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Compactness of protein molten globules: temperature-induced structural changes of the apomyoglobin folding intermediate

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Abstract. Apomyoglobin undergoes a two-step unfolding transition when the pH is lowered from 6 to 2. The partly folded intermediate (I) state at pH 4 and low ionic strength has properties of a molten globule. We have studied structural features of this state, its compactness, content of secondary structure, and specific packing of aromatic side chains, using dynamic light scattering, and small-angle X-ray scattering and far- and near-ultraviolet circular dichroism spectroscopy. Particular attention was paid to temperature-dependent structural changes. The results are discussed with reference to the native-like (N) state and the highly unfolded (U) state. It turned out that the I-state is most compact near 30 °C, having a Stokes radius 20% larger and a radius of gyration 30% larger than those of the N-state. Both cooling and heating relative to 30 °C led to an expansion of the molecule, but the structural changes at low and high temperatures were of a different kind. At temperatures above 40 °C non co-operative melting of structural elements was observed, while the secondary structure was essentially retained on cooling. The results are discussed in context with theoretical predictions of the compactness and the stability of apomyoglobin by Alonso et al. [Alonso, D. O. V., Dill, K. A., and Stigter, D. (1991) Biopolymers 31:1631–1649]. Comparing the I-state of apomyoglobin with the molten globules of α-lactalbumin and cytochrome c, we found that the compactness of the molten globule states of the three proteins decreases in the order α-lactalbumin > apocytochrome c > apomyoglobin. While α-lactalbumin and cytochrome c are rather homogeneously expanded, apomyoglobin exhibits a non uniform expansion, since two structural domains could clearly be detected by small-angle X-ray scattering.

Key words: Protein folding – Molecular dimensions – Small-angle X-ray scattering – Dynamic light scattering – Circular dichroism

Introduction

Studies of partly folded intermediate states of proteins are essential to the understanding of the mechanism and the main forces of protein folding. In general, stable intermediate states are only weakly populated in the transition from a highly unfolded to the unique native conformation of the polypeptide chain. This phenomenon is caused by co-operative interactions (Privalov 1979; Murphy and Freire 1992; Murphy et al. 1992). Accordingly, those proteins that are of particular interest are those that exhibit substantial populations of partly folded intermediates under certain conditions, such as low pH or moderate concentrations of denaturants. Most attention has been focused on compact denatured states, now commonly called molten globule states. The structural characteristics and the role in protein folding of the molten globule state have been discussed by Kuwajima (1989), Christensen and Pain (1991), Ptitsyn (1987, 1992), and Haynie and Freire (1993). It should be noted that over the past few years the term molten globule has been employed in a broad context. Thus, it is worthwhile to qualify this term in particular cases, as was recommended by Ptitsyn (1992).

Apomyoglobin, myoglobin with its heme group removed, retains a compact, native-like conformation at room temperature and near neutral pH (Griko et al. 1988; Hughson et al. 1990). Therefore, this state is called the native state (N-state) of apomyoglobin. A two step unfolding transition is observed when the pH is lowered. The conformation of apomyoglobin under acidic conditions depends on both pH and ionic strength. Goto and Fink (1990) have constructed a phase diagram for the pH and ionic strength-dependent conformation. At pH 2 and

Abbreviations: CD, circular dichroism; DLS, dynamic light scattering; SAXS, small-angle X-ray scattering; N, I, and U, the native, intermediate, and unfolded forms of apomyoglobin

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low ionic strength, the protein adopts a highly unfolded conformation (U-state). At pH 4 and any ionic strength, the protein is in a rather compact intermediate state (I-state) with a significant amount of secondary structure but without rigid, native-like tertiary structure. Thus, the I-state reveals essential characteristics of a molten globule state. The I-state is also observed at pH < 4 and sufficiently high ionic strength. Essential structural details of the I-state of apomyoglobin have been obtained by determining the location of protected amide protons and by measuring their protection factors (Hughson et al. 1990). These studies revealed that out of the 8 helices (A–H) existing in myoglobin (Kendrew et al. 1960; Kuryian et al. 1986) only the A, G, and H helices are detectable in the I-state. Testing the stability of the I-state by site-directed mutagenesis, the same group (Hughson et al. 1991) concluded that the compact subdomain formed by the A, G, and H helices is stabilised by non-native interactions. This conclusion was confirmed by a three-state analysis (Barrick and Baldwin 1993 a), by studying urea- and acid-induced conformational transitions. Recently, an early structured intermediate was found in kinetic folding experiments (Jennings and Wright 1993). This intermediate has protected NH groups in the A, G, and H helical regions, thus being similar to the equilibrium I-state. This supports the hypothesis that molten globules are universal intermediates on kinetic folding pathways (Ptitsyn et al. 1990; Barrick and Baldwin 1993 b) and further emphasises the importance of a detailed understanding of the nature of molten globule states.

Furthermore, apomyoglobin is of particular interest to protein folding investigations, because myoglobin as well as apomyoglobin can be denaturated by both heating and cooling within attainable temperature regions (Privalov et al. 1986; Griko et al. 1988). The thermodynamics of apomyoglobin were studied by Griko et al. (1989) and Griko and Privalov (1994).

