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Compact state of a protein molecule with pronounced small-scale mobility: bovine α-lactalbumin

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Abstract. We describe a novel physical state of a protein molecule which is nearly as compact as the native state and has pronounced secondary structure, but differs from the native state by the large increase of thermal fluctuations (in particular, by the large mobility of side groups). This state has been characterized in detail for the acid form of bovine α-lactalbumin as a result of the study of physical properties of this state by a large variety of different methods (hydrodynamics, diffuse X-ray scattering, circular dichroism and infrared spectra, polarization of the luminescence, proton magnetic resonance, deuterium exchange and microcalorimetry). It has been shown that bovine α-lactalbumin can be transformed into a similar state by thermal denaturation. This process is thermodynamically two state (i.e. all-or-none transition), which means that this state differs from the native one by a phase transition of the first order.

Key words: Protein denaturation, protein folding, α-lactalbumin, molten globule state of proteins, phase transitions in proteins

Introduction

For an experimental study of protein folding it is especially important to understand the physical nature of thermodynamically stable states of protein molecules intermediate between the completely ordered native state and completely unordered statistical coil. The study of these states can clarify many general problems of protein structure and protein folding as these states may correspond to the "switching off" of some interactions which stabilize the native protein structure leaving other interactions basically unaltered. As a result, some levels of protein structure may be destroyed while some others may remain unchanged.

It is well known that proteins denatured by different agents have different structures (Tanford 1968). The proteins denatured by high concentrations of urea or Gu·HCl usually have the completely unordered structure of the statistical coil, while those denatured by high temperatures and sometimes those denatured by acid or basic pH are not completely unordered (Tanford 1968). On the other hand, it has been shown that various denatured states of a protein have similar enthalpy and entropy when extrapolated to the standard conditions (Privalov 1979). The physical nature of partly unordered denatured states of proteins remains a field of debate.

A new approach to this problem arose when it was shown that some proteins undergo *two* different conformational transitions with an increase of concentration of strong denaturing agents (e.g. Gu·HCl). The first one can usually be detected by a drastic change of CD in the near UV region while the second one is accompanied by a drastic change of CD in the far UV region. This means that the environment of aromatic side chains can alter independently of protein secondary structure. The currently studied proteins with these properties include e.g. carbonic anhydrase (Wong and Tanford 1973), growth hormone (Holladay et al. 1974), β-lactglobulin (Ananthanarayanan and Ahmad 1977), bovine (Kuwajima et al. 1976) and human (Nozaka et al. 1978) α-lactalbumins etc. The properties of at least some of these proteins at acid pH are similar to

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Abbreviations: Bα-LA, bovine α-lactalbumin; Gu·HCl, guanidine hydrochloride; CD, circular dichroism; UV, ultraviolet; IR, infra-red; NMR, nuclear magnetic resonance. Different forms of Bα-LA are abbreviated as follows: N – native form, A – acid form, T – temperature-denatured form, U – unfolded form (by 6 *M* Gu·HCl or 8 *M* urea). All forms have intact S-S bonds.

those at Gu·HCl concentrations between the two transitions.

The best-studied example of these proteins is bovine α-lactalbumin (Kronman et al. 1964, 1965, 1966, 1967, 1971, 1981; Kronman and Holmes 1965; Robbins and Holmes 1970; Takesada et al. 1973; Kuwajima et al. 1975, 1976, 1981; Kita et al. 1976; Kuwajima 1977; Maruyama et al. 1977; Kuwajima and Sugai 1978; Sommers and Kronman 1980; Hiraoka et al. 1980; Permyakov et al. 1981 a, b). It has been shown that bovine α-lactalbumin (Bα-LA) denatured by acid (Kronman et al. 1964, 1965; Kuwajima et al. 1976; Sommers and Kronman 1980) or by basic (Kronman et al. 1967; Kuwajima et al. 1981) pH, by high temperatures (Takesada et al. 1973; Kuwajima and Sugai 1978; Sommers and Kronman 1980), by intermediate concentrations of Gu·HCl (Kuwajima et al. 1976) and by perchlorate salts (Maruyama et al. 1977) is not as unordered as Bα-LA denatured by high concentrations of Gu·HCl. For example, the far UV CD spectrum of the acid (A-) form of Bα-LA is much more intense than that of the completely unordered Bα-LA and even more intense than the spectrum of the native protein (Robbins and Holmes 1970; Kronman et al. 1971; Kuwajima et al. 1975, 1976). On the other hand, the near UV CD spectrum of the A-form is drastically reduced in intensity as compared with the native state and is similar to the spectrum in 6 *M* Gu·HCl (Kronman et al. 1971; Kuwajima et al. 1976, 1981; Maruyama et al. 1977). It has been shown that some physical properties of Bα-LA denatured by basic pH (Kronman et al. 1967; Kuwajima et al. 1981), high temperatures (Kuwajima and Sugai 1978; Sommers and Kronman 1980), intermediate concentrations of Gu·HCl (Kuwajima et al. 1976) and perchlorate salts (Maruyama et al. 1977) are similar (though not identical) to those of the acid-denatured α-LA.

Based on these data together with some results suggesting a rather large expansion of the A-form of Bα-LA as compared with its native state (Kronman et al. 1964; Kuwajima et al. 1975), Kuwajima (1977) proposed a model of this form as an unfolded (or at least strongly expanded) molecule with the α-helical content even higher than that of the native protein. Kuwajima also suggested that this model may be valid for at least some other "intermediate" or "partly folded" (Sommers and Kronman 1980) forms of this protein. However this model cannot explain some experimental data, e.g. the low solvent accessibility of tryptophan residues in the A-form (Kronman and Holmes 1965).

We feel that α-lactalbumins are good models for the study of the general properties of "partly folded" states of globular proteins, i.e. of their states intermediate in structure between the native and completely unfolded states. Therefore we have studied the physical properties of bovine and human α-lactalbumins in different partially folded forms by a large variety of physical techniques giving complementary information on the protein structure (see also a previous paper, Dolgikh et al. 1981). This permits us to propose a model for these forms as a novel state of a protein molecule which is nearly as compact as a native protein and has a pronounced secondary structure, but differs from the native state by the large increase of thermal fluctuations (in particular, by the large mobility of side groups). In this paper we shall describe in detail the properties of this state, taking the acid and temperature-denatured forms of bovine α-lactalbumin as examples.

2. Materials and methods

Bα-LA was prepared from fresh cow milk by the method described previously (Armstrong et al. 1967, method IIb; Armstrong et al. 1970) and was completely homogeneous according to disc-electrophoresis with and without sodium dodecyl sulphate and according to the analysis of five N-terminal amino acids. The protein concentrations were determined by the extinction coefficient $E^{1\%,1\,cm}_{280\,nm} = 20.9$, obtained by Wetlaufer (1961) and confirmed by us with the CHN-analyzer.

