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Molten globule versus variety of intermediates: influence of anions on pH-denatured apomyoglobin

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Abstract The molten globule state was shown to be the third thermodynamic state of protein molecules in addition to their native and unfolded states. On the other hand, it was reported that optical and hydrodynamic properties of pH-denatured apomyoglobin depend on the nature of anions added to the protein solution. This observation was used to conclude that there are many 'partly folded' intermediates between the native and unfolded states rather than one distinct molten globule state. However, little is known on the structures of pH-denatured apomyoglobin in the presence of different anions. Two tyrosine residues in horse apomyoglobin have been successively modified by the reaction with tetranitromethane. This approach was employed to measure the distances between tryptophans and modified tyrosines in different states of apomyoglobin by the method of direct energy transfer. Experimental data show that the distance between the middle of the A-helix and the beginning of the G-helix and/or the end of the H-helix in 'anion-induced' states are very close to those in the native holo- and apomyoglobins. This suggests that the AGH helical complex, being the most structured part of apomyoglobin in the molten globule state, exists also in pH-denatured apomyoglobin in the presence of different anions. Consequently, all non-native forms of apomyoglobin studied so far share the common important feature of its native structure.

Key words: Apomyoglobin; Fluorescence energy transfer; Molten globule; Protein folding

1. Introduction

The molten globule (MG) state has been predicted as a folding intermediate [1], and has been revealed both as an equilibrium state under mild denaturing conditions [2] and as a kinetic intermediate of protein folding [3,4]. Although a number of small single-domain proteins fold without any detectable intermediates [5], the MG still is a kinetic intermediate for many other proteins [4]. Furthermore, it is expected that the MG exists in a living cell and participates in physiological processes: e.g. protein translocations through membranes or protein recognition by chaperones [6–9].

The equilibrium intermediates between the native (N) and unfolded (U) states usually are observed at mild denaturing conditions (at moderately low pH, moderately low concentrations of denaturants and so on). Different denaturants, however, might perturb the structure and properties of intermediates, and the number of reports testifying to the existence of a variety of equilibrium intermediates in denaturant-induced (un)folding increases steadily. These studies have raised new questions with respect to the MG state. For example: How many intermediates can be covered by the term 'molten globule'? Clearly, this issue relates to the concept of MG being the third thermodynamic state of proteins in addition to their native and unfolded states. Indeed, general laws of molecular physics claim that any thermodynamic state of the macroscopic system should be separated from the others by barriers high enough to ensure the 'all-or-none' transition between these states. In macroscopic systems, like protein molecules, it means that each molecule can be transformed from one state to another as a whole. Therefore, we can speak of the different thermodynamic states of a protein molecule only if these states are separated by 'all-or-none' transitions.

The experiments on the heat denaturation of small proteins revealed the 'all-or-none' nature of both the N↔U and N↔MG transitions [4,10–12]. In fact, the existence of the MG state suggests that the large increase of protein volume and the cooperative destruction of protein secondary structure are not necessary conditions of 'all-or-none' denaturation. The imperative condition appears to be a rather small increase of volume leading to the destruction of tight packing of side chains [13]. This conclusion is consistent with the idea that not only N↔U, but also N↔MG, is the intramolecular analogy of the first order phase transition in macroscopic systems.

As to another potential transition in proteins, i.e. MG↔U, its character remains disputable. Originally it was postulated that there are only two thermodynamic states of protein molecules, i.e. native and denatured, and no 'all-or-none' transitions occur between denatured states of the proteins [10,11]. From this point of view, the MG is no more than a squeezed coil. However, the strict evidence for the 'all-or-none' nature of the MG↔U transition in small proteins is the molecular weight (M) dependence of the transition slope. Clearly, if the transition embraces the entire molecule, its slope should be sharper when the molecular weight is greater. The analysis of 60 proteins has shown that the slopes of the N↔MG and MG↔U transitions (induced by urea or guanidinium chloride) are proportional to $M^{1.02}$ and $M^{0.89}$, respectively [4,14]. These results completely exclude both the *transitions in local parts* of small proteins as well as an intramolecular

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[1] Oleg Ptitsyn passed away on March 22, 1999, one hour before his lecture, surrounded by his students and friends. Such an amazing finale! We will remember him as a Great Scientist, Teacher, and Friend.