A statistical mechanical theory that aims to predict protein stabilities as a function of temperature, pH, and salt concentration was presented by Alonso et al. (1991). As will be shown below, these theoretical calculations predict most of the experimental data very well. A structural thermodynamic framework of the molten globule state was recently published by Haynie and Freire (1993). Particularly, these authors have calculated stability surfaces of apomyoglobin as a function of temperature and pH.

In this work we investigate temperature-dependent structural changes of sperm whale apomyoglobin at pH 4 and low ionic strength. Evidently, the protein is in the I-state at temperatures near and below 30 °C. From the structural changes observed at low (near 0 °C) and high temperatures, conclusions will be drawn regarding the stability of the I-state and the main forces that are responsible for its stability. It is of particular interest to compare these results with theoretical predictions of the compactness (or density) of the protein (Alonso et al. 1991). For the sake of completeness, we also report on structural features of the N- and the U-states. Moreover, little is known of how proteins in the molten globule state unfold further as a function of temperature. The existing data are controversial (Ptitsyn 1992). It is worthwhile to compare the structural data of the molten globule intermediate of apomyoglobin (I-state) with that of other proteins, e.g., α-lactalbumin (Dolgikh et al. 1981; Damaschun et al. 1986; Gast et al. 1986; Timchenko et al. 1986), in order to qualify the term molten globule with respect to particular structural properties.

The experimental techniques used in this work are dynamic light scattering (DLS), small-angle X-ray scattering (SAXS), and far- and near-UV circular dichroism spectroscopy (CD). These methods are capable of determining essential structural features of the molten globule as its compactness, the amount of secondary structure and the presence or absence of specific packing of aromatic side chains.

Materials and methods

Sample preparation

Apomyoglobin was prepared from sperm whale myoglobin (SERVA Lot E9, FRG) as described by Teale (1959) with one modification. We used histidine-HCl buffer, pH 2, instead of 10 mM HCl, pH 2. The residual heme content of apomyoglobin was determined photometrically by comparing the absorbances at 408 nm and at 280 nm; it was found to be less than 1%. The protein was precipitated by ammonium sulphate and stored at 4 °C until use. For sample preparation of the I-form of apomyoglobin, ammonium sulphate precipitated protein was dissolved in 10 mM HCl, pH 2, and then exhaustively dialysed against 10 mM sodium acetate buffer, pH 4, at 4 °C. The sample concentrated by ultrafiltration was applied to a 1.6 cm × 100 cm Sephacryl S-100 HR column (Pharmacia, LKB Biotechnology). Only peak fractions were taken for the measurements. For SAXS measurements, dialysed samples were concentrated in collodium bags (Sartorius, Germany). The N-form of apomyoglobin was prepared in the same manner using 10 mM sodium acetate buffer, pH 6.5. The U-form measured in 10 mM HCl, pH 2 was gel filtered on a 1 cm × 100 cm Sephadex G-75 column (Pharmacia, LKB Biotechnology) before use. Apomyoglobin concentrations were determined from the absorbance at 280 nm using $A^{1\%}_{1\,cm} = 8.9$ (Harrison and Blout 1965).

Circular dichroism

CD spectra were obtained on a J-720 spectrometer (JASCO, Japan) equipped with a temperature control system (NESLAB, USA) in 10-mm (320–260 nm) or 1-mm cells (260–190 nm) at protein concentrations of about 1.5 or 0.2 g/L, respectively. Mean residue ellipticities were calculated using a value of 112.4 for the mean residue weight of apomyoglobin. The spectrometer was calibrated with (+)-10-camphorsulfonic acid at 290.5 and 192.5 nm (Johnson 1990).

Dynamic light scattering

The DLS spectrometer has been described previously (Gast et al. 1992; Damaschun et al. 1993 a). All DLS measurements were made at a scattering angle of 90° using 100-µl flow-through cells (Hellma, Germany) and an argon laser operating at $\lambda = 514.5$ nm. The solvent and the protein solutions were filtered through 20 nm-pore-size filters (Protein Solutions Ltd., U.K.) directly into the scattering cells.

The homodyne autocorrelation functions, $G^2(\tau)$, were calculated by a 90-channel multibit multiple-τ correlator and then fed into an on-line coupled PC/AT equipped with a transputer board, ALV-800 (ALV Laser-Vertriebsgesellschaft mbH, Germany), for fast data evaluation using the program CONTIN (Provencher 1982 a, b).

The translational diffusion coefficients, D, obtained from the autocorrelation functions, were converted into hydrodynamic effective radii, R_S, via the Stokes-Einstein equation, $R_S = kT/(6\pi\eta_0 D)^{-1}$, where k is Boltzmann's constant, T, the temperature in Kelvin, and η_0, the solvent viscosity. The temperature dependence of η_0 was determined by means of a viscometer, Viscoboy 2 (Lauda, Germany), and a digital density meter, DMA 58 (Anton Paar KG, Austria).