The native and the temperature-denatured forms of Bα-LA have been studied in solutions containing Tris·HCl buffer (pH varying from 7.0 to 7.6) and 0.05 *M* KCl in all experiments except H-D exchange and IR-spectroscopy (see below). The A-form of Bα-LA has been studied in 0.05 *M* KCl·HCl buffer (pH 2.0).

Tris and Gu·HCl (analytical grade), specially prepared for optical measurements, were from "Biokhimreaktiv" (USSR), EDTA (disodium salt) was from Serva Feinbiochemica, other reagents (analytical grade) were from "Reakhim" (USSR). All solutions were prepared with glass-distilled water. The Gu·HCl concentration was determined from the refractive index at 589 nm (Nozaki 1972). pH of solutions was determined by the Radiometer pH-meter (model 26) using the expanded scale.

Intrinsic viscosity was measured by the automatic capillary viscometer, "Viscomatic", thermostatted within 0.01 °C. Differences between flow times (measured by electronic timer) for the solution (t) and the solvent (t_0) were from 0.4 to 8.0 s. Several time readings, reproducible to within 0.02 s, were taken for each solution. The intrinsic viscosity was determined by the linear extrapolation of $(t-t_0)/t_0 c$

values to zero protein concentration in the concentration range, $c \approx 2-8$ mg/ml.

Diffuse X-ray scattering curves were measured using the equipment described earlier (Serdyuk and Grenader 1975) and recalculated to point collimation according to Shchedrin and Feigin (1966). The Fourier transform of scattering curves was made according to standard calculations (Guinier and Fournet 1955).

A Spinco Model E (Beckman) ultracentrifuge was used for determining the sedimentation constant and a MOM 3170 ultracentrifuge was used for sedimentation equilibrium measurements. A partial specific volume value of 0.719 was used (measured by an Anton Paar densitometer at the Institute of Physical Chemistry of Graz University, Austria). The apparent molecular weights were obtained from more than 10 sedimentation equilibrium measurements in the concentration range from 0.2 to 0.8 mg/ml (the initial concentration in the cell). No concentration dependence of the apparent molecular weight was observed in this concentration range.

Luminescence polarization and the average lifetime of the excited state of Trp residues were measured as described by Semisotnov et al. (1981), using unpolarised excitation.

NMR spectra were recorded on a Brucker WH360 spectrometer operating in the Fourier transform mode. D_2O (99.8%) and DCl were used for preparing 0.02 *M* Tris·DCl buffer (pH** 7.6 at 20 °C) for the investigation of N- and T-forms and 0.05 *M* KCl·DCl buffer (pH* 2.0 at 20 °C) for the investigation of the A-form.

CD spectra were measured by the Mark III-S dichrograph (Jobin Ivon). The mean residue molecular weight of 115 (Brew et al. 1970) was used for calculating the values of the molar ellipticity.

IR spectra were recorded on a Perkin Elmer 180 spectrophotometer in solutions of D_2O as described by Chirgadze et al. (1973) and Chirgadze & Brazhnikov (1974). The samples were prepared by dissolving the lyophilized Bα-LA in D_2O buffers, the pH* values being adjusted with 0.1 *M* DCl or 0.1 *M* NaOD.

Microcalorimetric measurements were carried out by the differential adiabatic microcalorimeter DASM-1 *M* (Privalov et al. 1975) according to the method described there.

The H-D exchange was studied in D_2O solutions by the IR spectrometer IR-20 (Carl Zeiss, Jena, DDR) as described by Abaturov et al. (1976). Full deuteration of Bα-LA peptide NH-groups was achieved by incubating the protein in D_2O at 50 °C for 10 min. The values for the ratio of Amide II/Amide I intensities for completely unexchanged peptide NH-groups was taken as 0.45 for Bα-LA (Takesada et al. 1973) and 0.42 for oxidized ribonuclease (Lebedev et al. 1976).

Calcium binding was studied by measuring the radioactivity of Bα-LA labeled by radioactive ^{45}Ca after passing through a Sephadex G-25 column (volume ~ 1 ml). Labeling was done by an addition of an excess of radioactive $CaCl_2$ to a solution of Bα-LA in 0.02 *M* Tris·HCl buffer, pH 7.5 with 5 m*M* EDTA. The radioactivity of the protein solution was nearly 500 counts/min per 0.05 ml aliquot used for radioactivity determination.

All experiments were made at room temperature if not otherwise indicated.

3. Results

A. Acid form: "native-like" properties

I. Compactness. The characteristic of a molecule most sensitive to its molecular volume is, of course, its intrinsic viscosity. The intrinsic viscosity, $[\eta]$, of the A-form (3.1 cm^3/g) coincides within experimental error (± 0.4 cm^3/g) with the intrinsic viscosity of the N-form (3.4 cm^3/g) and is typical for globular proteins. At the same time, for Bα-LA unfolded by 6 *M* Gu·HCl (with intact S-S bonds), i.e. for the U-form, $[\eta] = 6.1$ cm^3/g. These results clearly indicate the compactness of the A-form of the protein though due to the experimental errors in measuring the intrinsic viscosities we cannot exclude a possible difference of ~30% in the molecular volumes of the N- and A-forms.

This conclusion has been confirmed by a study of diffuse X-ray scattering. Figures 1a and b show Guinier plots for the N- and A-forms of Bα-LA at various concentrations. The downward curvature of the Guinier plots for the A-form at high concentrations and small scattering angles could be due to the electrostatic repulsion of charged Bα-LA molecules (cf. Vainstein and Feigin 1965). From the curves recalculated to point collimation and extrapolated to zero concentration the molecular weights, *M*, and gyration radii, R_g, for both forms have been obtained. For the N-form we have obtained $R_g = 15.5$ Å which is between the values of 14.5 Å (Pessen et al. 1971) and 16.7 Å (Krigbaum and Kügler 1970) published earlier. The differences between these values may reflect the presence of a small amount of associated Bα-LA molecules in the N-form under some experimental conditions (see Pessen et al. 1971). In fact the molecular weights of the N-form obtained from X-ray diffuse scattering data are

* The pH values of all D_2O solutions are reported as pH* without addition of 0.4 pH unit to the pH-meter reading

