. In the first intermediate a secondary structure fluctuating around its native position is already formed, whereas the second intermediate has much more stable secondary structure and the main features of the native tertiary fold (i.e. the crude mutual positions of α-helices and β-strands).

Both these kinetic intermediates have been observed experimentally and described in detail. First, it was shown that secondary structure (Robson & Pain 1976) and a compact state (Creighton 1980) are formed before the tertiary structure. Secondly, in 1987 it was shown that protein folds through at least two kinetic intermediates. The first intermediate is formed in less than 10 ms and has a substantial fluctuated secondary structure (Gilmanshin & Ptitsyn 1987; Kuwajima *et al.* 1987; Elöve *et al.* 1992; Radford *et al.* 1992). The second intermediate is formed within 0.1–1 s and has a globular shape (Semisotnov *et al.* 1987, 1992) and much more stable secondary structure (Elöve *et al.* 1992; Radford *et al.* 1992). By using the pulsed hydrogen exchange method (Baldwin 1993; Roder & Elöve 1994) and site-specific mutagenesis (Fersht 1993) it has been shown that the second kinetic intermediate (usually just preceding the rate-limiting step of folding) has many features of the three-dimensional structure of the native protein.

It was shown (Dolgikh *et al.* 1984; Baldwin 1993; Jennings & Wright 1993) that the second kinetic intermediate shares many features with an equilibrium intermediate, the molten globule state (Dolgikh *et al.* 1981), which is a typical state of protein molecules under mild denaturing conditions (for review, see Ptitsyn 1992). It was shown also that the molten globule state is involved into some important physiological processes (for review, see Bychkova & Ptitsyn 1993).

This paper summarizes our recent results on the molten globule state and other equilibrium folding intermediates.

2. THE MOLTEN GLOBULE IS A SPECIFIC THERMODYNAMIC STATE OF PROTEIN MOLECULES

One of the most important questions about the molten globule state is whether it is a specific thermodynamic state of protein molecules or whether it is similar to either a slightly disordered native protein or a 'squeezed coil'. The criterion of a specific thermodynamic state is the presence of phase transition between that state and other ones. The most important are first-order phase transitions ('all-or-none' transitions) which are coupled with drastic change of at least one of the first derivatives of free energy (like enthalpy, number of 'absorbed' solvent molecules, etc.). It was shown that protein denaturation (i.e. loss of its activity accompanied by the loss of its rigid tertiary structure) is of all-or-none character, presenting the first example of intramolecular first-order phase transition (Privalov 1979). However, it was assumed (Privalov 1979, 1992) and widely accepted that all denatured states of proteins are identical or similar from the thermodynamical point of view.

To determine whether the molten globule is separated by all-or-none transitions from other states, we applied the well-known statistical physics concept that the slope of all-or-none transition in a small system is proportional to a number of units in that system (Hill 1968). Applied to this investigation, it means that the slope of an intramolecular transition must be proportional to molecular mass of a macromolecule.

Figure 1 shows experimental data on the slopes of urea- and guanidinum chloride (GdmCl)-induced N⇔MG and MG⇔U transitions (N, native state; MG,

