α-LACTALBUMIN: COMPACT STATE WITH FLUCTUATING TERTIARY STRUCTURE?

D. A. DOLGIKH, R. I. GILMANSHIN, E. V. BRAZHNIKOV, V. E. BYCHKOVA, G. V. SEMISOTNOV, S. Yu. VENYAMINOV and O. B. PTITSYN*

Institute of Protein Research, USSR Academy of Sciences, 142292 Poustchino, Moscow Region, USSR

Received 1 November 1981

1. Introduction

The denaturation of some globular proteins by Gu–HCl shows a non-simultaneous change of different optical properties: the CD spectrum in near UV (aromatic) region changes at smaller concentrations of Gu–HCl than the CD spectrum in far UV (peptide) region [1–4]. This suggests the existence of stable, partly denatured (intermediate) forms of these proteins with a more or less symmetrical environment of aromatic groups but with a more or less native-like secondary structure. For both bovine [2] and human [4] α-lactalbumins as well as for some other proteins [5] a similar state exists also at acid pH. While the partly denatured (P) form at moderate Gu–HCl concentrations usually can be obtained only in mixture with native (N) and/or unfolded (U) forms, the acid (A) forms of these proteins can be studied in pure state. It was shown that for bovine α-LA U → A transition takes <1 ms while A → N transition needs as much as 0.1–1 s which suggests that the intermediate state similar to the A-form can be also a kinetic intermediate in the course of protein folding [6].

Basing on the small time of U → A transition for Bα-LA and on CD spectra of its A-form it has been suggested [6] that this form is an unfolded one but its secondary (helical) structure is even more pronounced than that of the native protein. This paper presents the results of investigation of acid and some similar forms of bovine and human α-lactalbumins which show that these forms are compact, have secondary structure similar to the native one but their tertiary structure can slowly fluctuate. More detailed experimental data and their discussion will be published later.

Abbreviations: Bα-LA, bovine α-lactalbumin; Hα-LA, human α-lactalbumin; Gu–HCl, guanidine hydrochloride; CD, circular dichroism; UV, ultraviolet

Different forms of protein are abbreviated as: N, native form; A, acid form; P, partly denatured by Gu–HCl form; Apo, form with Ca^{2+} removed by EDTA; T, temperature denatured form; U, completely denatured by Gu–HCl (unfolded) form; all forms have intact S–S bonds

* To whom correspondence should be addressed

2. Materials and methods

Bovine and human α-LAs were isolated as in [7,8] with small modifications. The homogeneity of the samples were proven by disc-electrophoresis in polyacrylamide gels with and without SDS. Protein concentration was determined by extinction coefficients $E^{1\%}_{1\,cm,\,280\,nm}$ equal to 20.9 for Bα-LA and 18.2 for Hα-LA. Native forms of proteins were studied in Tris–HCl buffer (pH 7.0–7.4) and acid forms in KCl–HCl buffer, (pH 2.0).

Intrinsic viscosity has been measured by capillary viscometer Viscomatic, CD spectra by dichrograph J-41A using standard procedures. Descriptions of other methods are given in the following papers: diffuse X-ray scattering [9], IR spectra [10,11], polarized luminescence [12], deuterium exchange [13] and microcalorimetry [14].

3. Results

Using an 'unfolded-like' CD spectrum in the aromatic region and a 'native-like' CD spectrum in the peptide region as an empirical test for intermediate forms of α-lactalbumins we have shown that these forms can be obtained not only at moderate Gu-HCl concentrations (P-form) or at acid pH (A-form) but

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.75

also by temperature denaturation of these proteins (T-form) and by removing Ca^{2+} from human α-LA at room temperature and neutral pH (Apo-form). The conditions at which these forms can be obtained are shown in table 1. All these forms have qualitatively similar properties which will be described below.

3.1. *'Native-like' properties*

3.1.1. Compactness

The values of intrinsic viscosities [η] of A-, Apo- and T-forms of both bovine and human α-LAs shown in table 1 are typical for globular proteins (3–4 cm^3/g) and very near to the values for native α-LAs (3.1–3.4 cm^3/g). On the other hand, the [η] values for unfolded forms of both bovine and human α-LAs are equal to 6.1–6.6 cm^3/g. It follows that all these intermediate forms are nearly as compact as the native one (the maximal possible increase of the molecular volume does not exceed 25%). It was impossible to estimate the compactness of the P-form by measuring its [η] (due to aggregation at >1 mg/ml) but the data on the polarized luminescence (see below) give evidence of the compactness of this form.