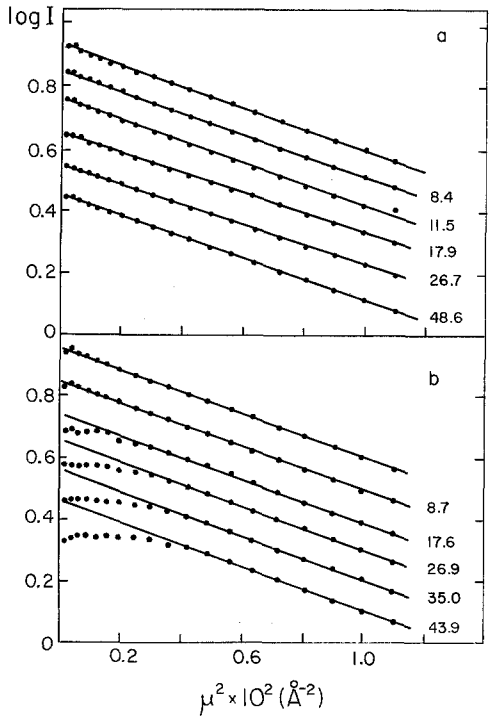

Fig. 1 a and b. Dependence of $\log I$ on μ^2 for the native (**a**) and acid (**b**) forms of Bα-LA. I – scattering intensity, $\mu \equiv (4\pi/\lambda)\,\mathrm{Sin}\,\theta$ – scattering vector (λ – X-ray wavelength, 2θ – scattering angle). Protein concentrations (in mg/ml) are shown at the curves. The top curves in Fig. 1 a and b are obtained by extrapolation to zero concentration. All curves are shown without recalculation to point collimation

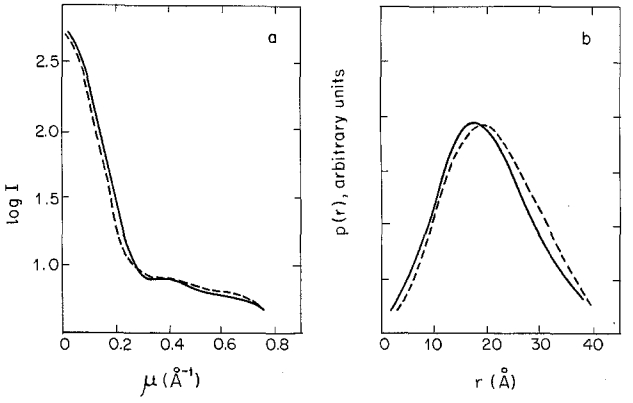

Fig. 2. a Dependence of the logarithm of scattering intensity, $\log I$, on the scattering vector μ for Bα-LA in the native (——) and acid (– – –) forms. Scattering intensity is extrapolated to zero concentration and recalculated to point collimation. **b** Distance distribution functions $p(r)$ for Bα-LA in the native (——) and acid (– – –) forms

equal to 13,000 (Pessen et al. 1971), 15,500 (Krigbaum and Kügler 1970) and 15,800 (this work) as compared with the value of 14,200 calculated from the amino acid content (using the sedimentation equilibrium method we have obtained $M = 14{,}900$). For the A-form we have obtained $R_g = 15.7$ Å and $M = 14{,}100$ (by X-ray diffuse scattering) and 13,600 (by the sedimentation equilibrium method). Thus R_g values for the N- and A-forms of Bα-LA either coincide or differ by not more than ~10% which again shows the similar compactness of both forms.

A comparison of the large-angle X-ray scattering curves for the N- and A-forms of Bα-LA is shown in Fig. 2a. Although the curves do not coincide in detail, they have similar shapes typical of globular proteins. Distance distribution functions, $p(r)$, for the N- and A-forms obtained by the Fourier transform of their scattering curves (Fig. 2b) show only a very slight shift (~2 Å) of $p(r)$ to larger distances for the A-form. Thus, large-angle diffuse X-ray scattering also confirms the compactness of Bα-LA molecules in the A-form.

Sedimentation coefficients of Bα-LA (extrapolated to zero concentration from the concentration range of 1.5 – 12.0 mg/ml) are equal to 1.67 S at pH 7 and 1.40 S at pH 2. Earlier, Kronman et al. (1964) obtained the values of 1.93 S (pH 8.55) and 1.67 S (pH 2) at higher ionic strength, which can lead to an increase in the degree of protein association (Kronman et al. 1964). In both cases the sedimentation coefficient of the A-form is ~15 – 20% smaller than that of the N-form. This does not invalidate our conclusion that the molecular volume of the A-form is not substantially larger than that of the N-form, as this difference can be, at least partly, due to the small degree of association of the protein in the N-form (see above).

Summarizing all these data, we can conclude that there is no large increase in the volume of Bα-LA molecules in the A-state as compared with the N-one. However, these data do not exclude (though do not prove) the relatively small (up to ~30%) increase of this volume.

II. Secondary structure. Far UV CD spectra of Bα-LA at pH 2 and pH 7 are presented in Fig. 3a. These spectra are very similar to those published earlier (Robbins and Holmes 1970; Kronman et al. 1971; Kuwajima et al. 1975, 1976) and demonstrate a rather significant difference between the CD properties of Bα-LA at neutral and acid pH. However it is clear that CD spectra of both N- and A-forms reflect a high content of secondary structure and are quite different from the CD spectrum of the U-form of Bα-LA (in 6 M Gu·HCl) shown in Fig. 3 for comparison.

A qualitative analysis of the far UV CD spectra of the N- and A-forms (Robbins and Holmes 1970) has led to the conclusion that the A-form is even more α-helical (and less β-structured) than the N-form. The quantitative analysis of these spectra