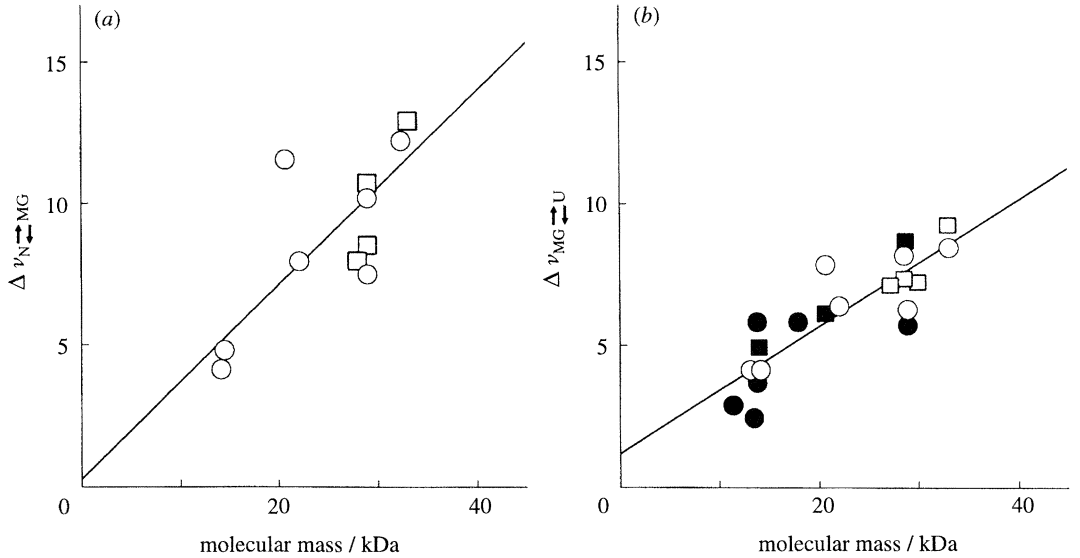

Figure 1. Molecular mass dependence of slopes for urea-induced (squares) and GdmCl-induced (circles) N⇔MG (*a*) and MG⇔U (*b*) transitions. Open symbols refer to three-state unfolding of native proteins (N⇔MG⇔U), while filled symbols refer to two-state unfolding of pH-induced MG state (MG⇔U). Slopes are presented as $\Delta\nu = \partial \ln K/\partial \ln a$ in the middle of transition, where K is equilibrium constant between two states of a protein molecule and a is activity of urea or GdmCl. Adapted from Ptitsyn & Uversky (1994).