Abbreviations: N, native state; MG, molten globule state; U, unfolded state; apoMb, apomyoglobin; Trp, tryptophan; Tyr, tyrosine

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analogy of the *second order phase transition* in protein [15]. In fact, the former requires the slope to be independent of M, while for the latter it should be proportional to $M^{0.5}$ [16]. Both options completely contradict the experimental data. It appears that both the N↔MG and MG↔U transitions are of the 'all-or-none' type, and the existence of three distinct thermodynamic states of protein molecules is a well-established experimental fact. Evidently, small size and heterogeneity of protein molecules might lead to the deviations of simple laws suggested for a large homogenous system. These deviations appear as a rather large scattering in 'transition slope versus M' plots [4]. Some proteins might be heterogeneous enough to force their folding out of the 'all-or-none' principle. For instance, it was reported that the intermediate state of apomyoglobin (induced by urea or pH) is split into two slightly different intermediates [17]. Another example is the multi-step melting of α-lactalbumin by urea [18]. Perhaps the most dramatic differences between *numerous intermediates* were revealed by the study of pH-denatured apomyoglobin (apoMb) in the presence of anions [19]. It was shown that the helix content in non-native forms of apoMb generated by the anion additions is 1.9–1.2 times smaller than that of the native protein; whereas the molecular volume of these forms is 3–7-fold larger than in the native state. Certainly, such large differences can not be ignored.

Here, we study the influence of anions on pH-denatured apoMb by the fluorescence direct energy transfer technique, which is the most detailed method to probe the overall structure of non-native proteins. Recently we have shown [20] that in the 'classical' molten globule state of apoMb (pH 4.2, low salt concentrations [21–23]), two key distances between the middle of the A-helix and the beginning of the G-helix and the end of the H-helix remain very close to those in the crystal X-ray structure of the holoprotein. These helices are formed at the early stage of folding [24] and remain relatively stable in the equilibrium MG state [25]. The side chains at the interfaces of these helices in MG state appear to be more or less tightly packed [26]. Variety of helicity degree and especially molecular volume of pH-denatured apoMb in the presence of anions should be reflected in substantial differences of these AGH distances. This paper presents the data that no such differences can be observed.

2. Materials and methods

2.1. Preparation of apomyoglobin

Horse heart myoglobin was purchased from Sigma. Apomyoglobin was obtained and characterized by routine procedures described previously [20]. Protein concentration was estimated by absorbance with a Hewlett-Packard 8452A diode array spectrophotometer. The molar extinction coefficient (ε_{280nm}) was calculated from the tryptophan and tyrosine composition and the standard values of 5690 and 1280 M^{-1} cm^{-1} reported for those amino acids [27].

Tyrosine residues in horse apomyoglobin were modified by the reaction with tetranitromethane. The reaction with native protein allows for selective nitration of the tyrosine (Tyr) residue located at position 146 [28]; whereas, the reaction with unfolded protein produces an entirely modified sample with nitration of both Tyr-103 and Tyr-146 [20]. To characterize the selectively modified protein, we performed amino acid analysis as described in [28]. Only fragments containing Tyr-146 showed the increase of 45 a.u., in accordance with the mass of the NO_2 group. The extent of tyrosine modification was assessed by spectroscopic means, namely by spectrophotometry and mass spectroscopy. Fig. 1 demonstrates the changes in protein absorbance following the modification of tyrosine. The absorption peak of nitrotyrosine (Tyr(NO_2)) at 360 nm is clearly visible. The molar excess of Tyr(NO_2) per protein molecule was calculated with $\varepsilon_{381nm} = 2200$ M^{-1} cm^{-1} [29]. The mass spectrum of horse apoMb corresponded to the theoretical value of 16951. The mass spectra of selectively and entirely modified samples showed the shift of 45 and 90 a.u., respectively. The efficiency of tyrosine modification in apoMb samples was $>90\%$.

2.2. Non-native states of apomyoglobin

To prepare the non-native forms of apoMb induced by the presence of anions we followed the procedure previously reported [30]. Anion solutions were prepared by dissolving the appropriate sodium salt in 10 mM HCl (pH 2). The denatured apoMb in 10 mM HCl (pH 2) was mixed with the anion solutions to prepare the desired anion concentrations of 500 mM NaCl, 100 mM $NaClO_4$, 100 mM NaTFA, and 50 mM NaTCA. The measurements were carried out 20 min afterwards to ensure that the anion-induced folding of apoMb was completed [30]. The native and molten globule states were reached by dissolving the protein in 10 mM sodium acetate-10 mM sodium phosphate buffer mixture at pH 6.5 and 4.2, respectively. The unfolded conformation was reached by dissolving salt free apoMb in 10 mM HCl (pH 2). The experiments were also carried out in deionized water (at correspondent pH) in salt free solutions and in the presence of 30 mM NaCl meant to stabilize the MG state [31]. This variation in conditions showed no effect on the experimental parameters. The pH was measured using a Radiometer PHM83. All experiments were done at 20 ± 0.05°C controlled by an RTE-111 Neslab water bath. The refractive index was measured on an AO Abbe refractometer.