The same results have been obtained for the A-form of Bα-LA also by diffuse X-ray scattering. Gyration radii of the N- and A-forms obtained from Guinier plots are very near to each other (~15.5 Å) and typical for globular proteins.

Similar results can be obtained from the polarization of intrinsic luminescence of Trp residues to both proteins. The mean values of relaxation times τ of tryptophan movements presented in table 1 show that τ for the Apo- and P-forms of both proteins is near to τ of their N-forms (~20 ns) and for the A-form of human α-LA it is even greater (33 ns). At the same time τ values for unfolded forms of both proteins are much smaller (8–9 ns). This confirms that intermediate forms of α-LAs certainly are not unfolded and the intramolecular movements (in the interval of 10^0–10^2 ns which can be registered by polarized luminescence) are not less restricted than in the N-form.

3.1.2 Secondary structure

CD spectra in far UV (peptide) region of all studied intermediate forms of both bovine and human proteins shown in fig.1 are qualitatively similar to the CD spectra for the A-form of Bα-LA in [2]. They are typically even more pronounced than CD spectra of the native forms however the difference spectra cannot be interpreted in terms of change of α-or β-content in these proteins. Amide I bands of infrared spectra of the A- and N-forms of Bα-LA are similar which confirms the suggestion that the secondary structure remains mainly unaltered. Some peculiarities of the difference CD and infrared spectra allow us to suggest that conformational changes may involve mainly irregular chain regions.

Table 1
Physical properties of different forms of bovine and human α-lactalbumins

Form	Conditions	Sample	Native-like CD spectra		[η] (cm^3/g)	τ_{Trp} (ns)	Cooperative temperature-transition
			Near UV	Far UV			
Native (N)	pH ~ 7, 20°C	Bovine	+	+	3.4	16	+
		Human	+	+	3.1	20	+
Acidic (A)	pH ~ 2, 20°C	Bovine	–	+	3.1	19	–
		Human	–	+	4.2	33	–
Partly denatured (P)	~2 M Gu–HCl, pH ~ 7, 20°C	Human	–	+		22	–
Temperature denatured (T)	pH ~ 7, 90°C	Bovine	–	+	3.0[a]		
Apo	pH ~ 7, 20°C, 10 mM EDTA	Human	–	+	3.6	17	–
Unfolded (U)	~6 M Gu–HCl, pH ~ 7, 20°C	Bovine	–	–	6.1	8[b]	
		Human	–	–	6.6	9	

[a] Measured at 50°C in the presence of 10 mM EDTA which shifts the temperature transition from 65–37°C
[b] Measured in 8 M urea

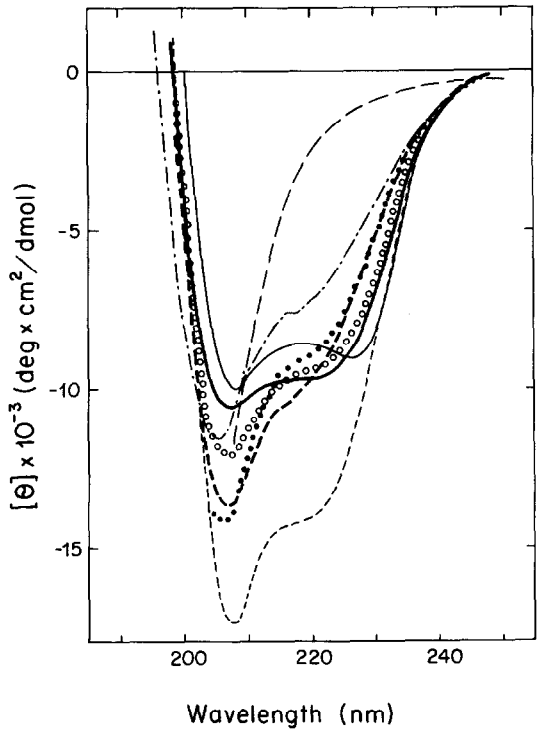

Fig. 1. CD spectra in far UV region: bovine α-LA, (——) N-form; (– – –) A- form; (–·–) T-form; human α-LA (——) N-form; (– – –) A-form; (...) P-form; (ooo) Apo-form; (– – –) U-form.