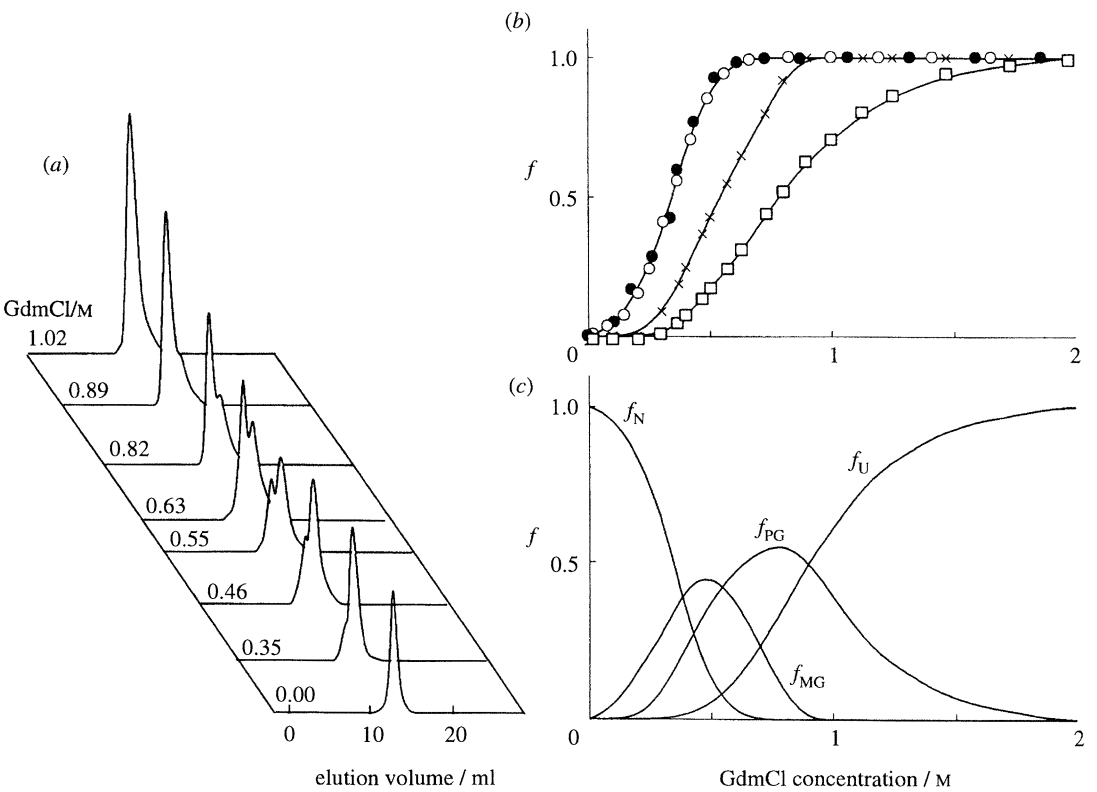

Figure 2. GdmCl-induced unfolding of *Staphylococcal* β-lactamase. (*a*) Elution profiles at different concentrations of GdmCl. (*b*) GdmCl dependence of activity (open circles) and molar elipticity at 270 nm (filled circles), as well as fraction of 'less compact' molecules (x) and their elution volume (open squares) in relative units. (*c*) GdmCl dependence of fractions of native (N), molten globule (MG), pre-molte globule (PG) and unfolded (U) protein molecules. Adapted from Uversky & Ptitsyn (1994).

molten globule state; U, unfolded state) in all small (one-domain) proteins studied up-to-date (Ptitsyn & Uversky 1994). The figure demonstrates that in both cases the slopes are proportional to the molecular masses of proteins. Thus, both N⇔MG and MG⇔U transitions are of all-or-none character, i.e. are intramolecular first-order phase transitions. The all-or-none character of N⇔MG transition was indeed demonstrated even earlier for a temperature denaturation of α-lactalbumins which are in the MG state at high temperatures (Dolgikh *et al.* 1981, 1985).

The all-or-none character of MG⇔U transition has been demonstrated by the most direct method: bimodal distribution of protein elution volumes upon

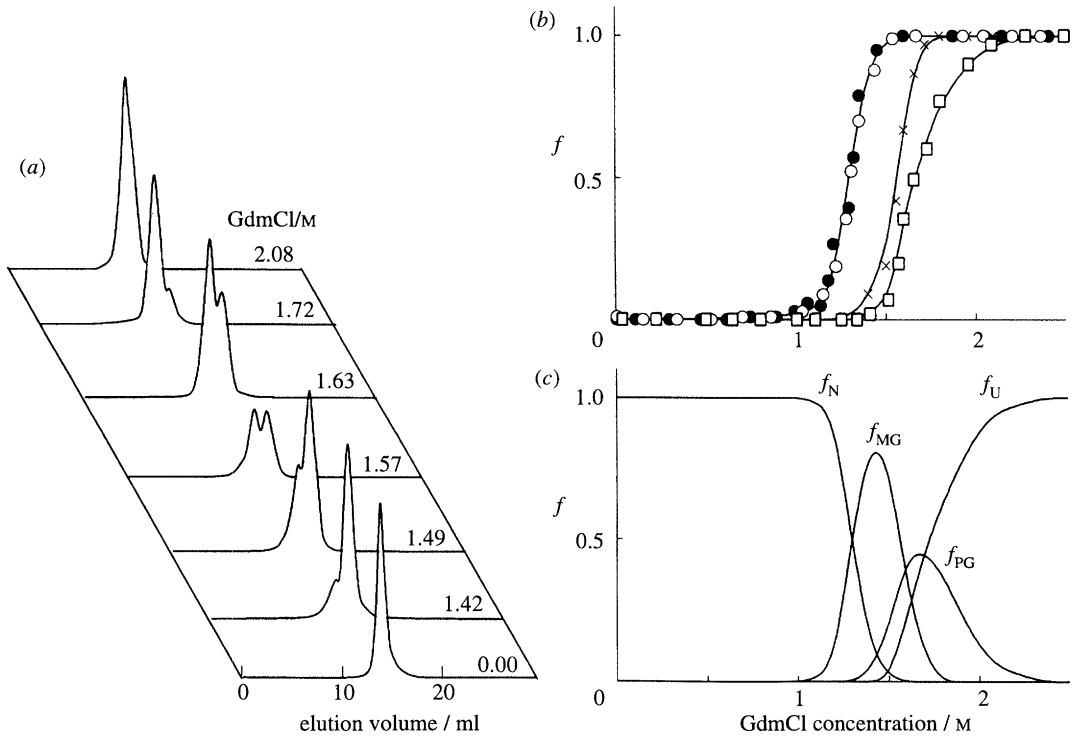

Figure 3. GdmCl-induced unfolding of bovine carbonic anhydrase B. All notations are the same as in figure 2. Adapted from Ptitsyn & Uversky (1995).