Small-angle X-ray scattering

SAXS was measured on a small-angle/wide-angle X-ray diffractometer (Freiberger Präzisionsmechanik GmbH, Germany) with slit geometry (Damaschun et al. 1991 a). The CuK_α-radiation was collimated by five vertical slits and horizontal Soller slits. The methods of data handling have been previously described (Müller et al. 1986). The samples were mounted in a temperature-controlled holder.

Results

Circular dichroism

The circular dichroism of the I-form of apomyoglobin was measured in steps of 10 °C between 1 °C and 80 °C. Representative near-UV and far-UV spectra measured at protein concentrations of 1.25 g/L and 0.2 g/L are shown in Fig. 1 a and Fig. 1 b, respectively. For comparison, we also show the CD spectra of the N-form. The far-UV spectra of the N-form and the I-form at 30 °C differ mainly in their amplitude, while the CD spectrum of apomyoglobin in the near-UV region is remarkably altered in shape and amplitude on the transition from the N-form to the I-form. Temperature increases from 1 to 80 °C was accompanied by a gradual decrease of the near-UV CD amplitude, particularly within the wavelength range between 270 and 295 nm. The CD spectrum in the far-UV region was found to be essentially unaffected by temperature changes between 1 and 40 °C. Above 40 °C, a gradual decrease of the ellipticities was observed. To demonstrate this, we show in Fig. 2 the temperature dependences of the mean residue ellipticities, $[\Theta]$, measured at 193 and

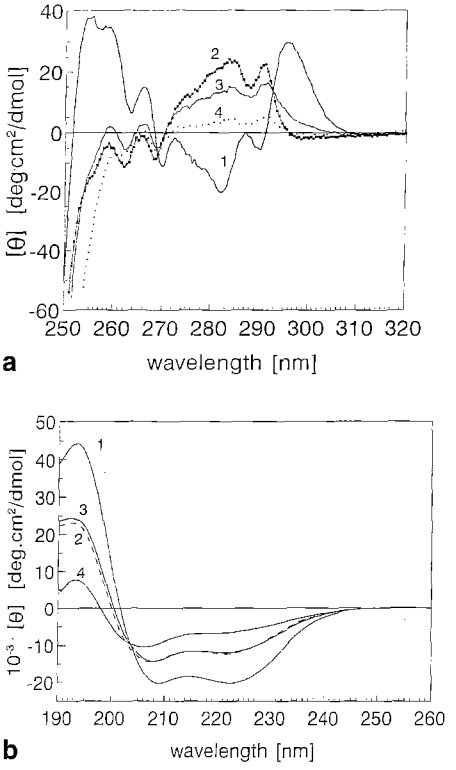

Fig. 1. Near-ultraviolet **a** and far-ultraviolet **b** CD spectra of apomyoglobin. 1: N-form at 30 °C; 2: I-form at 1 °C; 3: I-form at 30 °C; 4: I-form at 80 °C

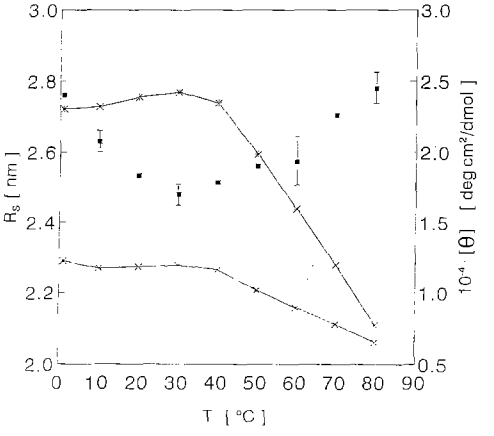

Fig. 2. Temperature dependence of R_S (■), $[\Theta]_{193}$ (*), and $-[\Theta]_{222}$ (×) for apomyoglobin in 10 mM sodium acetate, pH 4.0

222 nm. All temperature-induced changes of the CD spectra of the I-form were found to be reversible.

Dynamic light scattering

In order to measure temperature-dependent changes of the hydrodynamic effective dimensions of apomyoglobin in the I-state, DLS experiments were performed over the temperature range from 1 °C to 80 °C. The translational diffusion coefficient corrected to standard conditions,