2.3. Steady state fluorescence experiments

Fluorescence spectra were recorded on a SPEX Fluorolog-2 spectrofluorometer supplied with DM-3000 software (data interval 0.5 nm, scan speed 50 nm/min). Fluorescence emission was measured in the ratio mode and corrected for the appropriate solvent-blanks, as well as for wavelength dependent bias of the optics and detection system. The excitation of fluorescence was set at 295 nm. The emission was scanned from 300 to 450 nm and integrated within this range for further analysis. The fluorescence quantum yield, ϕ, was calculated by comparing the absorbance and the emission of a protein with a standard, which was a twice recrystallized *N*-acetyltryptophanamide in water [20]. The protein concentration was less than 0.2 g/l in most of the experiments. To study apoMb in the presence of NaTCA, the measurements were done at protein concentrations varying from 0.15 to 0.4 g/l.

2.4. Direct energy transfer data analysis

To estimate the average donor-acceptor distance in the system of interest, one has to measure the efficiency of the energy transfer from a donor to an acceptor. Direct energy transfer efficiency E is defined as a relative loss of the donor fluorescence due to the interaction with the acceptor [32]:

$$E = 1 - \frac{\phi_{D,A}}{\phi_D} \tag{1}$$

Here, ϕ_D and $\phi_{D,A}$ are the fluorescence quantum yields of the donor in the absence and presence of the acceptor, respectively. Importantly, the energy transfer efficiency is directly related on the donor-acceptor distance R_{DA} [32]:

$$E = \frac{1}{1 + \left(\frac{R_{DA}}{R_o}\right)^6} \tag{2}$$

Here, R_o is the characteristic donor-acceptor distance, when the probability of the spontaneous donor fluorescence and that of the energy transfer are equal to each other. This parameter can be computed from the donor-acceptor spectral properties:

$$R_o^6 = \frac{9000 \ln 10}{128\pi^5 N} \frac{\langle k^2 \rangle \phi_D}{n^4} \int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda \tag{3}$$

The parameter ϕ_D is as defined above, n is the refractive index of the medium, N is the Avogadro's number, and λ is the wavelength. $F_D(\lambda)$ is the fluorescence spectrum of the donor with the total area normalized to unity, and $\varepsilon_A(\lambda)$ is the molar extinction coefficient of the acceptor. The overlap integral, $\langle J \rangle = \int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda$, expresses the

spectral overlap between the donor emission $F_D(\lambda)$ and the acceptor absorption $\varepsilon_A(\lambda)$. These parameters were directly obtained from the independent experiments on each protein state of interest. The $\langle k^2 \rangle$ parameter represents the effect of the relative orientations of the donor and acceptor transition dipoles on the energy transfer efficiency. For each donor-acceptor couple, the orientation factor can be calculated as follows [32]:

$$k^2 = (\cos \alpha - 3\cos \beta \cos \gamma)^2 \quad (4)$$

Here, α is the angle between transition moments of the donor and the acceptor, and β and γ are the angles between the donor and acceptor transition moments and the donor-acceptor vector, respectively. In an unfolded protein chain, a large number of random donor-acceptor orientations are possible, resulting in $\langle k^2 \rangle = 0.67$ as a statistical average [32,33]. In native and molten globules of apoMb, the $\langle k^2 \rangle$ was recently found to be 0.18 [20]. The latter was used to analyze the energy transfer data obtained for 'anion-induced states', which are more helical than the 'classical' molten globule [19,22,28]. It is important that R_{DA} depends on $\langle k^2 \rangle^{1/6}$; i.e. it is a very slow function. Therefore, the uncertainty associated with this assumption should be small for $\langle k^2 \rangle$ varying from 0.18 (native) to 0.67 (unfolded). The direct energy transfer parameters needed for the donor-acceptor distance evaluation are listed in Table 1.