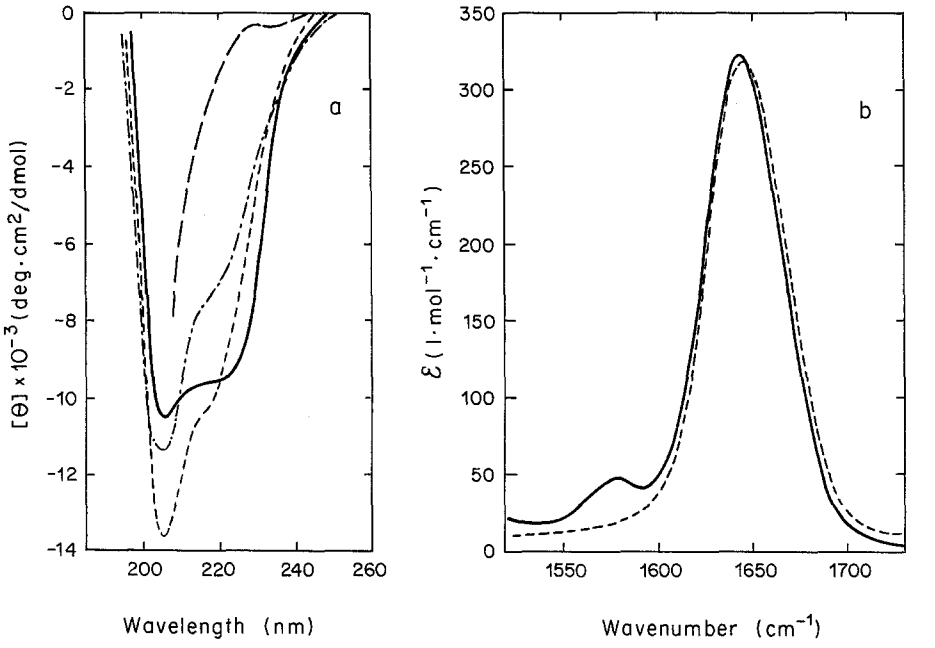

Fig. 3. **a** Far UV CD spectra for Bα-LA in the native (——), acid (– – –), temperature-denatured (– · – · –) and unfolded (— — —) forms. **b** Infrared spectra for Bα-LA in the N (——) and A (– – –) forms. CD spectra for the N- and A-forms have been measured at pH 7 and pH 2, and infrared spectra at pH* 6.4 and pH* 1.7. CD spectrum for the T-form has been measured at pH 7 and 90 °C and that for the U-form in 6 *M* Gu·HCl. Protein concentrations were 0.4 to 0.6 mg/ml (**a**) and 5 to 20 mg/ml (**b**). Pathlengths of the optical cells used were 0.5 mm (**a**) and 0.08 to 0.12 mm (**b**)

made by us using the approach of Provencher and Glöckner (1981) has shown that the α-content in the A-form is ~10% larger than in the N-form, while the β-contents of both forms are the same. On the other hand, Kronman et al. (1966, 1971) have assumed that the differences between the far UV optical properties of the N- and A-forms of Bα-LA are mainly due to the different contributions of aromatic side chains (having different environment in the N- and A-forms – see below) in the far UV region (see also Sears and Beychok 1973).

Therefore we have also studied the difference between the secondary structures of the A- and N-formes of Bα-LA by their infrared spectra, which permits one to isolate the contribution of peptide groups from that of the side groups (Chirgadze et al. 1975). Figure 3b presents infrared spectra of the N- and A-forms in the Amide I region after subtraction of side group contribution according to Chirgadze et al. (1975). One can see that the IR spectra of peptide groups in the Amide I region practically coincide for the A- and N-forms, which is evidence for the absence of significant changes in the secondary structure. Quantitative estimates show that the very small differences between the IR spectra of the N- and A-forms are consistent with the change of α-content, no more than ~10%, and β-content no more than ~5%.

Thus, our data do not confirm the suggestion that the secondary structure contents of N- and A-forms differ substantially though we cannot exclude small changes, especially in the α-helical content.

B. Acid form: "unfolded-like" properties

In contrast to the above mentioned experimental data which demonstrates the "native-like" properties of the A-form of Bα-LA, some other experimental parameters show strongly different behaviour.

I. Environment of side groups. Bα-LA contains four tryptophans, four tyrosines and four phenylalanines in the chain which includes, in total, 123 amino acid residues (Brew et al. 1970). As has been shown earlier (Kronman et al. 1971; Kuwajima et al. 1976), the near UV (aromatic) CD spectrum of Bα-LA in the A-form is drastically reduced in intensity as compared with the N-form, and is similar to the spectrum of the protein unfolded by 6 *M* Gu·HCl (Fig. 4). This observation was one of the main arguments in favour of Kuwajima's model of the unfolded (largely expanded) structure of Bα-LA in the A-form, because low intensity CD spectra in the near UV are typical for unfolded proteins in which aromatic groups have little contact either one with another, or with the other groups of the protein molecule.

In order to check if this is the case for the A-form of Bα-LA, we have applied the technique of tryptophan residue luminescence polarization (see Semisotnov et al. 1981) to study the relaxation properties of these residues (which make a large contribute to the near UV CD spectrum of the native Bα-LA). The average relaxation time, φ, of the movements of tryptophan residues can be determined (Weber 1952) from the linear dependence of

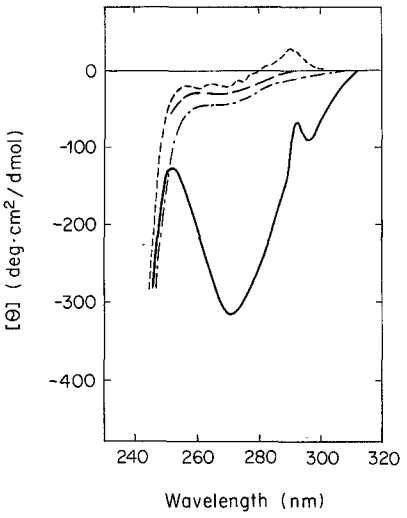

Fig. 4. Near UV CD spectra for Bα-LA in the native (——), acid (– – –), temperature-denatured (– · – · –) and unfolded (– –) forms. The spectra were obtained using a 10-mm path-length at the same conditions as the spectra in Fig. 3 a

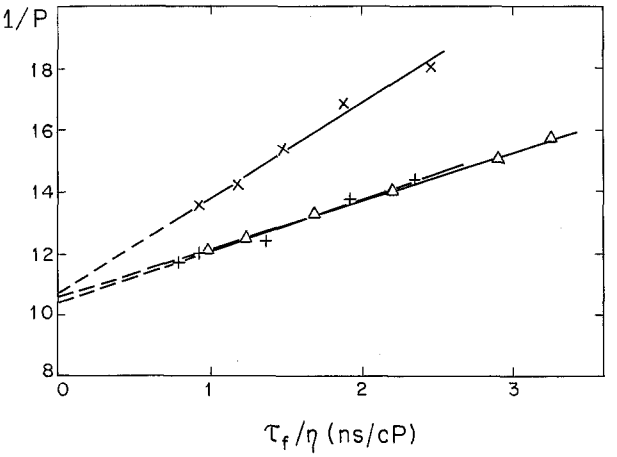

Fig. 5. Dependence of the inverse luminescence polarization, $1/P$, on τ_f/η (τ_f – average life-time of the excited state of luminescence groups, η – solvent viscosity) for Bα-LA in the native (+++), acid (ΔΔΔ) and unfolded by 8 *M* urea (×××) forms at 25 °C. Solvent viscosities, η, have been varied from 0.89 cP (buffer) to ~ 3 cP by adding sucrose up to ~ 30%. The τ_f values do not depend on sucrose concentration and are equal to 2.1, 2.9 and 3.7 ns for the N-, A- and U-forms, respectively. The protein concentration was 0.5 mg/ml. Excitation wavelength was 296 nm, emission wavelength was 350 nm

$1/P$ (P is the polarization of tryptophan luminescence) *versus* τ_f/η (τ_f is the average life-time of the excited state of tryptophan indole rings, η is the solvent viscosity). It should be noted that all processes which do not depend on solvent viscosity (including the energy transfer between different tryptophan residues) do not influence the *slope* of this dependence.