$D_{20,w}$, was measured at protein concentrations of 0.49 and 0.64 g/L. The relative changes of $D_{20,w}$ with respect to the value at 20 °C were the same at both concentrations. This shows that the concentration dependence of $D_{20,w}$ does not noticeably change within the investigated temperature range. Therefore, the exact concentration dependence $D_{20,w} = D^0_{20,w}(1 + B'c)$ was measured only at 20 °C using concentrations between 0.3 and 1.5 g/L. B' is the diffusive virial coefficient and c is the protein concentration in g/L. This value of B' was then further used to extrapolate $D_{20,w}$ measured at different temperatures to zero protein concentration. R_S was calculated from $D^0_{20,w}$ via the Stokes-Einstein equation. The values of R_S and B' for the I-form at 20 °C are shown in Table 1. To give an idea of the differences in the hydrodynamic dimensions of the various forms of apomyoglobin, we have included the corresponding data of the N-form and the U-form in Table 1. The state at pH 5 was termed N′, because the native state is predominantly populated at this pH. The ratio of the Stokes radii to that of the N-form at pH 6.5 are also shown in Table 1 for easier comparison of the dimensions of the apomyoglobin molecules in different states. The increase of B' with decreasing pH clearly reflects the increase in the electrostatic repulsion between the protein molecules. This is mainly due to the increase in the net charge of the protein molecules also causing the expansion of the molecules when the pH is lowered. The temperature dependence of R_S of the I-form is shown in Fig. 2. The changes in R_S were found to be reversible. This was checked by measuring R_S at 20 °C after stepwise heating of the sample from 1 °C to 80 °C. In contrast to this, attempts to measure R_S of the N-form at high temperatures were unsuccessful, because the protein aggregated irreversibly on thermal unfolding above 60 °C. The Stokes radii of both the N- and N′-forms were constant within the experimental error of about ±1% at temperatures between 1 °C and 50 °C (data not shown) with the following exception. At 1 °C and pH 5, an increase in R_S of about 3% as compared to its value at 20 °C was measured which might be an indication of the onset of cold denaturation. In the case of the I-form, the minimum of R_S in the vicinity of 30 °C and the increase at both low and high temperatures is clearly visible.

Small-angle X-ray scattering

SAXS measurements give information about the size and, additionally, about the shape or the conformation statistics of the investigated molecules. The radius of gyration of the molecule, R_G, is the second moment of the electron density distribution within the molecule. It can be calculated from the innermost part of the scattering curve and is a sensitive measure of the molecular size. The course of the scattering curve up to larger scattering angles is a characteristic indicator of the molecular shape and of the type of conformational fluctuations of chain molecules (Glatter and Kratky 1982; Feigin and Svergun 1987).

Scattering curves of apomyoglobin were measured at pH 6.5 in 10 mM sodium acetate buffer at 20 °C, at pH 4 in 10 mM sodium acetate buffer at 20 °C and 5 °C, and at pH 2 in 10 mM HCl at 20 °C over concentration ranges of 7.8–65.4 g/L, 3.85–19.8 g/L and 4.0–15.7 g/L, respectively. All parameters were extrapolated to zero protein concentration. Figure 3 shows the Guinier plots of scattering curves of apomyoglobin in the N-state (pH 6.5), in the I-state (pH 4), and in the acid-unfolded U-state (pH 2).

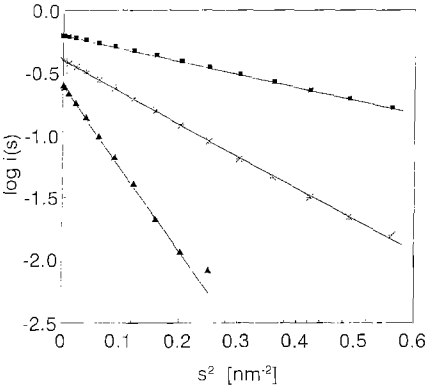

Fig. 3. Guinier plots of SAXS curves for apomyoglobin in the N-state (■), the I-state (*), and the U-state (▲). The full lines represent the Guinier approximations of the inner parts of the scattering curves. $i(s)$: scattered intensity, s: magnitude of the scattering vector. The plots have been arbitrarily shifted along the ordinate

Table 1. Stokes radii, R_S, and diffusive virial coefficients, B', for different forms of apomyoglobin at 20 °C

Form	pH	R_S [nm]	$R_S/R_S(N)$	B' [L/g]
N	6.5	2.09 ± 0.02	1	0.026
N′	5.0	2.15 ± 0.02	1.03	0.069
I	4.0	2.53 ± 0.03	1.21	0.104
U	2.0	4.29 ± 0.04	2.05	0.52

The scattered intensity at small angles can be approximated by the function $I(s) = I_0 \exp(-R_G^2 s^2/3)$. R_G is the radius of gyration, and s is the magnitude of the scattering vector $s = 4\pi \sin \Theta/\lambda$, where 2Θ is the scattering angle and λ the wavelength of the X-rays. The radii of gyration for the N-, I-, and the U-states, determined from the concentration dependences of R_G by linear extrapolation to zero concentration are given in Table 2. The value of $R_G = 4.67$ nm, obtained for the U-state by linear extrapolation, is possibly somewhat smaller than its true value. This can be expected, since we have observed slightly curved dependences $R_G(c)$ for other acid-denatured proteins at low concentrations (Damaschun et al. 1991 a, b).

The largest diameter of the molecules, L, was determined from the distance distribution function $P(r)$ of the excess electron density. This function was calculated from the scattering data by a Fourier transformation (Feigin and Svergun 1987). The distance distribution functions for the N- and the I-states are shown in Fig. 4.

The shape of $P(r)$ of the N-state is typical of a globular protein. By contrast, the function $P(r)$ of the I-state has a shape typical of a protein consisting of two subunits with non-coinciding centres of gravity. Such functions $P(r)$ have been calculated for models by Glatter (1982) (see Fig. 9 in Glatter 1982).