3. Results

Recently we proposed a method to probe the 3-dimensional (3D)-structure of proteins, which is based on measurements of average distances between groups of the protein chain by means of the direct energy transfer technique [20]. For these experiments, the tyrosine residues should be modified by reaction with tetranitromethane to be converted to a Tyr(NO_2) form. This reaction results in substantial changes of the tyrosine spectral properties: e.g. a red shift of the absorption and lack of the fluorescence [29]. As a consequence, nitrotyrosine becomes an acceptor of the tryptophan electronic excitation energy [29]. The horse myoglobin contains Trp-7 and Trp-14 in the middle of the A-helix, Tyr-103 at the beginning of the G-helix and Tyr-146 at the end of the H-helix (Fig. 2). Both tyrosine residues can be successively converted to the nitro-form [20,28]. If one can measure the decrease of tryptophan fluorescence in the presence of nitrotyrosine, then one can determine the average distance between these residues of the protein chain. In other words, one can measure the distance from the middle of the A-helix to the N-terminus of the G-helix as well as the distance between the middle of the A-helix and the C-terminus of the H-helix.

Fig. 3 shows the fluorescence spectra of apoMb samples with differing extent of tyrosine modification. The data of the A-series refer to non-modified protein. The B-series shows the spectra obtained for selectively modified apoMb, containing Tyr-146(NO_2). The fluorescence of the entirely modified samples, containing Tyr-146(NO_2) and Tyr-103(NO_2), is indicated by the C-series. Each series includes the spectra obtained in molten globules, anion-induced forms and native state. The first observation to be made is that the tryptophan fluorescence in the non-native forms generated by NaCl, NaTFA, and $NaClO_4$ is about 10% higher than that in the native state (A-series). In contrast, the fluorescence recorded for the TCA-induced state is about 80% less than that in the native protein (Fig. 3, inset). It appears that the tryptophan residues in the anion-induced states may be involved in intramolecular interactions different from those in the native and molten globules. The second and most important observation is that the modification of tyrosine results in a sharp decrease of the tryptophan fluorescence. Fig. 3 illustrates that the fluorescence of non-modified samples (A-series) is higher than that of selectively (B-series), and entirely (C-series) modified samples. Clearly, the extent of quenching increases in accordance with the nitrotyrosine content. We find that the higher the nitrotyrosine content per protein molecule, the shorter the lifetime of tryptophan fluorescence (data not shown). This means that the quenching observed by the steady state method results from the interaction of the excited tryptophan-donor with the unexcited nitrotyrosine-acceptor and, therefore, may be interpreted in terms of non-radiative transfer of the

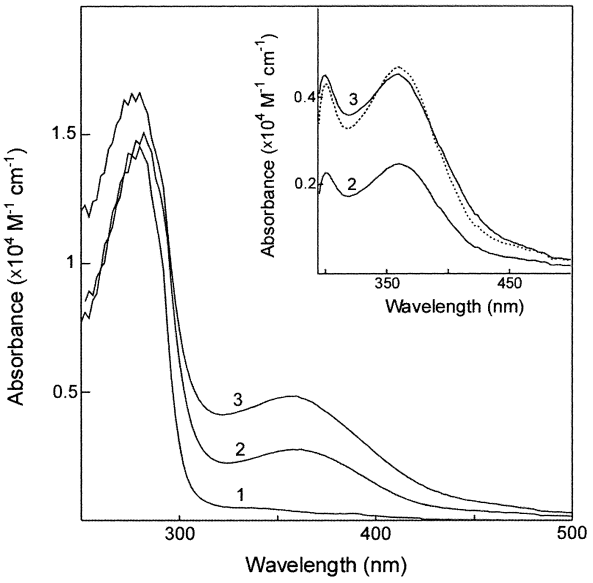

Fig. 1. Absorption spectra of unmodified (1), selectively (2) and entirely (3) modified apomyoglobin. Inset shows the absorbance of 3-nitrotyrosine in samples 2 and 3. Dotted curve simulates two-fold increase of the absorbance of sample 2. Native state: 10 mM sodium acetate-10 mM sodium phosphate buffer with 30 mM NaCl, pH 6.5.

Table 1
Parameters of Trp → Tyr(NO_2) energy transfer in apomyoglobin

System	Conditions	ϕ_D (%)	n	$\langle k^2 \rangle_{eff}$	$\langle J \rangle$ ($\times 10^{-14}$ M^{-1} cm^3	R_o (Å)
Native, apoMb[a]	pH 6.5	8	1.333	0.18	2.9	22
Intermediate 1	pH 2 (50 mM NaTCA)	1.6	1.335	0.18	2.8	17
Intermediate 2	pH 2 (100 mM NaTFA)	8.8	1.334	0.18	2.85	22
Intermediate 3	pH 2 (100 mM $NaClO_4$)	8.3	1.334	0.18	2.85	22
Molten globule[a]	pH 4.2 (30 mM NaCl)	13	1.341	0.18	3.0	24
Intermediate 4	pH 2 (500 mM NaCl)	8.5	1.338	0.18	2.85	22
Acid unfolded	pH 2 (10 mM HCl)	7	1.341	0.67	3.5	28

[a]Protein was dissolved in 10 mM sodium acetate-10 mM sodium phosphate buffer.