Figure 5 shows the plots of $1/P$ *versus* τ_f/η for the N-, A- and U-forms of Bα-LA. The figure shows that the slopes of the plots for the N- and A-forms are very similar and correspond to apparent average correlation times $\varphi_N = 16$ ns and $\varphi_A = 19$ ns. On the other hand, the slope of the plot for the U-form is twice as great as that for the N- and A-forms and corresponds to $\varphi_U = 8$ ns (all φ values are reduced to the water viscosity at temperature 25 °C).

These results suggest that the average rotational relaxation of *solvent accessible* tryptophans is closely similar in the A-form and in the N-form, but is substantially faster in the U-form.

It follows that the surface tryptophan residues (and even more so, the buried ones) are not free from intramolecular contacts with other groups in the A-form of Bα-LA. These data are in accordance with the results of Kronman and Holmes (1965) who showed that the perturbant accessibility of aromatic side chains in the A- and N-forms of Bα-LA is similar. Therefore the practical absence of the CD spectrum of aromatic side groups in the A-form of Bα-LA cannot be explained by the absence of their intramolecular contacts. Instead, we have to assume that their intramolecular contacts are time-averaged (due to the movements much slower than the rotation of a molecule as a whole) in such a way that these groups have lost their fixed intramolecular environment typical for native proteins.

The absence of the specific native environment of side groups in the A-form of Bα-LA demonstrated by near UV CD spectra for aromatic side groups is confirmed by the comparison of proton NMR spectra of the N- and A-forms (see Fig. 6). The ^{1}H NMR spectrum of native Bα-LA is typical for globular proteins; it contains many resonance lines through the whole spectral region with chemical shifts which are not observed in the individual amino acids or in completely unfolded proteins. However the ^{1}H NMR spectrum of the acid form is much simpler than that of the native protein and resembles much more the typical spectra of amino acids or of completely unfolded proteins. For example, no resonance lines remain in the region between −0.7 and +0.8 ppm. As these lines are most likely due to the shifting of resonances of aliphatic side groups by the ring currents of the neighbouring aromatic groups, we can conclude that the specific mutual positions of aliphatic and aromatic side chains are lost in the A-form. No resonance lines remain in the region between 5.0 and 6.5 ppm (these lines are most likely due to the protons of aromatic rings as well as to some C_α-protons). It follows that many side chains (including aromatic ones) have lost their specific native environment in the A-form.

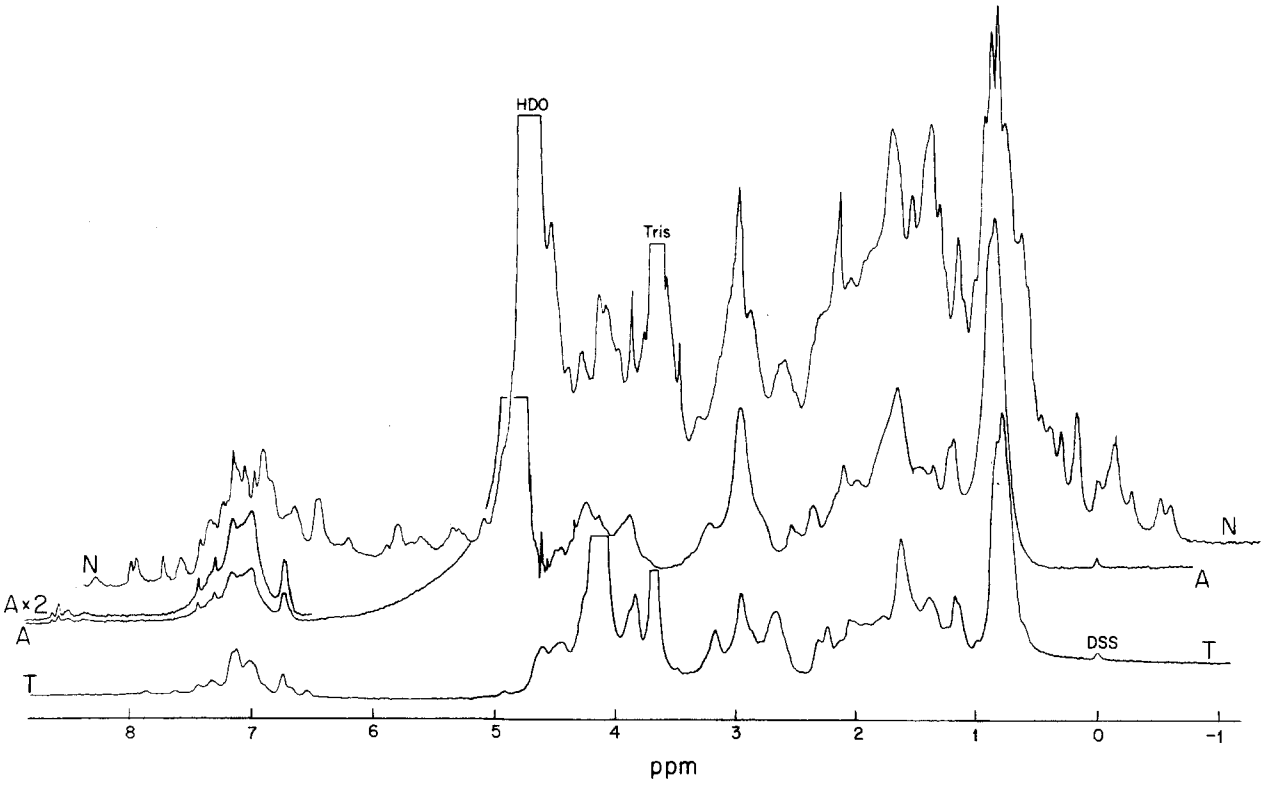

Fig. 6. Proton NMR spectra of the native (N), acid (A) and temperature-denatured (T) forms of Bα-LA. Resonance line at 0 ppm corresponds to sodium 2,2′-dimethylsilapentane-5-sulphonate (DSS) used as the internal standard. Thick lines at 3.7 ppm and between 4.0 and 4.9 ppm correspond to Tris and HDO protons, respectively. Spectra for the N- and A-forms were measured at pH 7 and 2 and 20 °C and for the T-form at pH 7 and 80 °C. Protein concentrations were 3 to 5 mg/ml

On the other hand, some resonance lines remain in the ^{1}H NMR spectrum of the A-form, e.g. several distinct resonances near 8.5 ppm (which are most probably due to C2 imidazole ring protons of histidines). Though one cannot exclude the possibility that these resonances are due to different states of histidine ionization, it may also mean that the A-form of Bα-LA still preserves some traces of the specific environment of histidine side groups.