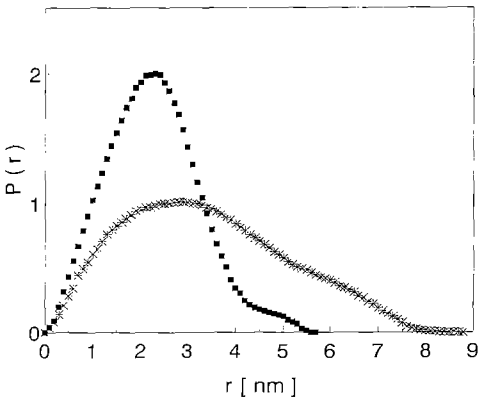

Fig. 4. Distance distribution functions of apomyoglobin in the N-state (■), pH 6.5, 20 °C, and in the I-state (*), pH 4.0, 5 °C

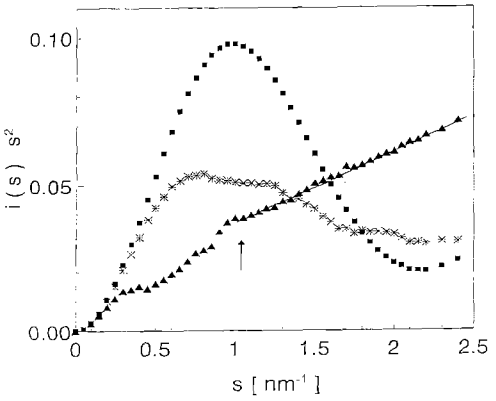

Fig. 5. Kratky plots of the SAXS data for apomyoglobin in the N-state (■), the I-state (*), and the U-state (▲). In the case of the U-state, the transition from coil-scattering behaviour to rod-scattering behaviour is marked by arrow

Table 2. Radii of gyration, R_G, and largest diameters, L, for different states of apomyoglobin

State	pH	T [°C]	R_G [nm]	L [nm]
N	6.5	20	1.75 ± 0.02	5.60 ± 0.30
I	4.0	20	2.35 ± 0.02	9.05 ± 0.50
		5	2.53 ± 0.02	8.75 ± 0.50
U	2.0	20	$\geq 4.67 \pm 0.08$	≥ 14

The values of L for the different states of apomyoglobin are given in Table 2. To facilitate the discussion of the shape of chain molecules, it is useful to convert the scattering data into a Kratky plot (Glatter and Kratky 1982). We have applied this method to denatured protein molecules for the first time in the case of acid-unfolded apocytochrome c to determine directly the persistence length and the statistics of the shape fluctuations (Damaschun et al. 1991 a). Kataoka et al. (1993) have used the same method for characterising the molten globule state of cytochrome c. Figure 5 shows the Kratky plots of the scattering curves, extrapolated to zero concentrations, for the N-, I-, and U-states. Apomyoglobin in the N-state exhibits a scattering curve typical of a globular protein. In contrast to this curve, that for apomyoglobin in the U-state shows characteristics of a polymer molecule with the conformation and the fluctuations of a statistical coil. We have observed scattering curves of this type previously for acid-unfolded apocytochrome c (Damaschun et al. 1991 a) and for cold-denatured phosphoglycerate kinase (Damaschun et al. 1993 a). The persistance length, a, of acid-unfolded apomyoglobin was determined from the transition – marked by an arrow – of the scattering curve from the range where $I \propto s^2$ to the range $I \propto s$. We obtained $a = 1.83$ nm. Apomyoglobin in the I-state exhibits a scattering curve with novel features, being different from the curve which was observed by Kataoka et al. (1993) for the molten globule state of cytochrome c. The curve for apomyoglobin in the I-state is atypical of a homogeneously expanded globule. It exhibits two maxima at $s = 0.78\ \text{nm}^{-1}$ and at $s = 1.16\ \text{nm}^{-1}$. These two maxima indicate that the apomyoglobin molecule consists of two domains at pH 4. Furthermore, these two domains with non-coinciding centres of gravity cause the shoulder in the distance distribution function at the right-hand side of the maximum (Fig. 4). The molecular parameters determined from the SAXS curves are summarized in Table 2.

Discussion

Characteristics of the I-form in comparison with the N-form

To begin with, we summarise essential features of the N-form of apomyoglobin from sperm whale. The struc-

Table 3. Compactness of different proteins in molten globule states

Protein	Native			Molten globule			q_X	q_X^3	q_D	q_D^3
	R_G [nm]	R_S [nm]	ϱ	R_G [nm]	R_S [nm]	ϱ				
α-Lactalbumin	1.55	1.77	0.88	1.57	1.99	0.79	1.01	1.03	1.12	1.42
Cytochrome c	1.35	1.66 [a]	0.81	1.74	2.01	0.87	1.29	2.14	1.21	1.77
		(1.98) [b]	(0.68)						(1.02)	(1.05)
Apomyoglobin	1.75	2.09	0.84	2.35	2.53	0.93	1.34	2.40	1.21	1.77