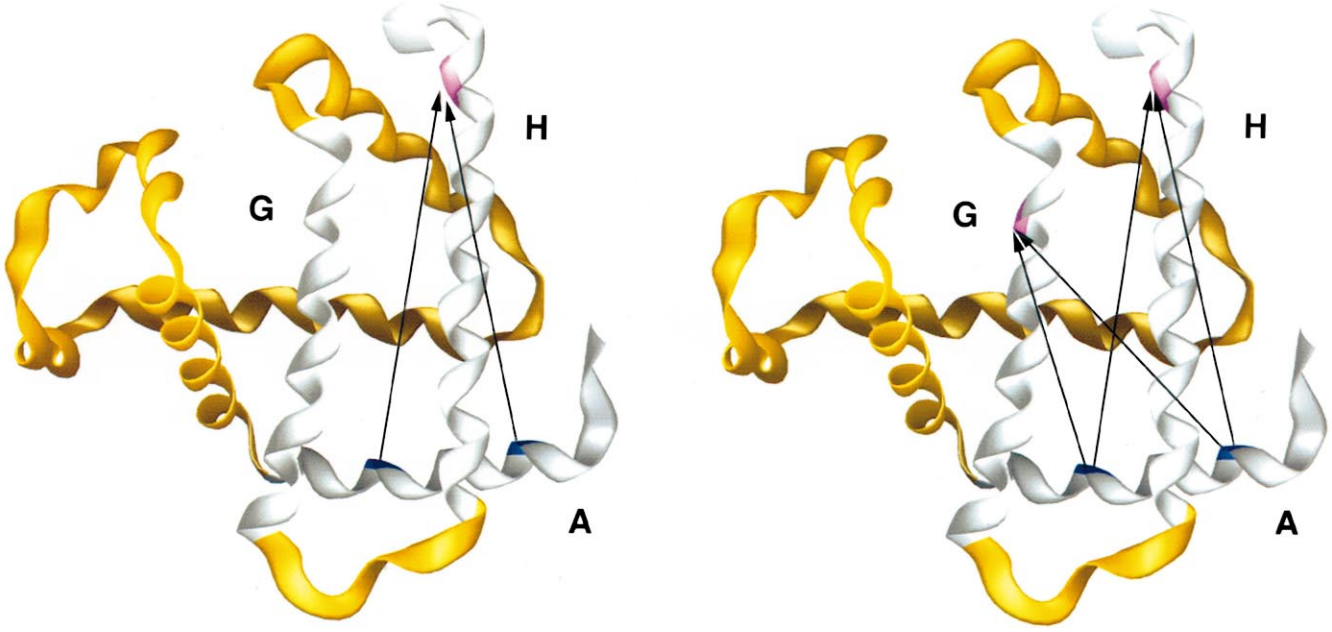

Fig. 2. Schematic picture of apomyoglobin selectively (left) and entirely (right) modified with tetranitromethane. Arrows show the direct energy transfer from tryptophan-donor (blue) to 3-nitrotyrosine-acceptor (pink).