GdmCl-induced protein unfolding (Uversky *et al.* 1992; Uversky & Ptitsyn 1994, 1995). Figures 2*a* and 3*a* show this bimodal distribution for two studied proteins and present a clear evidence for an all-or-none transition. This transition occurs at concentrations of GdmCl substantially higher then the concentrations which lead to denaturation of these proteins (see figures 2*b* and 3*b*). Therefore it is not connected with protein denaturation but rather is the transition between two denatured states: a compact and a less compact. The first denatured state is non-active, has no rigid tertiary structure, but is compact, has a large content of secondary structure and strongly binds 1-anilino-naphtalene-8-sulphonate (ANS), thus meeting all usual criteria of the MG state. As to the second (less compact) denatured state, it is a mixture of at least two states with quite different volumes (see below).

The possible explanation of two equilibrium phase transitions in protein molecules is the existence of two levels of their three-dimensional structure: a large-scale order ('tertiary fold') which can exist already in the MG state (Peng & Kim 1994), and a short-scale order (rigid tertiary structure) which exists only in the native state. It is quite possible that two phase transitions in proteins correspond to the formation (or destruction) of these two levels of protein structures (Ptitsyn 1994).

3. EQUILIBRIUM 'PRE-MOLTEN GLOBULE' STATE: A POSSIBLE ANALOGUE OF THE FIRST KINETIC INTERMEDIATE

Figures 2 and 3 demonstrate an additional important feature of the equilibrium folding or unfolding of proteins. They show that an elution volume of less compact molecules substantially decreases with the increase in GdmCl concentration, which corresponds to a further increase of a hydrodynamic volume (cf. Palleros *et al.* 1993). This increase is much larger than the normal swelling of unfolded proteins in good solvents (Uversky & Ptitsyn 1994) and continues even at those GdmCl concentrations at which all compact (MG) molecules disappear. It can be explained only by the existence of two different 'less compact' states which are in a fast equilibrium: the partly folded 'pre-molten globule' (PG) state and the really unfolded state (Uversky & Ptitsyn 1994, 1995). For both studied proteins a hydrodynamic volume (V) of the pre-molten globule state $V_{PG} \lesssim 2.4\ V_N$ (and $\lesssim 1.5\ V_{MG}$), while $V_U \simeq 12 V_N$. Far ultraviolet circular dichroism (UV CD) spectra show that the pre-molten globule state has a substantial secondary structure, i.e. the secondary structure of the MG state decreases in two steps: upon MG → PG and PG → U transitions. The pre-molten globule state also binds ANS although less strongly than the MG state. Thus, this new state belongs to the family of relatively compact and partly structured states of protein molecules. We have suggested that the pre-molten globule state may include an 'embryo' of the native-like tertiary fold (Uversky & Ptitsyn 1995).

Properties of this novel equilibrium state are similar to those of the first kinetic intermediate. In fact, this kinetic intermediate also is partly condensed (Kawata & Humaguchi 1991; Elöve *et al.* 1992), has a substantial amount of secondary structure (Kuwajima *et al.* 1993) and binds ANS (Semisotnov *et al.* 1991).

It is known that both the first kinetic intermediate and the kinetic MG state accumulate upon protein folding, i.e. are separated by high potential barriers both from each other and from the native state. The existence of two equilibrium phase transitions in proteins and the accumulation of two kinetic inter-

mediates upon protein folding therefore perhaps have a common purpose: the independent formation of two levels of three-dimensional structure (Ptitsyn 1994).