[a] Value calculated by Müller (1991)
[b] Experimental value (Ohgushi and Wada 1983)
The errors in our experimental values of R_G and R_S are ±1% resulting in an uncertainty of ±2% in ϱ, q_X, and q_D

ture of native apomyoglobin ($M_R = 17200$) closely resembles that of myoglobin. It was shown (Hughson et al. 1990) that the amide proton protection pattern observed in myoglobin is largely preserved in apomyoglobin. The α-helix content is reduced from 78% in myoglobin to 65% in apomyoglobin. The value for apomyoglobin was calculated from our CD data using the method of Johnson (1990). Hydrodynamic investigations (Crumpton and Polson 1965; Griko et al. 1988) have shown that native apomyoglobin is slightly expanded compared to myoglobin. In particular, the Stokes radii calculated from the translational diffusion coefficients measured by Crumpton and Polson (1965) are 1.98 and 2.15 nm for myoglobin and apomyoglobin, respectively. The value for apomyoglobin agrees well with our data (Table 1) and with that for horse apomyoglobin, $R_S = 2.15$ nm, as measured by gel-exclusion chromatography (Goto et al. 1990). In spite of the observed swelling of the molecule, the pronounced CD spectrum in the near-UV region (Fig. 1 a) hints at an essentially rigid environment of the aromatic amino acid side chains. According to micro calorimetric studies (Griko et al. 1988), apomyoglobin is most stable near 30 °C and undergoes co-operative unfolding transitions at high and low temperatures. At pH 5, the transitions are found near 60 °C and slightly below 0 °C. All these data support the idea that apomyoglobin is in a rather compact, native-like conformation at room temperature near neutral pH and justify the use of the term N-form.

In contrast to this close structural similarity of myoglobin and the N-form of apomyoglobin, the structural differences between the N-form and the I-form are more pronounced. According to the phase diagram of Goto and Fink (1990), the compact intermediate state (I-form) of apomyoglobin exists at pH values below 4.5 and at an ionic strength depending on the particular pH. In our investigations, we have chosen pH 4 and low ionic strength for two main reasons. First, preliminary DLS investigations revealed that aggregation is minimal under these conditions. Second, the results obtained at low ionic strength can be more easily compared with theoretical predictions by Dill and coworkers (Alonso et al. 1991). The results shown in Figs. 1 and 2 clearly indicate temperature-dependent structural changes of apomyoglobin at pH 4 and low ionic strength. Thus, it is necessary to refer to a particular temperature in discussing structural data of the I-form. If not otherwise stated, we will call the state at pH 4, low salt concentration ($C_S = 0.01$ M), and near 30 °C the I-form of apomyoglobin.

The molecular dimensions are considerably altered on the transition from the N-state to the I-state. This is reflected in both the increase in the Stokes radius, R_S, of 21% and the increase in the radius of gyration, R_G, of 32%. It must be emphasised that R_G and R_S are different measures of compactness displaying the geometric and the hydrodynamic dimensions, respectively. The ϱ-factor, $\varrho = R_G/R_S$, is a sensitive indicator of the molecular conformation. Its value is 0.775 for a compact sphere, 0.8 ± 0.05 for an "average" globular protein consisting of a single chain (Damaschun et al. 1993 b), and 1.51 for a polypeptide chain with the conformation of a Gaussian coil (Tanford 1961). The ϱ-factor of the I-form of apomyoglobin is 0.93.

The far-UV CD spectrum of the I-form points to significantly lower helix content when compared to the N-form. Secondary structure analysis of the CD data by the method of Johnson (1990) yielded 45% α-helix. Calculating the helix content from the ellipticity at 222 nm, $[\Theta]_{222}$, (Moss et al. 1976) we found 36%. The latter value agrees with that reported in the literature (Goto et al. 1990; Hughson et al. 1990). The strongly altered CD spectrum in the near-UV region reflects considerable changes of the environment and/or of the mobility of aromatic side chains. It is unfeasible to relate these spectral changes unequivocally to alterations of the tertiary structure. The reduced near-UV CD amplitude is indicative of the loss of rigid packing in the global structure of the apomyoglobin molecules. Nevertheless, the differences in the spectra at 30 and 60 °C hint at the existence of some rigid structure at 30 °C. These findings are consistent with the assumption of an essentially rigid hydrophobic cluster formed by the A, G, and H helices (Hughson et al. 1990, 1991). The CD band at 295 nm is due to tryptophans, Trp7 and Trp14 in the present case. Both tryptophans are located within the A helix. The spectral differences at 295 nm between the N-form and the I-form indicate that the native environment of Trp7 and Trp14 is lost. This supports the model of the I-form assuming a remaining hydrophobic cluster formed by the A, G, and H helices but with non-native packing of these helices. It should be noted that most of the investigations leading to this model (Hughson et al. 1990, 1991; Barrick and Baldwin 1993 a) were performed at temperatures near or at 0 °C. Structural changes occurring between 30 °C and lower temperatures will be discussed in the next section.