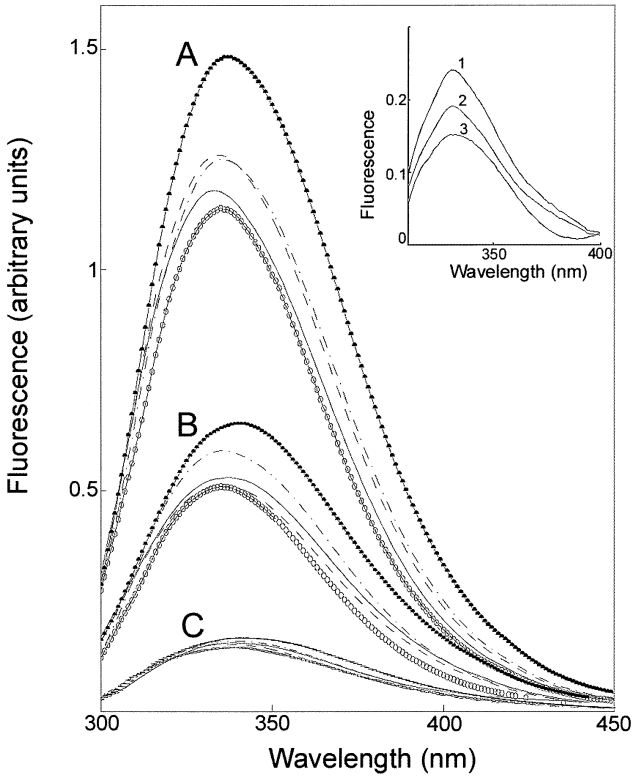

Fig. 3. Fluorescence spectra of unmodified (A), selectively (B) and entirely (C) modified apomyoglobin in different conformational states: native (○), molten globule (●), NaCl (dashed line), $NaClO_4$ (line), TFANa (dot-line-dot). Inset shows the spectra measured in TCA-induced state for unmodified (1), selectively (2), entirely (3) modified apomyoglobin. Native state: 10 mM sodium acetate-10 mM sodium phosphate buffer with 30 mM NaCl, pH 6.5. Molten globule state: 10 mM sodium acetate-10 mM sodium phosphate buffer with 30 mM NaCl, pH 4.2. Anion-induced states (10 mM HCl, pH 2): NaCl (500 mM), $NaClO_4$ (100 mM), TFANa (100 mM), NaTCA (50 mM) at 20°C.

electronic excitation energy of the tryptophan to the nitrotyrosine.

In these experiments we measure the apoMb fluorescence and calculate the energy transfer efficiency due to the particular acceptor present. Specifically, the energy transfer in the selectively modified samples reflects the interactions of Trp residues with Tyr-146(NO_2) only; whereas, in the entirely modified samples it reflects the interaction with both Tyr-146(NO_2) and Tyr-103(NO_2). In view of the additive character of the energy transfer [32,33], the efficiency for each acceptor may be calculated as follows:

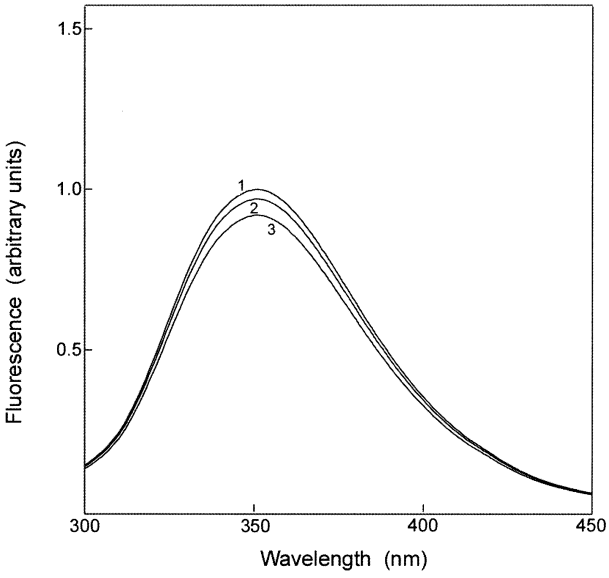

Fig. 4. Fluorescence spectra of unfolded apomyoglobin (10 mM HCl) with different extents of tyrosine modification: unmodified (1), selectively (2), entirely (3).

Table 2
Trp→Tyr(NO_2) direct energy transfer in apomyoglobin

System	Conditions	Sample	$\phi^{a}_{relative}$	R/R_o	$\langle R_{(7-14)-146}\rangle$ (Å)	$\langle R_{(7-14)-103}\rangle$ (Å)
Holomyoglobin					22	24
Native, apoMb	pH 6.5	*Non-modified*	1.14			
		Selectively	0.51	0.97	22	
		Entirely	0.14	1.11		24
Intermediate 1	pH 2, 50 mM NaTCA	*Non-modified*	0.23			
		Selectively	0.19	1.29	22	
		Entirely	0.16	1.47		25
Intermediate 2	pH 2, 100 mM NaTFA	*Non-modified*	1.25			
		Selectively	0.59	0.98	22	
		Entirely	0.16	1.13		25
Intermediate 3	pH 2, 100 mM $NaClO_4$	*Non-modified*	1.18			
		Selectively	0.53	0.96	21	
		Entirely	0.14	1.12		25
Molten globule	pH 4.2, 30 mM NaCl	*Non-modified*	1.48			
		Selectively	0.55	0.92	22	
		Entirely	0.17	1.12		27
Intermediate 4	pH 2, 500 mM NaCl	*Non-modified*	1.26			
		Selectively	0.51	0.94	21	
		Entirely	0.14	1.14		26
Unfolded	pH 2	*Non-modified*	1.0			
		Selectively	0.97	1.78	> 50	
		Entirely	0.92	1.63		> 50