4. FOLDING INTERMEDIATES UPON PHYSIOLOGICAL CONDITIONS

Physical studies of the molten globule state suggest that the molten globule preserves the main elements of native secondary structure and their crude mutual positions in three-dimensional space, but differs from the native state by a less tight packing of side chains (Shakhnovich & Finkelstein 1989), and by partial unfolding of loops and ends of a chain (Ptitsyn 1992). This state is almost ideal for a protein which have to adapt itself to different external conditions (like the conditions in a living cell), maintaining a memory on its overall architecture. It was the basis of our prediction that the molten globule state can exist in a living cell and can be involved in a number of physiological processes (Bychkova *et al.* 1988). Part of these predictions – especially recognition of the MG state by chaperones (Martin *et al.* 1991) and the role of the MG state in insertion of proteins into membranes (van der Goot *et al.* 1991) – has been confirmed experimentally. More recently a number of other physiological applications of the MG state have been shown or suggested (for a review, see Bychkova & Ptitsyn 1993).

In many cases folding intermediates in a cell are the kinetic intermediates trapped by chaperones just after protein biosynthesis before proteins can completely fold. It is known that chaperones transfer proteins in these intermediates states to corresponding compartments of a cell where they are released from chaperones, are translocated across membranes into the compartment and then acquire their completely folded structures (Schatz 1993; Hendrick & Hartl 1993). Another possible case may be proteins with mutations which prevent them from complete folding (see below).

However, there are also proteins, or their domains, which normally exist in the rigid ('native') state but must denature to fulfil their function. Examples include pore-forming domains of some toxins (van der Goot *et al.* 1992), as well as proteins that act as carriers for large non-polar ligands (see below). How these proteins can denature at physiological conditions (at usually neutral bulk pH, large ionic strength and normal temperatures) is not clear. However, we have to remember that a cell is transpired by membranes and cytoskeleton and contains a lot of proteins, nucleoprotein complexes and organelles. Thus, it can be compared to a thick Russian soup rather than to salted water which often is believed to be a proper model for a natural environment of proteins.

There are at least two factors which can lead to a protein denaturation near membrane surfaces. The first is that negative charges of membrane attract protons which leads to a local decrease of pH (van der Goot *et al.* 1991). The second is that an organic moiety of membranes decreases an effective value of local dielectric constant (Bychkova & Ptitsyn 1993).

A very crude model of a 'concert' action of these two effects may be a protein denaturation in water–organic mixtures at moderately low pH, although the states of denatured proteins in these cases may be different from their 'normal' folding intermediates (see below).

5. PROTEIN DENATURATION AND RELEASE OF NON-POLAR LIGANDS

A good example of this situation are protein carriers of large non-polar ligands. These ligands often are deeply buried into a rigid protein and tightly packed with non-polar groups of its core as it is the case, for instance, for retinol-binding protein (RBP). The release of these ligands may become possible only in the molten globule or other denatured state where ligands are less tightly packed with protein groups (Bychkova & Ptitsyn 1993).

It was shown (Bychkova *et al.* 1992) that retinol can be released from RBP at low pH (in the interval from 5.5 to 3.0 with the middle point about 4.5) and that the release of retinol is coupled with the protein transition into the MG state. This was the first evidence that the MG state can be involved in a target release of non-polar ligands. Of course, in water solutions both transitions occur at pH which are much lower than their physiological values. However, recently we have shown (V.E. Bychkova, A. Fantuzzi, A.E. Dujsekina, G.-L. Rossi & O.B. Ptitsyn, unpublished data) that both release of retinol and denaturation of RBP can be achieved at substantially higher pH in water–methanol mixtures. For example, the increase of methanol content from 0 to 30 and 50% shifts the ends of both these transitions from pH 3.0 to 4.5 and 7.0, respectively. As a result, a native RBP-retinol complex is stable only at pH > 5.5 and at methanol content less than 35%, which corresponds to average dielectric constants larger than 60. These experiments clearly demonstrate the importance of a 'concert' action of pH and organic environment on a function of protein coupled with its denaturation.

Another interesting example of a protein carrying large non-polar ligands is α-fetoprotein, which transfers unsaturated fatty acids and estrogens to embryonal and some cancer cells (for a review, see Abelev 1993). It was shown (Uversky *et al.* 1995) that α-fetoprotein also can be transformed into MG state at pH 3 in water solutions. In fact, figure 4 shows that this protein at pH 3.1 has a native-like far UV CD spectrum and native-like spectrum of Trp fluorescence, but has no co-operative temperature melting and strongly binds ANS, i.e. meets all usual requirements of the MG state. It is quite possible that the release of unsaturated fatty acids and other ligands from α-fetoprotein also is connected with the transformation of rigid 'native' molecules into molten globule or other denatured state.