It should also be noted that the resonance lines in the spectrum of the A-form are usually broader than those in the typical spectra of completely unfolded proteins. This broadening can reflect both the slower movements of side groups in the compact A-form compared to the completely unfolded state and the difference of the environments of side groups.

II. Deuterium exchange. Figure 7 shows the kinetic curves of H−D exchange of Bα-LA at pH* 4.0 (N-form) and pH* 1.9 (A-form). As the exchange rate of the completely exposed NH-groups has the minimum near pH* 2.5, the exchange rate of these groups at pH* 1.9 is about ten times lower than at pH* 4.0 (Englander et al. 1972). This is reflected in the kinetic curves of Bα-LA measured at pH* 1.9 and 4.0 at the same temperature of 10 °C (see Fig. 7). These curves show that on initial stages of the exchange reaction (up to ~ 50 min), influenced mainly by the exchange of exposed NH-groups, the

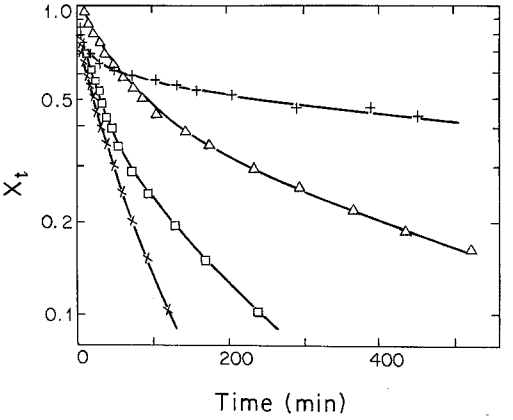

Fig. 7. Time dependence of the unexchanged fraction of NH-groups, X_t, in the course of H−D exchange for the native form of Bα-LA at pH* 4.0 and 10 °C (+++) as well as for the acid form at pH* 1.9 and 10 °C (ΔΔΔ) and 20 °C (□□□). For comparison the exchange curve for oxidized ribonuclease at pH* 1.9 and 10 °C is also presented (×××). Protein concentrations were 15 to 25 mg/ml

exchange rate at pH* 1.9 is really lower than at pH* 4.0. However, at times > 50 min the exchange rate of Bα-LA at pH* 1.9 is much *higher* than at pH* 4.0. The difference between the H−D exchange curves increases with the increase of time. After about seven hours only approximately a half of the protons in the NH-groups of native protein remain unchanged and this amount seems to decrease only slightly with time. On the other hand, in the acid form of the protein more than 80% of protons in these groups are changed and this number seems to decrease rapidly with a further increase of time.

This means that in the acid form the dynamic accessibility of incompletely exposed NH-groups is much higher than in the native one. It should be noted that pH* 4.0 is near the pH of the N → A transition of Bα-LA (Kronman et al. 1965) and the increase of pH* from 4.0 to 6.6 (Takesada et al. 1973) leads to an additional decrease of the dynamic accessibility of peptide groups to the solvent.

It is interesting that the kinetic curve of the H−D exchange at pH* 1.9 is more homogeneous than that at pH* 4.0. This suggests that the division of peptide NH-groups into those easily accessible to the solvent and those partly buried is less pronounced in the A-form than in the N-form.

For comparison, Fig. 7 shows the H−D exchange curve for oxidized ribonuclease at pH* 1.9 and 10 °C. Its structure is characterized as unordered and unfolded and the H−D exchange of its peptide groups is similar to the exchange of peptide groups completely exposed to the solvent (Woodward and Rosenberg 1970; Molday et al. 1972). One can see that at the same pH* (1.9) and temperature (10 °C) the exchange of detectable NH-groups in the A-form of Bα-LA is about ten times slower than that in oxidized ribonuclease.

Thus, according to the H−D exchange study, the A-form of Bα-LA is characterized by an essential common increase of intramolecular mobility as compared with its N-form. At the same time the conformation of the A-form significantly differs from the hydrated coil by the kinetics of H−D exchange.

III. Temperature melting. As has been shown earlier by Pfeil (1981), the N-form of Bα-LA has the usual peak on the partial heat capacity C_p *versus* temperature curve (see Fig. 8). This peak corresponds to ~65 °C and the calorimetric heat of melting (determined from the area under the peak) is equal to the van't Hoff's heat of melting (determined from the width of the transition) which is evidence (Privalov 1976) for the "all-or-none" temperature transition of the native Bα-LA (see also Pfeil 1981). At the same time the A-form has a smooth gentle $C_p(T)$ curve without any indications of a cooperative transition. Moreover, the heat capacity of Bα-LA in the A-form is already anomalously high at room temperature (20 °C), being equal to 0.41 cal/g · degree (1.7 J/g · degree) compared with 0.30 cal/g · degree (1.3 J/g · degree) for the N-form.

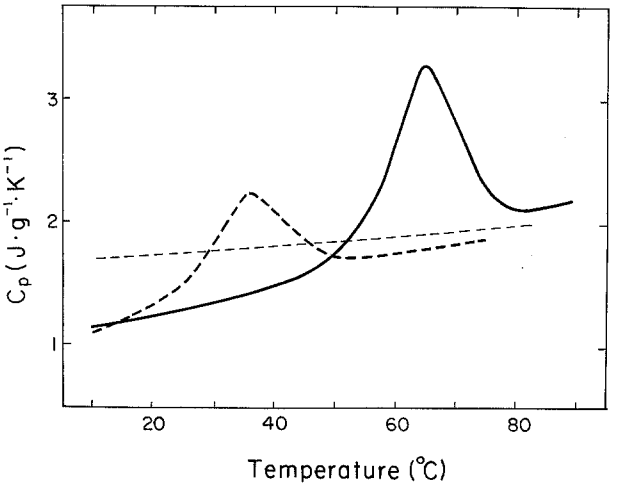

Fig. 8. Dependence of partial heat capacity, C_p, on temperature for Bα-LA in the native (——) and acid (– – –) forms as well as in the calcium-free (– – –) form (in 0.02 *M* Tris · HCl buffer, pH 7.4, 0.05 *M* KCl in the presence of 10 m*M* EDTA; the curves for 3 and 5 m*M* EDTA are similar). Protein concentrations were 1 to 4 mg/ml. The cell volume is 1 ml, the rate of heating was 1 deg/min

C. Temperature-denatured form

The absence of the cooperative temperature melting of the A-form suggests that it is already "melted" at room temperatures, i.e. that the temperature-denatured (T-form) and the A-form of Bα-LA are thermodynamically equivalent (cf. Privalov 1979). The question arises whether these forms are also similar from the structural point of view. To answer this question, we have studied the compactness, the secondary structure and the environment of side groups of the T-form and have compared them with the A-form.