[a]Relative quantum yields in refer to that in acid unfolded state, $\phi_{un} = 0.07$ [20].

$$E_{146} = E_{\text{selectively}} \quad (5)$$

$$E_{103} = E_{\text{entirely}} - E_{\text{selectively}} \quad (6)$$

Finally, with the fluorescence data summarized in Table 2 we calculate, using Eqs. (2)–(6) the average distance from the Trp-7-Trp-14 segment to the Tyr-103 and/or Tyr-146 residues for each protein state of interest.

As expected, in unfolded molecules the tryptophan fluorescence is almost unaffected by the presence of the nitrotyrosine (Fig. 4). The energy transfer efficiency is very small: it does not exceed 8% for entirely modified sample. This indicates that each of the donor-acceptor distances, $\langle R_{(7-14)-103}\rangle$ and $\langle R_{(7-14)-146}\rangle$, is more than 50 Å. In contrast, in the anion-induced states the energy transfer efficiency is 60% for Tyr-146(NO_2) and 30% for Tyr-103(NO_2). We find that the distances $\langle R_{(7-14)-146}\rangle$ in the anion-induced states are very close to each other being about 22 ± 1 Å. Similarly, the distances $\langle R_{(7-14)-103}\rangle$ were found to be 26 ± 1 Å. Unexpectedly, the nature of the anion has no effect on these two key distances of the AGH cluster. Furthermore, $\langle R_{DA}\rangle$ measured at low pH in the presence of anions is very close to that in the native and molten globule states. For instance, the average distances $\langle R$(7-14)-146$\rangle$ and $\langle R_{(7-14)-103}\rangle$ in native molecules are 22 ± 2 Å and 24 ± 2 Å, respectively. Whereas, for the 'classical' molten globule we obtain 22 ± 2 Å and 27 ± 3 Å. The corresponding molecular dimensions calculated from the crystallographic data reported for horse holomyoglobin ([34,35], 1ymb.pdb) have values of 22 ± 2 Å and 24 ± 3 Å. Consequently, the average distances from the Trp-7-Trp-14 segment to Tyr-146 and Tyr-103 in all non-native states of apoMb studied so far coincide with each other to an accuracy of 15%, and are very close to the corresponding dimensions in holomyoglobin.

4. Discussion

Protein molecules in equilibrium intermediate state(s) usually are nearly as compact as native proteins and have a native-like secondary structure [4]. However, some exceptions have been reported for 'anion-induced intermediates' of apoMb [19]. We employed the fluorescence energy transfer technique to measure the distances between α-helices comprising the AGH helical complex, which is considered to be the structured core of the protein. Experiments reveal that the two key distances between the A-, G-, and H-helices remain the same in 'classical' molten globules, anion-induced states, and the native holo- and apomyoglobins. These results do not support the conclusion that apoMb has quite different structures and dimensions in the presence of different anions [19]. Specifically, all non-native forms of apoMb studied so far contain the AGH-core whose overall 3D-structure is similar

Table 3
Properties of different states of apomyoglobin

System	Conditions	$-[\Theta]_{222}$($\times 10^3$ deg cm $dmol^{-1}$)	Refs	Relative volume [19]
Holomyoglobin		21–24	[38,39]	
Native, apoMb	pH (6.5–7.5)	17–23	[19,28,31,38–41]	1
Intermediate 1	pH 2, 50 mM NaTCA	17–19	[19,30]	2.8
Intermediate 2	pH 2, 100 mM NaTFA	14–15	[19,30]	4.3
Intermediate 3	pH 2, 100 mM $NaClO_4$	13	[30]	-
Molten globule	pH 4.2	10–11.5	[28,31]	1.7[a]
Intermediate 4	pH 2, 500 mM KCl	11–13	[19,30,31]	7.4
Unfolded	pH 2	3.5–4	[19,28,31]	11.6

[a]As reported in [21–23].