Of course, proteins denatured in water–alcohol mixtures can be quite different from the folding intermediates in water. In fact, all proteins studied up-to-date, including ubiquitin, lysozyme, monellin and α-lactalbumin (see, for example, Alexandrescu *et al.* 1994, and references therein), as well as cytochrome *c*, carbonic anhydrase B and RBP (our unpublished

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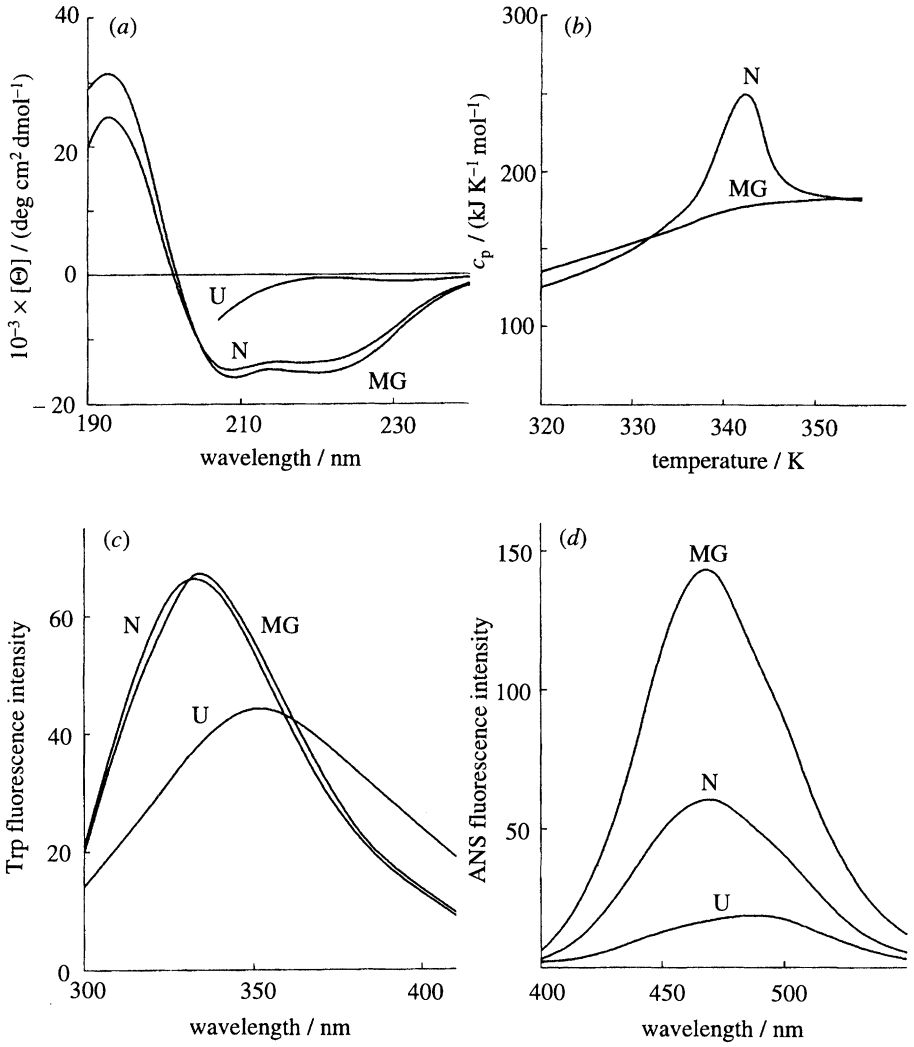

Figure 4. (*a*) Far UV CD spectra, (*b*) microcalorimetric recording, (*c*) Trp and (*d*) ANS fluorescence spectra for α-fetoprotein at pH 7.2 (N state), pH 3.1 (MG state) and in 9 M urea (U state). Adapted from Uversky *et al.* (1995).

