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Solvent and thermal denaturation of the acidic compact state of apomyoglobin

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Abstract

The stability of the acidic compact state of apomyoglobin toward the denaturant action of guanidinium hydrochloride and temperature was studied by examining the effects induced on the intrinsic tryptophanyl fluorescence and that of the adduct formed with 1,8-anilinonaphthalenesulfonate (ANS). The results indicated that the disorganization of tryptophanyl environments is caused by a cooperative discrete molecular transition, thus contrasting the assumption that the acidic compact form of apomyoglobin might be a molten globule state. The unfolding of the ANS binding regions was found to involve, at least, two stages over a wide range of denaturant concentrations.

Key words: Apomyoglobin; Molten globule state; Acidic compact state; Folding intermediates

1. Introduction

Proteins fold spontaneously into their native conformations. During the folding transition, especially in the case of small globular proteins, only two states are significantly populated, i.e. the folded state and the fully unfolded one. Nevertheless, several reports have pointed out the existence of partly folded intermediates during the unfolding pathway of many proteins. The structure of these intermediates often shares most of the properties attributable to the so-called 'molten globule state', a protein state characterized by compact globularity with native-like secondary structure but unfolded tertiary structure [1–4]. Much experimental evidence so far reported has shown that the compact denaturated conformation that proteins adopt under specific experimental conditions, i.e. strongly acidic or alkaline salt containing solutions can be regarded as a molten globule state [5–9]. The denatured compact states are currently object of intensive studies because of their possible implication in the folding process of several proteins [1,2,10].

Despite the relatively small molecular size of myoglobin, the guanidine- and acid-induced unfolding of this protein is not a true two-state transition [11–16]. The structural characteristics of the major partly folded intermediate, detected in the unfolding pathway of this protein, have been also described [17,18]. The main features were the presence of a certain amount of native like secondary structure and a fluctuating, although compact, tertiary structure [15–17,19]. Similar characteristics were also reported for the acidic compact form of apomyoglobin [5,7–9]. These findings led to the conclusion that the conformational state of the partly folded intermediate as well as that of the acidic compact form could be a 'molten state' [20].

The structural and dynamic properties of the salt-induced acidic compact state of apomyoglobin have been recently investigated [20,21]. This was done by analyzing the emission and anisotropy decay of the intrinsic tryptophanyl residues as well as that of the extrinsic fluorophore 1,8-anilinonaphthalenesulfonate (ANS). The binding of ANS to the acidic compact state determined the appearance of the characteristic fluorophore emission although with properties largely different from those detected upon binding to native apomyoglobin [20]. The results indicated that the compact state of apomyoglobin is an asymmetric globular but very flexible structure having an high solvent accessibility, at least for that concerning the fluorophore containing molecular regions.

In this report, we analyze the stability of the acidic compact state of apomyoglobin toward the denaturant action of guanidinum hydrochloride and temperature. The results show that the molecular region containing the tryptophanyl residues unfolds in a rather cooperative manner in contrast with the assumption that the acidic compact form of apomyoglobin might be a true molten

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Abbreviations: ANS, 1,8-anilinonaphthalenesulfonate; Gdn-HCl, guanidinum hydrochloride.

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globule state. In fact, in this case, no fixed tertiary interaction should be present with a consequent lack of cooperativity. By contrast, the unfolding of ANS binding region seems to be due to a progressive diffusion of denaturant into the protein matrix.

2. Materials and methods

2.1. Myoglobin

Horse myoglobin was purchased from Sigma; the protein was used after a run on Sephadex G-50 (2.5 × 100 cm) equilibrated with 0.05 M phosphate buffer, pH 7.0. The homogeneity of the preparations was controlled by sodium dodecyl sulfate gel electrophoresis with 15% gels and 5% stacking gels [22].

2.2. Apomyoglobin

The heme was removed from myoglobin by the 2-butanone extraction procedure of Teale [23]. The contamination of the apoprotein by myoglobin was assessed spectrophotometrically. In all cases no significant absorption was observed in the Soret region.

2.3. Protein concentration

The concentration of apomyoglobin was determined by absorbance at 280 nm on a Perkin-Elmer Lambda Array 3840 spectrophotometer. The molar excitation at 280 nm was calculated from the tryptophan and tyrosine content [24] by using molar excitation coefficients of 5500 and 1250, respectively [25].