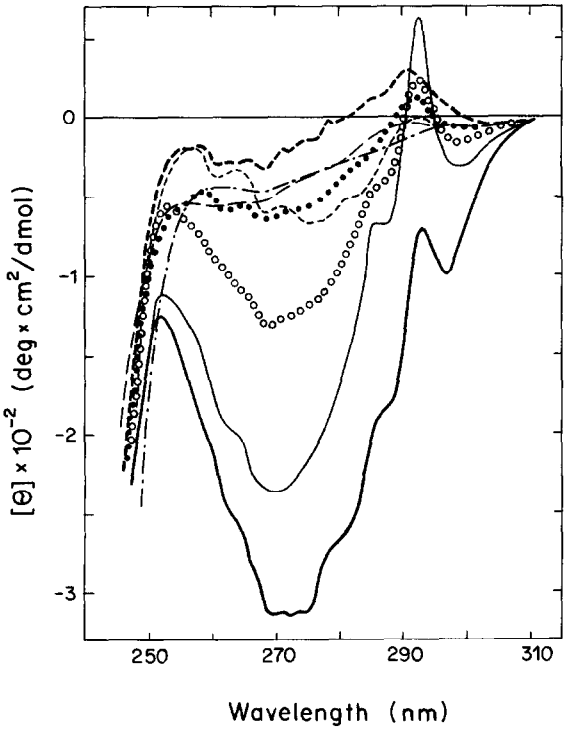

Fig.2. CD spectra in near UV region; for notation see fig.1.

3.2. *'Unfolded-like' properties*

3.2.1. Symmetrical environment of aromatic groups

CD spectra in near UV (aromatic) region of all intermediate forms of both proteins shown in fig.2 are very similar to CD spectra of their unfolded forms (the only exception is the CD spectrum for the Apo-form of Hα-LA which is between the N- and U-forms). This means that the environment of aromatic groups in all intermediate forms is much more symmetrical than in the native ones.

3.2.2. Absence of temperature transition

Native forms of both proteins have typical 'all-or-none' temperature transitions detected by microcalorimetry technique [15]. The removing of Ca^{2+} from Bα-LA shifts this transition from ~65–37°C. However, the A-form of both proteins as well as the P- and Apo-forms of Hα-LA have no cooperative temperature transitions: heat capacity smoothly increases with the temperature and its value at 20°C (0.4 cal/g . deg) is between the typical values for globular (0.3) and temperature denatured (0.5) proteins.

3.2.3. Deuterium exchange

The deuterium exchange study of Bα-LA in both N- and A-forms shows that the exchange is much faster in acid form than in the native one. In fact, the exchange rate in the A-form is between those in the N-form and in oxidized ribonuclease which is commonly used as model of an unfolded protein chain.

4. Discussion

It follows from the above that intermediate forms of α-LAs have a rather unusual mixture of 'native-like' and 'unfolded-like' properties. They are nearly as compact as native proteins and have a similar secondary structure. However, they have a symmetrical environment of aromatic groups, no cooperative temperature transition and fast deuterium exchange.

We have only one explanation of these unusual combinations of physical properties. The practical absence of CD spectra in near UV region could be

explained by the rapid rotation of the aromatic groups (for example due to exposure to water). This is certainly not the case for the intermediate forms of α-LAs as is evidenced by the polarized luminescence of tryptophan residues (see above). However the data on the polarized luminescence does not contradict the assumption on the existence of slow intramolecular fluctuations in $>10^2$ ns. These fluctuations which cannot be registered by polarized luminescence can lead to the time-averaged symmetrization of environment of aromatic groups and, as a result, to the practical absence of their CD spectra. The fast deuterium exchange in the A-form of Bα-LA confirms this assumption. It follows that all properties of the A-, P-, Apo- and T-forms of α-LAs are consistent with the model of a

compact globule with native-like secondary structure and with slowly fluctuating tertiary structure.

Our data cannot answer the question on the scale of these slow fluctuations. They can involve only slow rotation of side chain groups or also rearrangements of irregular loops of a protein molecule or even mutual movements of secondary structure regions. An interesting point is that this intermediate state can be obtained also by temperature denaturation of α-LA and at least in this case it is separated from the native state by the 'all-or-none' transition. This means that the entropy of these intermediate forms is substantially greater and their energy is substantially smaller than that for the native state. The fact that the temperature-denatured form of these proteins is similar to their acid, partly denatured and apo-forms explains the absence of cooperative transitions between these forms.