The compactness of the T-form of Bα-LA was evaluated by its intrinsic viscosity. To avoid measuring at a very high temperature (90 °C), which cannot be made accurately enough, we made use of the decrease in thermostability on removing the Ca^{2+} ion with 10 m*M* EDTA (Hiraoka et al. 1980; see below, Sect. D). In the presence of 10 m*M* EDTA the melting process is completed at a temperature as low as 50 °C (see Fig. 8) which allows

relatively accurate measurements of intrinsic viscosity. These measurements have shown that the $[\eta]$ value for thermally denatured Bα-LA at 50 °C is 3.0 ± 0.5 cm^3/g, i.e. it practically coincides with that for the A- (and also N-)form, while for the protein unfolded by 6 *M* Gu·HCl at 50 °C $[\eta]$ is equal to 7 ± 1 cm^3/g. This means that Bα-LA molecules in the T-form are practically as compact as in the N- and A-forms, and much more compact than in the U-form.

The secondary structure of the T-form of Bα-LA was compared with other forms by far UV CD spectra. Figure 3a shows the far UV CD spectrum for the T-form of Bα-LA at pH 7 and 90 °C as compared with the spectra of the N-, A- and U-forms. The CD spectrum at pH 2 and 90 °C (not shown in the figure) is very similar to that for pH 7 and 90 °C. It follows that the far UV CD spectrum of Bα-LA does not change as drastically upon temperature denaturation as it does upon unfolding by 6 *M* Gu·HCl (cf. the data of Takesada et al. 1973 on the temperature dependence of far UV CD spectrum of Bα-LA in D_2O). The far UV CD spectrum of the T-form of Bα-LA is more similar to the A-form than to the N-form, differing from the A-form spectrum only by a rather small decrease in the amplitude. This means that the content of secondary structure of the T-form is only a little smaller than that of the A-form, i.e. it is not destroyed to the same extent as in the U-form.

On the other hand, the environment of side groups in the T-form is very different from that in the N-form and is very similar to their environment in the A-form. First of all, Fig. 4 shows that the near UV CD spectrum of the T-form is similar to that of the A-form (and to that of the U-form) and is drastically reduced in intensity as compared to the spectrum of the N-form. Even stronger evidence for the similarity between the environment of side groups in the A- and T-forms of Bα-LA is given by a comparison of their NMR spectra. These spectra (presented in Fig. 6) are very similar and both show the absence of the many chemical shifts of resonance lines typical for the native protein.

Therefore the temperature-denatured form of Bα-LA is similar to its A-form by its compactness, secondary structure and the environment of the side groups. It is not surprising therefore that heating of the A-form of Bα-LA does not lead to any cooperative changes of the protein structure. However, the most interesting consequence of the structural similarity between the A- and T-forms of Bα-LA is that the state of the protein molecule common for both of these forms derives from the native state by the "all-or-none" transition (see above), i.e. by an intramolecular phase transition of the first-order.

D. The role of calcium in stabilization of the native structure of Bα-LA

It has been shown (Hiraoka et al. 1980) that Bα-LA is a Ca-binding metalloprotein. At neutral pH it strongly binds one Ca^{2+} ion per protein molecule. Kronman et al. (1981) and Permyakov et al. (1981b) have shown that the fluorescence properties of Ca-free Bα-LA at neutral pH and 20–25 °C are similar to those for the A-form and have assumed that the transition to the A-form is due to the loss of the Ca^{2+} ion at acid pH.

We have studied the binding of the Ca^{2+} ion by Bα-LA at pH 2 using radioactive ^{45}Ca and have found that under these conditions Bα-LA really does not bind the Ca^{2+} ion (the radioactivity of the A-form is as small as 6 ± 5 counts/min as compared with 500 ± 20 counts/min for the native protein). On the other hand, we have shown that the removal of Ca^{2+} ion from Bα-LA with EDTA does not significantly alter either the near or far UV CD spectra of Bα-LA at room temperature. The addition of 3, 5 or 10 m*M* EDTA under our conditions (Tris·HCl buffer, pH 7.4, 0.05 *M* KCl) leads only to the shift of the middle point of the temperature transition of Bα-LA from 65° to 37 °C (Fig. 8).

Thus, the removal of the Ca^{2+} ion from Bα-LA, taken by itself, is not sufficient to destroy the native structure of Bα-LA. At acid pH the native structure of Bα-LA is destabilized not only by the absence of the Ca^{2+} ion, but also by the electrostatic repulsion between positively charged groups. Therefore, at pH 2 the native structure of Bα-LA is "melted" even at room temperature, while at neutral pH (in the presence of salt) it is just at the beginning of its melting process (see Fig. 8). Small changes of the experimental conditions (e.g. the removal of salt) can shift the temperature melting region of Ca-free Bα-LA at neutral pH in such a way that it can lose the optical properties of the native protein even at room temperature (Kronman et al. 1981; Permyakov et al. 1981b), retaining them however at lower temperatures (Segawa and Sugai 1983).

4. Discussion

A. Physical nature of "intermediate" state

Previous studies of α-lactalbumins and some other proteins (see Introduction) have shown that these proteins can be transformed into an "intermediate" (or "partially folded") state in which their near UV optical properties are close to those of the unfolded state, while their far UV CD spectra are similar to or even more intense than those of the native state.

In principle, there are two possible explanations for these observations:
(1) The protein molecule completely or at least substantially unfolds without the drastic loss of its secondary structure.
(2) The protein molecule remains compact and has a pronounced secondary structure while its specific tertiary structure is lost either completely or at least locally (near the majority of aromatic side groups).

The first explanation (see e.g. Kuwajima 1977) is excluded by our data which show that the volume of the Bα-LA molecule does not increase in the "intermediate" state (at acid pH or high temperatures) by more than ~30% as compared with the native one. On the other hand, our NMR data show that not only aromatic but also many other side groups lose their specific environment in these forms. This suggests that the "melting" involves regions of specific tertiary structure throughout the molecule. We infer that the majority of side groups weaken their specific intramolecular contacts and possess motion inside the compact protein molecule. Moreover, our data on deuterium exchange show that the "intermediate" state differs from the native one not only by small-scale fluctuations of side groups but also by large-scale fluctuations of the protein molecule.