2.4. Chemical and solutions

All common chemicals were reagent grade and were purchased from British Drug Houses. Ultrapure Gdn-HCl[2] and urea were obtained from Schwarz/Mann. ANS was a product of Merck Co., and its Mg^{2+} salt was recrystallized twice with the method described by Weber and Young [26]; ANS concentration was determined spectrophotometrically using the following extinction coefficient: $5 \times 10^3\ M^{-1} \cdot cm^{-1}$.

2.5. Fluorescence measurements

Fluorescence and polarization measurements were performed on a Perkin-Elmer MPF-66 fluorescence spectrophotometer. The polarization was calculated from $P = (I_{VV} - G \cdot I_{VH})/(I_{VV} + G \cdot I_{VH})$ where $G = I_{HV}/I_{HH}$, I is the intensity, and the first and the second subscripts refer to the plane of polarization of the excitation and emission beams, i.e. V, vertical, and H, horizontal. In denaturation experiments, the spectral measurements were monitored through time until an apparent equilibrium was reached. Temperature was maintained constant at 20°C using an external bath circulator.

3. Results

At pH 2.0, in the presence of 0.15 M sodium chloride, the polypeptide chain of apomyoglobin is known to adopt a compact denatured conformation, called acidic compact state, which is characterized by the presence of a certain amount of secondary structure and a rather fluctuating tertiary organization [5,7–9].

It has been recently reported that ANS is able to bind the acidic compact state of apomyoglobin [20]; however, its emission properties largely differ from those detected when bound to the native protein. In fact, not only the fluorescence emission maximum shifts from 462 (native apomyoglobin) to 480 nm (acidic compact state) but also the fluorescence lifetime decreases from 13.6 to 7.4 ns [20,21]. The large red shift of the emission maximum could reflect either an increased accessibility of water molecule to the bound fluorophore due to a more open conformation or a major propensity of protein dipoles to fluctuate around the ANS excited state. The increase of protein flexibility is also consistent with the lack of dichroic activity observed for the ANS-apomyoglobin adduct in the longest ANS absorbing wavelength region [20]. Finally, it may not be excluded that the ANS binding region of the compact state is not structurally corresponding to the heme binding site of native apomyoglobin.