Structural changes of the I-form on cooling and heating

According to the measured Stokes radii, the I-form of apomyoglobin is most compact near 30 °C. Both cooling and heating lead to a gradual increase of the Stokes radius of about 10%. The maximum increase is not reached within the attainable temperature range. One might expect that the observed changes in compactness have the same energetic reasons as cold and heat denaturation. However, it must be emphasised that differential scanning calorimetry (Griko et al. 1988; Griko and Privalov 1994) did not reveal heat absorption peaks at low and high temperatures under the present conditions. Furthermore, the same authors (Griko and Privalov 1994) have shown that only a heat capacity increase is observed on heating apomyoglobin in the I-state from 30 °C up to 80 °C. Comparing the near-UV spectra at 1 °C and 80 °C in Fig. 1 a and inspecting the temperature dependence of the specific ellipticities at 193 and 222 nm in Fig. 2, it becomes evident that the structural changes on cooling and heating must be quite different.

At temperatures above approximately 40 °C, our experimental data point to a non-cooperative melting of structural elements of the I-form and a transition to an essentially unfolded conformation. While the near-UV

spectrum at 80 °C is very similar to that of the U-form (data not shown), the Stokes radius of about 2.8 nm in considerably smaller than $R_S = 4.3$ nm of the U-form. The far-UV spectrum of the U-form (data not shown) differs considerably from the spectrum of the I-form at 80 °C. The former exhibits a pattern more typical of a protein lacking regular secondary structure. Accordingly, the structure of apomyoglobin at pH 4 and 80 °C should be different from the structure in the U-state.

The structural changes occurring at temperatures below 30 °C must be of a totally different kind. There are only minor changes of the ellipticities at 193 and 222 nm, showing that the secondary structure is only slightly altered upon cooling. The near-UV CD amplitude increases, particularly in the wavelength range between 270 and 290 nm. Accordingly, some of the aromatic side chains, mostly those of tyrosines, are expected to be in a more rigid environment. On the other hand, the weak shoulder at 295 nm, seen in the spectrum at 30 °C, vanishes at 1 °C. The radius of gyration increases by 8% upon cooling from 20 to 5 °C. Taking a Stokes radius of 2.70 nm by interpolating the values measured at 1 and 10 °C, one gets a ϱ-factor of 0.94, which is practically the same as at 20 °C. This means that the hydrodynamic and the geometric dimensions are changing to the same extent in the low-temperature structural transition. The fact that the far-UV CD spectra at 1 and 30 °C are nearly identical while the Stokes radii and the near-UV CD spectra are quite different makes it very unlikely that the observed changes are only due to alterations in the population of the N-, I-, and U-states. While the high-temperature transition undoubtedly involves non-cooperative structural changes towards an unfolded conformation, the interpretation of its low-temperature counterpart is less straightforward. All in all, the experimental results are consistent with the following picture. The elements of secondary structure, mostly helices, are essentially retained. The hydrodynamic and geometric dimensions are increased by stiffening and/or reorientation of structural elements, e.g., helices or loops. According to the near-UV CD data, the tertiary structure is at most slightly changed, possible more rigid on average, but in no way lost upon cooling. The presence of such extended partially unfolded states at low temperatures was predicted for apomyoglobin by Freire and Murphy (1991). Similar partially unfolded states have been described for ribonuclease at subzero temperatures (Biringer and Fink 1988).

What is the driving force for the observed structural changes? The highest compactness and thus the minimum exposure of non polar surfaces of the protein is reached at temperatures near 30 °C. Both at low and high temperatures, structural changes are observed resembling heat and cold denaturation of native, compactly folded proteins. The appearance of both cold and heat denaturation is caused by the existence of a heat capacity difference ΔC_p between the native and the unfolded states. According to present knowledge (Privalov and Gill 1988), ΔC_p is predominantly due to the transfer of non polar surfaces into water. Recent calorimetric studies (Griko and Privalov 1994) revealed that the heat capacity of apomyoglobin in the I-state at 30 °C is larger than in the N-state but still smaller than in the U-state. Therefore, ΔC_p, observed on heating is obviously due to the exposure of remaining buried non polar surfaces to water. The latter must be responsible for weak hydrophobic interactions that stabilise the compact denatured state and cause the observed temperature dependence of its structure.

How do the results fit with theoretical predictions of the structure and stability of apomyoglobin?

Dill and coworkers have developed a statistical mechanical theory (Dill 1985; Dill et al. 1989; Stigter et al. 1991) to predict protein stability as a function of temperature and electrostatic interactions. The application of this theoretical framework to apomyoglobin (Alonso et al. 1991) should be discussed in context with our experimental results now. Characteristics of the protein which are used in applying the theory are the chain length, the fraction of non polar residues, Φ, and the number and pK_a values of titratable groups. In their work, Alonso et al. (1991) considered apomyoglobin and a hypothetical variant having a slightly higher content of non polar residues and a reduced number of ε-amino groups (12 instead of 19 in the native protein).