B. Phase transition between native and "intermediate" states

The drastic difference between the properties of the "intermediate" (or "partially folded") state and the native one is strongly confirmed by the fact that these two states are divided by a temperature phase transition of the first-order (see Sects. 3.B.III and 3.C). It means that the "intermediate" state has a much greater enthalpy and a much greater entropy than the native one ($\Delta H \approx 250\ \mathrm{kJ \cdot mol^{-1}}$ and $\Delta S \approx 700\ \mathrm{J \cdot mol^{-1} \cdot K^{-1}}$, see Pfeil (1981) and Kuwajima and Sugai (1978)) which reflects the large decrease of the intramolecular attraction and the large increase of thermal motions in the "intermediate" state. As the majority of the internal degrees of freedom of the protein molecule are connected with its small-scale fluctuations (e.g. with the fluctuations of side groups) the increase of these fluctuations can lead to a substantial increase of the entropy of a molecule which may make the "intermediate" state thermodynamically favourable under the new conditions (e.g. at high temperatures or low pH).

Earlier theoretical approaches to protein denaturation (Ptitsyn and Eizner 1965; Lifshitz et al. 1979) have treated it as an unfolding of a "liquid" globule without a specific tertiary structure. However it has been shown (Lifshitz et al. 1979) that the unfolding of this globule is usually a phase transition of the second- rather than of the first-order. Therefore, these theories failed to explain the all-or-none denaturation of protein molecules. According to the theory proposed recently by Shakhnovich and Finkelstein (1982), the all-or-none protein denaturation is due to the loss of the tight packing of side groups in the interior of a protein molecule (rather than to its unfolding or to the loss of its secondary structure). As many side groups are connected with a "structural frame" of a protein molecule consisting of α- and/or β-regions, the loss of the tight packing of these groups in each part of a protein or domain interior is possible only simultaneously with a shift (or with the destruction) of this "structural frame" as a whole, thus making this transition an "all-or-none" one.

The loss of the tight packing can take place with a small increase of the molecular volume which is insufficient for penetration of water into the protein interior and therefore cannot lead to the substantial decrease of hydrophobic interactions. Under some denaturating conditions (e.g. at high temperatures or upon a not very large increase of the total charge of the protein molecule) hydrophobic interactions are sufficient to make the compact "intermediate" state more stable than the unfolded one. Under other denaturating conditions (e.g. at high concentrations of strong denaturants or upon a very large increase of the total charge) the "intermediate" state becomes unstable and the protein molecule unfolds. Our experimental data are consistent with this explanation.

C. Other examples of the "intermediate" state

As has been shown in this paper, bovine α-lactalbumin can be transformed from its native state into the "intermediate" one in two quite different ways: by the decrease of pH and by heating. Moreover, it seems likely that similar forms of Bα-LA can be obtained also at intermediate concentrations of Gu·HCl (Kuwajima et al. 1976), at basic pH (Kronman et al. 1967; Kuwajima et al. 1981), in the presence of large amounts of $NaClO_4$ or $LiClO_4$ (Maruyama et al. 1977) and under some conditions upon the removal of Ca^{2+} at neutral pH and moderate temperatures (Hiraoka et al. 1980, Kronman et al. 1981; Permyakov et al. 1981b). We have shown also (Dolgikh et al. 1981) that human α-lactalbumin can be transformed into similar forms at acid pH, in the presence of 2 *M* Gu·HCl or by the removal of Ca^{2+} at neutral pH and moderate temperatures.

Of course, the different "intermediate" forms of α-lactalbumins are not completely identical, how-

ever they belong to the same type of structural and thermodynamic state, and differences between the properties of this state under different conditions are most likely conditioned by its lability.

There is also evidence for the existence of the "intermediate" state in some other proteins, including bovine pancreatic ribonuclease A in the presence of 3.5 *M* $LiClO_4$ at pH 3.0 (Denton et al. 1982) and bovine carbonic anhydrase B at pH 3.6 (Dolgikh et al. 1983; see also Wong and Hamlin 1974). It is important to note that carbonic anhydrase, unlike α-lactalbumin and ribonuclease, has no disulfide bridges which means that the "intermediate" state of globular proteins can exist even without them.

Recently Ohgushi and Wada (1983) have shown that cytochrome c at pH 2.0 in the presence of 0.5 *M* KCl is in a compact state with native-like far UV CD spectrum, but with a reduced near UV CD spectrum and an unfolded-like NMR spectrum in the aromatic region. They have concluded that their results coincide with the model of a "compact state with a fluctuating tertiary structure" proposed in our previous paper (Dolgikh et al. 1981) and have called this state a "molten-globule" one. We have shown however that cytochrome c under these conditions has a cooperative temperature melting and native-like NMR spectrum in the aliphatic region (unpublished) which does not confirm the conclusion that this state of cytochrome c is comparable to the "intermediate" forms described in this paper. Nevertheless, the ability of a number of different proteins to adopt an "intermediate" or "molten-globule" state suggests that this state may be of more general occurrence.

D. Possible role of "intermediate" state in protein folding

It was shown (Kuwajima et al. 1975; Kita et al. 1976; Kuwajima 1977) that the U → A transition in Bα-LA is much faster than the A → N one which suggests (Kuwajima 1977) that the "intermediate" form of Bα-LA may serve as a kinetic intermediate in protein folding. There are also other data which suggest that the protein secondary structure (Schmid and Baldwin 1979; McCoy et al. 1980; Kim and Baldwin 1980; Kato et al. 1981; Dolgikh et al. 1984) and the compactness of protein molecules (Creighton 1980; Creighton and Pain 1980; Schmid and Blaschek 1981; Dolgikh et al. 1984) are restored during their renaturation in vitro faster than their tightly packed tertiary structure (see also the review, Kim and Baldwin 1982). Therefore it is possible to suggest that the rearrangement of the "intermediate" or "molten-globule" state to the native one may be the rate-limiting stage of folding not only of Bα-LA but also of other proteins.

Ptitsyn (1973) postulated three main stages of protein folding in vitro: Stage I, the formation of a fluctuating secondary structure stabilized by hydrogen bonds; Stage II, the collapse of "blocks" of the secondary structure into the intermediate compact state (stabilized by hydrophobic interactions) which is still different from the native state; and Stage III, reconstruction of this intermediate compact state into the native one which is additionally stabilized by specific van-der-Waals and other interactions. It seems possible that the "intermediate" state of Bα-LA which has been described in this paper is just stage II of this model of the protein folding.

It should be noted finally that α-lactalbumins can be transformed from the native to the "intermediate" state by removing one Ca ion from the molecule at neutral pH and moderate temperatures. This shows that at least some proteins can be converted from the native to the "intermediate" state just by removing their ligands. It may permit them to bind their ligands very specifically but not very strongly, as the negative free energy of the ligand binding is partly compensated by the positive free energy of the fixation of their tertiary structure (see Schulz 1979).

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