Fig. 1 shows the guanidine-induced denaturation pat-

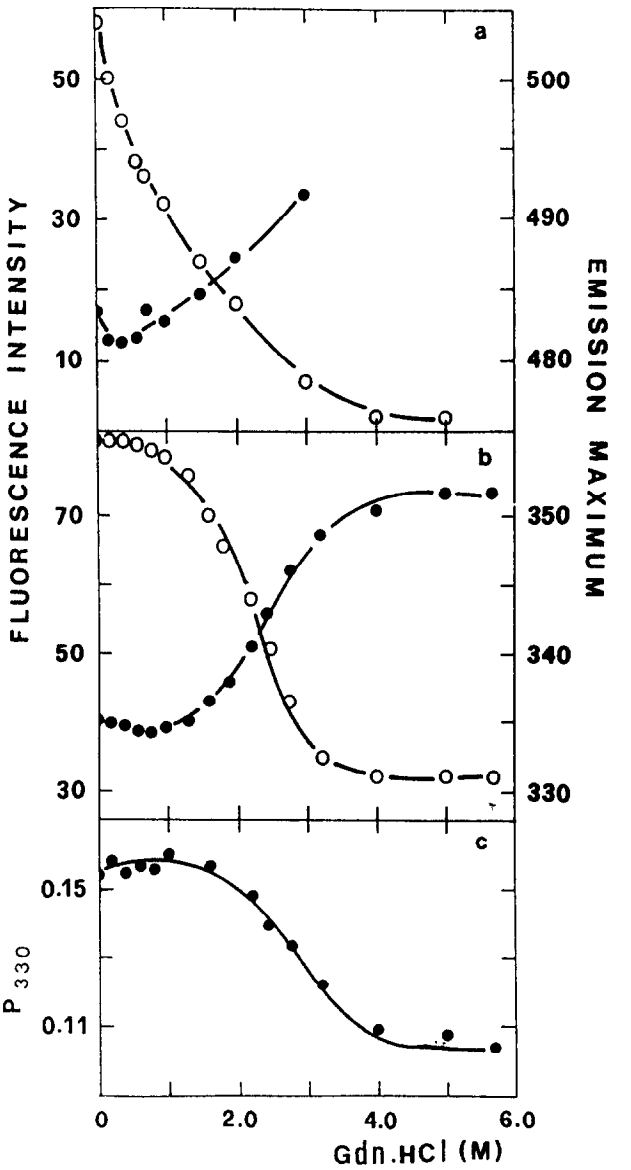

Fig. 1. The effect of increasing Gdn-HCl concentration on: (a) ANS fluorescence maximum (●—●) and intensity at 480 nm (○—○); (b) tryptophanyl fluorescence maximum (●—●) and intensity at 330 nm (○—○); (c) tryptophanyl fluorescence polarization at 330 nm of the acidic compact state of apomyoglobin at 20°C. Protein solutions contained 0.01 M sodium phosphate, 0.005 M sodium acetate, and 0.15 M sodium chloride pH 2.0. ANS/apomyoglobin molar ratio = 0.5. Protein concentrations were: (a) 4.0×10^{-5} M; (b) 0.88×10^{-5} M; (c) 0.88×10^{-5} M. Excitations were 295 nm for tryptophan and 350 nm for ANS.

tern of the acidic compact state of apomyoglobin obtained following the fluorescence changes of the two distinct spectroscopic probes, i.e. tryptophanyl residues and ANS. In the absence of denaturant, the tryptophanyl fluorescence emission of salt-refolded acidic apomyoglobin is centered at 334–335 nm, thus indicating that the environment of the two tryptophanyl residues, i.e. residues A5 and A12, is mainly hydrophobic. Moreover, the high value of tryptophanyl fluorescence polarization, i.e. 0.155, suggests that the indole residues are embedded into a structural environment possessing a large amount of organized structure. The increase of guanidine concentration produces a sigmoidal variation of emission intensity and maximum of tryptophanyl fluorescence, the midpoint of which is at about 2.4 M. The denaturation curve, obtained following the tryptophanyl fluorescence polarization, confirmed that the molecular transition responsible for the disorganization of tryptophanyl microenvironments occurs in the range of guanidine concentration between 1.0 and 4.0 M.

The denaturation curves, obtained following the fluorescence emission of ANS bound to the acidic compact form of apomyoglobin, do not overlap those observed for the intrinsic fluorophore. This suggests that the unfolding of the acidic compact state is not a two-state transition. More precisely, the decrease of ANS fluorescence is not throughly concomitant with the loss of structure in the N-terminal region of the molecule, where both tryptophans are located. In fact, the variations of tryptophanyl fluorescence intensity and emission maximum start at 1.0 M guanidine, a denaturant concentration at which about 50% of the initial fluorescence intensity of the extrinsic fluorophore is lost.

The denaturation curves showing the dependence of tryptophanyl fluorescence on guanidine concentration were analyzed in order to obtain the free energy of unfolding of the acidic compact state. At each concentration of denaturant, the free energy of unfolding, ΔG, was calculated using the following equation:

$$\Delta G = -RT \ln[(x - x_i)/(x_i - x_u)]$$

where x_i is the numerical value of the structure-sensitive parameter at the ith denaturant concentration; x and x_u are the numerical values of the same parameter relative to the initial and final states, respectively. Fig. 2 shows the dependence of the free energy of unfolding of the acidic compact apomyoglobin on guanidine concentration in comparison with that of apomyoglobin in its native form, i.e. at pH 8.0. For the native form, the ΔG values were calculated from the denaturation curves, reported by Irace et al. [17], obtained following the same spectroscopic parameters. For both the salt-refolded acidic and native apomyoglobin, the dependence of ΔG on guanidine concentration was found linear. The simplest method of estimating the conformational stability