The theoretical results have been presented in terms of the average density of the protein relative to the native state, $\langle \varrho \rangle$. Accordingly, the data can be easily converted into average radii, $\langle R \rangle$, which are directly related to radii of gyration, R_G, or – if the ϱ-factor is known – to Stokes radii, R_S. On the whole, the theoretical predictions are in fair agreement with our and other experimental results. In particular, the calculated relative average density, $\langle \varrho \rangle \approx 0.5$, for the compact denatured state (I-state) at temperatures near 30 °C corresponds to a relative increase in the radius of 25%. This value compares well with the experimentally determined increases in the radius of gyration of about 30% and in the Stokes radius of about 20% on the transition from the N- to the I-state. Furthermore, the model predicts a gradual decrease in the density of the compact intermediate state on either heating or cooling relative to 30 °C. There is a fair quantitative agreement with the observed temperature-dependent changes of the Stokes radius. The predicted density of the acid-unfolded U-state, $\langle \varrho \rangle \approx 0.02$ appears to be somewhat too low compared to the experimentally observed values of R_G and R_S. But, the problem of overestimating the electrostatic coil expansion has already been discussed by Stigter et al. (1991). The model predicts steep changes of the average density as a function of pH. This is confirmed by the observed changes of different experimental parameters. For the apomyoglobin model, the predicted pH values of the transitions, N- → I-state and I- → U-state, are somewhat higher than the experimentally determined ones. The observed positions of the transitions are in better agreement with the model variant having a reduced number of charges. It is obvious that it is difficult to estimate the effective number and the apparent pK_a values of titratable groups correctly. The results of pH titrations of histidine residue, the protonation of which is probably important for the unfolding transitions, were reported by Cocco et al. (1992).

An important prediction of the theory (Alonso et al. 1991) is that a compact denatured state can be expected for proteins having a sufficiently high content of non polar amino acids. This condition is fulfilled in the case of apomyoglobin.

Comparison of the molten globule of apomyoglobin with other molten globule forms

The term molten globule is a generic term of more or less compact intermediate states appearing during protein folding (Baldwin 1991), and it is a controversial subject whether the molten globule is a specific or an unspecific intermediate during protein folding. Up to now, detailed studies of the compactness of these I-states by means of small-angle X-ray scattering and dynamic light scattering have been made only on three proteins. Combining these two methods provides useful measures of the compactness of proteins (see Table 3). These studies concern the A-form of α-lactalbumin (Damaschun et al. 1986; Gast et al. 1986; Timchenko et al. 1986), the NaCl-induced molten globule state of acid-unfolded cytochrome c (Ohgushi and Wada 1983; Kataoka et al. 1993), and apomyoglobin at pH 4 (this work). The parameters being a measure of the compactness of these so-called molten globule states are compared in Table 3. As will be shown below, this table clearly indicates that the molten globule states of the three proteins are related but not identical.

The ϱ-factors for these proteins in the molten globule state are in the range 0.79–0.93, thus being nearer to those of compact proteins, $\varrho = 0.80$, than to those of statistical coils, $\varrho = 1.51$.

The values of $q_X = R_{G,MG}/R_{G,N}$ and $q_D = R_{S,MG}/R_{S,N}$ are measures of changes in the geometric and hydrodynamic radii, respectively. Correspondingly, q_X^3 and q_D^3 indicate the changes in the geometric and hydrodynamic molecular volumes. According to these parameters in Table 3, the compactness of the molten globule states of the three proteins decreases in the order α-lactalbumin > cytochrome c > apomyoglobin. The ratios of the geometric volumes in the molten globule states to those in the native states amount to a maximum of 2.4, thus corresponding to about doubling of the volume upon the transition folded state → molten globule state. This value is more than one order of magnitude smaller than the volume increases observed in experiments with acid-unfolded proteins and cold-denatured phosphoglycerate kinase where we found about thirty fold increases in the geometric volumes (Damaschun et al. 1991a, 1993a). The so-called molten globule state of apomyoglobin differs from the molten globule states of α-lactalbumin and cytochrome c in such a way that apomyoglobin consists of two domains with non coinciding centres of gravity, while α-lactalbumin and cytochrome c exhibit rather homogeneously expanded compact structures.

Conclusions

The results obtained in this work support the idea that the I-form of apomyoglobin has characteristics of a molten globule. Compared to other molten globules found for α-lactalbumin and cytochrome c, the I-state of apomyoglobin is least compact. It has typical features of a non uniformly expanded protein as it was proposed some years ago (Ptitsyn et al. 1986; Damaschun et al. 1986). This conclusion can be drawn from the SAXS data pointing to the existence of two structural domains. This is also consistent with previous findings (Hughson et al. 1990, 1991) which demonstrate the existence of a subdomain consisting of the A-, G-, and H-helices, whereas other parts of the molecule are essentially unfolded. From our structural investigations within the temperature range from 1 °C and 80 °C, the following conclusions can be drawn. Apomyoglobin at pH 4 and low ionic strength retains properties of a molten globule between 1 °C and 30 °C. Nevertheless, the molten globule undergoes minor structural changes within this temperature range, particularly changing its tertiary fold. Above 30 °C, the molten globule unfolds non-cooperatively.

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