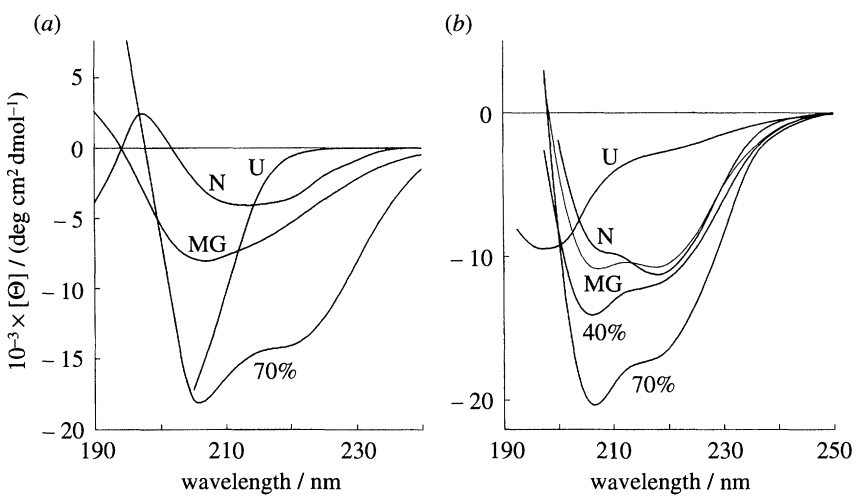

Figure 5. (*a*) Far UV CD spectra of bovine carbonic anhydrase B in 70% CH_3OH at pH 2.0 compared with those in N (pH 7.5, 0.5 M NaCl), MG (pH 3.6 in 10 mM phosphate buffer) and U (7 M GdmCl) states. (*b*) Far UV CD spectra of horse cytochrome *c* in 40 and 70% CH_3OH (pH 4.0, 0.5 M NaCl) compared with those in N (pH 4.0, 0.5 M NaCl), MG (pH 2.0, 0.5 M NaCl) and U (pH 2.0, water) states.

data), have much more pronounced far UV CD spectra in mixtures of water with large amounts of alcohols than in the native or in the molten globule states (see figure 5). In a similar way the addition of methanol from 0 to 60% changes $[\Theta]_{220}$ of RBP from +700 at pH 7.5 (N state) and −6000 at pH 2.0 (MG state) to −9200 and −20000 deg cm^2 dmol^{-1}, correspondingly. This suggests that alcohol-denatured state of proteins

may be substantially more helical than the N or the MG states. This conclusion is supported by NMR data suggesting alcohol-induced transitions of some β- or irregular chain regions of active proteins into α-helical state (see Alexandrescu *et al.* 1994, and references therein).

However, at smaller concentrations of methanol and moderately low pH, proteins have less pronounced far UV CD spectra. For example, for cytochrome *c* in 40% CH_3OH (figure 5*b*) or for RBP in 20% CH_3OH (data not shown), far UV CD spectra at pH 4 are close to those for the MG state of these proteins obtained without methanol at extreme pH ($\sim$ 2). The physical state of proteins at different alcohol concentrations requires more detailed investigation.

6. MOLTEN GLOBULES AND GENETIC DISEASES

As mentioned above, it is possible to obtain stable folding intermediates at physiological conditions by other means: some gene mutations can lead to a synthesis of mutant proteins which could not be completely folded under normal conditions. These mutations have been already reported (see, for example, Craig *et al.* 1985; Lim *et al.* 1992) and it was concluded that protein folding is stopped in these cases at the molten globule stage.

On the other hand, there are genetic diseases which can be provoked by point mutations in some proteins leading to their mislocation in a cell. This mechanism was well established for cystic fibroses (Yang *et al.* 1993) but is likely the case for some other diseases, for example for hypercholesterolemia and emphysema. This has led us (Bychkova & Ptitsyn 1994) to the assumption that mutations which cause genetic diseases by changing the intracellular pathway of proteins also inhibit the last stage of protein folding. These mutations may cause proteins to be trapped in the molten globule state which can either be associated with chaperones or aggregate. This prevents mutant proteins from normal trafficking in the cell and leads to mislocation and degradation of these proteins.

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