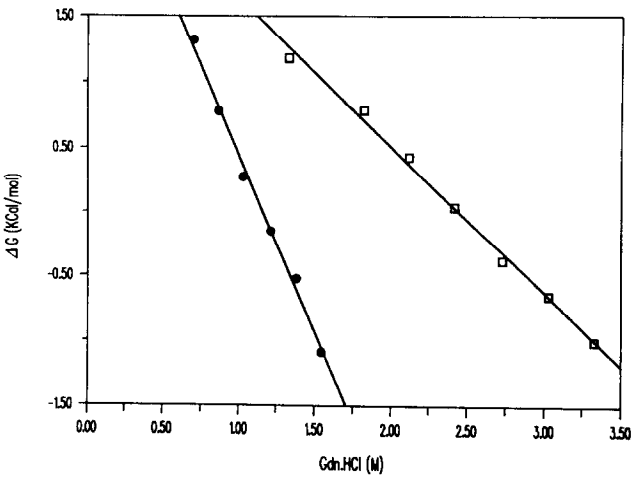

Fig. 2. The dependence of the free energy of unfolding, ΔG, on guanidinium hydrochloride concentration at 20°C; (□), salt-refolded acidic apomyoglobin; (●), folded apomyoglobin at pH 8.0. The data were calculated from the denaturation curves obtained following the variations of tryptophanyl fluorescence. Solid lines were obtained by least squares analysis according to the equation:

$$\Delta G(H_2O) = \Delta G - m(\text{Gdn-HCl})$$

in the absence of denaturant, $\Delta G(H_2O)$, is to assume that this linear dependence continues to zero concentration and to use a least-squares analysis to fit the data to the following equation:

$$\Delta G = \Delta G(H_2O) - m[\text{Gdn-HCl}]$$

where m is a measure of the dependence of ΔG on denaturant concentration and hence, an evaluation of the denaturation cooperativity. The $\Delta G(H_2O)$ at 20°C and m values were 3.16 kcal/mol and -2.73 M^{-1} for the native protein and 2.76 kcal/mol and -1.13 M^{-1} for the salt-refolded acidic compact form.

The effect of increasing temperature on the position of tryptophanyl emission maximum of salt-refolded acidic apomyoglobin is shown in Table 1 in comparison with that observed for apomyoglobin at neutral pH. No change is detected for native apomyoglobin between 15 and 50°C. This is consistent with the observation that the thermal unfolding at neutral pH begins above 60°C [27]. The behaviour of the acidic compact form is quite different since the tryptophanyl emission starts to shift at 40°C, thus revealing a major susceptibility toward thermal denaturation.

Fig. 3 shows the thermal dependence of the fluorescence emission maximum of ANS bound to native and acidic compact form of apomyoglobin as well as in alcohol solution, i.e. ethanol and *n*-butanol. In the latter solutions, the emission maximum of ANS remains practically constant between 15 and 60°C. In the same range of temperature, the emission maximum of ANS bound to apomyoglobin at neutral pH undergoes a very small

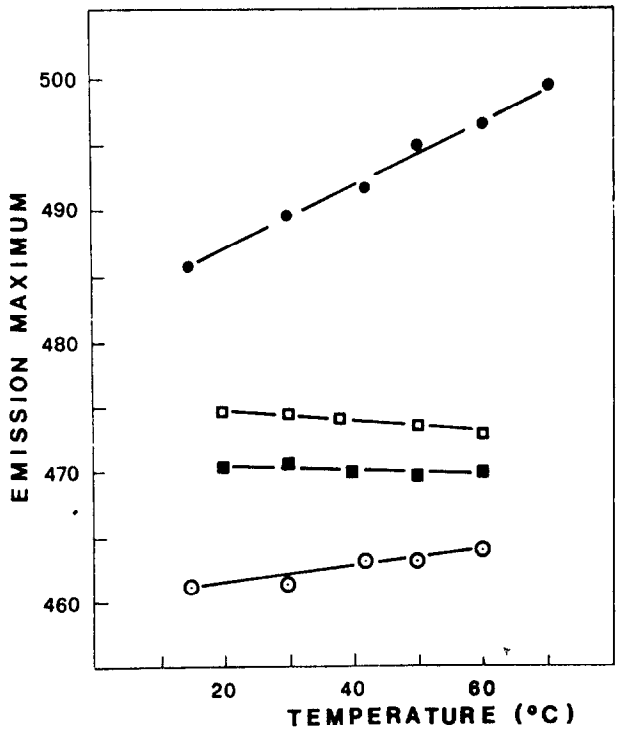

Fig. 3. Thermal dependence of the fluorescence emission maximum of ANS bound to the salt-refolded acidic apomyoglobin (●), folded apomyoglobin at pH 7.0 (○), and in alcohol solution: ethanol (□), *n*-butanol (■). ANS/apomyoglobin molar ratio = 0.5, apomyoglobin concentration = 4.0×10^{-5} M in 0.01 M sodium phosphate, 0.005 M sodium acetate, and 0.2 M sodium chloride. Excitation was at 350 nm.