The existence of an 'intermediate' compact state of globular proteins with slowly fluctuating tertiary structure can be important both for their folding and for their function. There is evidence that the rate-limiting intermediates of protein folding lie at the end rather than at the beginning of the folding process and are much more similar to the native form than to the unfolded one [16–19]. Moreover, it has been shown for bovine carbonic anhydrase that kinetic intermediates observed at the early stages in the course of renaturation of the protein from the completely unfolded form by Gu–HCl revealed a native-like CD spectrum in the far UV and unfolded-like CD spectrum in the near UV [20]. On the other hand, the polarized luminescence of Trp residues reaches a maximum during the early stage of the folding process [21]. It is possible that these kinetic intermediates are similar to the thermodynamically stable forms described above for α-LA.

Moreover, proteins forming native nucleoproteins with DNA or RNA may have a partly disordered structure in their free state and the corresponding entropy increase contributes to the equilibrium of the association–dissociation reactions [22]. The possibility cannot be excluded therefore that some DNA- and RNA-binding proteins in their free state may have structures similar to the above.

Acknowledgements

We thank I. A. Bolotina, Yu. N. Chirgadze, A. V. Finkelstein and E. I. Shakhnovich for fruitful discussions of the results. We acknowledge L. V. Abuturov and Yu. O. Lebedev for carrying out the deuterium exchange experiments and discussion. We thank E. I. Tiktopulo for providing the experimental data on microcalorimetry and discussion. We thank also I. G. Ptitsyna for help with the CD measurements.

References

[1] Wong, K.-P. and Tanford, C. (1973) J. Biol. Chem. 248, 8518–8523.
[2] Kuwajima, K., Nitta, K., Yoneyama, M. and Sugai, S. (1976) J. Mol. Biol. 106, 359–373.
[3] Ananthanarayanan, V. S. and Ahmad, F. (1977) Can. J. Biochem. 55, 239–243.
[4] Nozaka, M., Kuwajima, K., Nitta, K. and Sugai, S. (1978) Biochemistry 17, 3753–3758.
[5] Wong, K.-P. and Hamlin, L. M. (1974) Biochemistry 13, 2678–2683.
[6] Kuwajima, K. (1977) J. Mol. Biol. 114, 241–258.
[7] Armstrong, J. McD., Hopper, K. E., McKenzie, H. A. and Murphy, W. H. (1970) Biochim. Biophys. Acta 214, 419–426.
[8] Phillips, N. I. and Jennes, R. (1971) Biochim. Biophys. Acta 229, 407–410.
[9] Serdyuk, I. N. and Grenader, A. K. (1975) FEBS Lett. 59, 133–136.
[10] Chirgadze, Yu. N., Shestopalov, B. V. and Venyaminov, S. Yu. (1973) Biopolymers 12, 1337–1351.
[11] Chirgadze, Yu. N. and Brazhnikov, E. V. (1974) Biopolymers 13, 1701–1712.
[12] Semisotnov, G. V., Anufrieva, E. V., Zikherman, K. Kh., Kasatkin, S. B. and Ptitsyn, O. B. (1981) Biopolymers, in press.

[13] Abaturov, L. V., Yakobashvily, N. N., Jinoria, K. Sh., Molchanova, T. P. and Varshavsky, Ya. M. (1976) FEBS Lett. 70, 127–130.
[14] Privalov, P. L., Plotnikov, V. V. and Filimonov, V. V. (1975) J. Chem. Thermodynam. 7, 41–47.
[15] Privalov, P. L. (1976) Pure Appl. Chem. 47, 293–304.
[16] Cook, K. H., Schmid, F. X. and Baldwin, R. L. (1979) Proc. Natl. Acad. Sci. USA 76, 6157–6161.
[17] Creighton, T. E. (1980) J. Mol. Biol. 137, 61–80.
[18] Schmid, F. X. (1981) Eur. J. Biochem. 114, 105–109.
[19] Kato, S., Okamura, M., Shimamoto, N. and Utiyama, H. (1981) Biochemistry 20, 1080–1085.
[20] McCoy, L. F., Rowe, E. S. and Wong, K.-P. (1980) Biochemistry 19, 4738–4743.
[21] Stein, P. J. and Henkens, R. W. (1978) J. Biol. Chem. 253, 8016–8018.
[22] Schulz, G. E. (1979) in: Molecular Mechanisms of Biological Recognition (Katchalsky, E. ed) Elsevier/North-Holland, Amsterdam, New York.