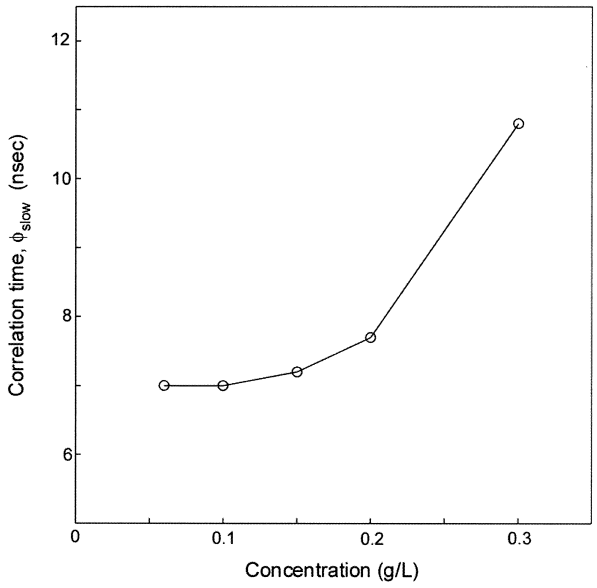

Fig. 5. Effect of apomyoglobin concentration on correlation time ϕ_{slow}, characterizing the protein global rotation. Increase of time shows the increase of the hydrodynamic volume (or molecular weight) of the rotating particle. Conditions are as in Fig. 1.

to that in the native state, i.e. *they share the most important feature of native structure*.

It has been assumed that the 'anion-induced intermediates' (A_k) are discrete states of the folding: i.e. the protein folds through many 'partially folded' states [19,30,36–38]. However, the entirety of all the data on apoMb summarized in Table 3 suggests to us a different scenario. If we agree with the idea that different anions can mimic the different stages of protein folding (A_k), then according to the helicity the folding pathway should be as follows: $N \leftrightarrow A_k \leftrightarrow MG \leftrightarrow U$. Yet, the hydrodynamic data testify to something quite different. The A_k forms have molecular dimensions that are remarkably larger than those of the molten globules. Consequently, the folding pathway is expected to be the following: $N \leftrightarrow MG \leftrightarrow A_k \leftrightarrow U$. This discrepancy prompts us to examine the issue in more detail. Fig. 5 presents our data on concentration dependence of the overall rotation time of native protein obtained by fluorescence anisotropy kinetic experiments. The rotation time shows no significant dependence on the protein concentration in the range up to 0.2 g/l, and then begins to grow with the increase of concentration. This finding indicates that native apoMb has a strong tendency toward aggregation even in highly dilute solutions. As to intermediate forms, their tendency to aggregate is even stronger than that of the native protein [19]. Therefore, for the present study we exclusively used protein concentrations less than 0.2 g/l. Most other techniques employed to investigate apoMb require much higher concentrations of 0.3–40 g/l [19]. Obviously, any type of protein aggregation appears in experimental data as an increase of molecular dimensions. In addition, different molecular characteristics respond to aggregation in different ways. For instance, intrinsic viscosity, being related to the specific molecular volume, is independent of aggregation, which does not effect the specific volume of a protein molecule. In contrast, mean molecular dimensions measured by X-ray and dynamics light scattering are extremely sensitive to any aggregation. Thus, it appears that the unusually large molecular dimensions of A_k forms of apoMb reported in [19] are most likely artifacts due to aggregation.

Ultimately, the advisability of using different anions as a method to study protein folding needs to be addressed. Typically, protein (un)folding is studied in water (pH 7) and at physiological ionic strength ($\sim$0.15 M NaCl). To compare these data with those obtained in the presence of anions it must be demonstrated that the anions do not interact directly with the protein molecule. Yet, by no means is this the case for anions, which can specifically bind to the protein. Indeed, it was shown that the SO_4^{2-} anion causes the 'intrachain bridge' formations ($N^{\oplus}...SO_4^{2-}...^{\oplus}N$), which provoke the collapse of macromolecule [42,43]. Similar effects have been observed for acetate anions [43]. The ClO_4^- anion is known as a stabilizer of the proton complex between nitrogen atoms of the polymer chain [44]. Finally, the occurrence of specific anion binding to the unfolded chain is supported by the facts [30] that the refolding of the pH-denatured apoMb is generated by a remarkably low anion concentration, and that the anion concentration at the midpoint of transition follows the series: $Fe(CN)_6^{3-} < SO_4^{2-} < TCA^-$. Thus, the stronger the anion ability to form the 'intrachain bridges', the smaller the anion concentration required to provoke the collapse. It is hardly possible to consider these specific forms of protein molecules, partly folded by interactions with 'specific anions', as a model for protein folding.

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