shift from 462 to 465 nm, probably because of the increased motion of dipolar groups around the excited state of the fluorophore. Since the fluorescence emission of ANS in aqueous solution is centered at 510–520 nm, the results shown in Fig. 3 relative to apomyoglobin at neutral pH indicates that the structure of the ANS binding site is retained up to 60°C although the internal motion might be much increased. The behaviour of the acidic compact state is largely different. In this case, the emission maximum of the extrinsic fluorophore shifts linearly from 482 to about 500 nm between 15 and 70°C.

4. Discussion

The data reported in this paper indicate that the unfolding of the acidic compact state of apomyoglobin is a very complex phenomenon, which cannot be considered either a two-state process or a gradual melting. We have already shown that no substantial difference exists between the acidic compact state of the holo- and apoprotein [20]; therefore, the results obtained on apomyoglobin can be reasonably extrapolated to myoglobin.

The denaturation curves of salt-refolded acidic compact form of apomyoglobin, obtained following the loss of ANS fluorescence on increasing the denaturant concentration, shows that the decrease of ANS fluorescence occurs at least in two stages. Between 0 and 1.0 M, the decrease of ANS fluorescence, i.e. about 50%, is not associated with variation of the emission maximum, which remains centered around 482 nm. At a denaturant concentration higher than 1.0 M, both the emission maximum and the fluorescence intensity change. This behaviour does not seem to be consistent with a cooperative unfolding of the protein segments forming the ANS binding site in the acidic compact form. This can be deducted comparing the denaturation pattern of the acidic compact state with that of native apomyoglobin, the ANS binding site of which unfolds cooperatively [11,12,17,28]. A similar conclusion can be drawn from the data concerning the thermal dependence of ANS fluorescence. In fact, the large linear thermal dependence observed for the emission maximum of ANS bound to the acidic compact state, is consistent with a gradually enhanced solvent penetration into the protein core.

The denaturation curves obtained following the position of the emission maximum and the degree of fluorescence polarization of the intrinsic tryptophanyl residues do not overlap the curves observed for the extrinsic fluorophore. The disorganization of tryptophanyl microenvironments seems not to be gradual and the experimental data are consistent with the occurrence of a molecular transition. The thermodynamic analysis of the denaturation curves revealed that the salt induced acidic compact state of apomyoglobin is less stable and cooperative than native protein. However, the $\Delta G(H_2O)$ values must be considered with caution since they do not reflect the overall protein unfolding but only the disorganization of tryptophanyl environment. In fact, even in the case of the apomyoglobin at neutral pH, the unfolding of this region is produced by an independent transition well separated from the disorganization of other protein regions [11,17,28].

It has been suggested that the salt-refolded acidic state of apomyoglobin consists of a compact structured subdomain, formed namely by A, G, and H helices, while

Table 1
The effect of increasing temperature on tryptophanyl fluorescence maximum of native and salt-refolded acidic apomyoglobin

Temperature (°C)	Apomyoglobin	
	pH 7.0	pH 2.0
15	334	336
30	334	336
40	334	338
50	334	339
58	335	340

Protein concentration was 7.4×10^{-6} M in 0.01 M sodium phosphate, 0.005 M sodium acetate, and 0.15 M sodium chloride. Excitation was at 295 nm.

the remainder of the protein is esentially unfolded [14,19]. In this respect, the ANS binding site in the acidic compact state could be formed by the contact surfaces of these two regions. This would explain most of the observed features, i.e. the gradual disorganization of the ANS binding site and the occurrence of a molecular transition determining the unfolding of the structured subdomain where both tryptophans are located. In conclusion, the acidic compact state of apomyoglobin can be regarded as a partly folded state which mimes some of the properties of the 'molten globule' state.

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