ISSN 0006-2979, Biochemistry (Moscow), 2008, Vol. 73, No. 6, pp. 693-701. © Pleiades Publishing, Ltd., 2008.
Published in Russian in Biokhimiya, 2008, Vol. 73, No. 6, pp. 863-873.

pH-Induced Equilibrium Unfolding of Apomyoglobin: Substitutions at Conserved Trp14 and Met131 and Non-conserved Val17 Positions

A. E. Dyuysekina[1], D. A. Dolgikh[2], E. N. Samatova (Baryshnikova)[1], E. I. Tiktopulo[1], V. A. Balobanov[1], and V. E. Bychkova[1]*

[1]*Institute of Protein Research, Russian Academy of Sciences, ul. Institutskaya 4, 142290 Pushchino, Moscow Region, Russia; fax: (495) 632-7871; E-mail: protres@protres.ru; bychkova@vega.protres.ru*
[2]*Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya 16/10, 117871 Moscow, Russia; fax: (495) 336-2000; E-mail: batch2k@yandex.ru*

Received September 4, 2007
Revision received October 18, 2007

Abstract—A number of residues in globins family are well conserved but are not directly involved in the primary oxygen-carrying function of these proteins. A possible role for these conserved, non-functional residues has been suggested in promoting a rapid and correct folding process to the native tertiary structure. To test this hypothesis, we have studied pH-induced equilibrium unfolding of mutant apomyoglobins with substitutions of the conserved residues Trp14 and Met131, which are not involved in the function of myoglobin, by various amino acids. This allowed estimating their impact on the stability of various conformational states of the proteins and selecting conditions for a folding kinetics study. The results obtained from circular dichroism, tryptophan fluorescence, and differential scanning microcalorimetry for these mutant proteins were compared with those for the wild type protein and for a mutant with the non-conserved Val17 substituted by Ala. In the native folded state, all of the mutant apoproteins have a compact globular structure, but are destabilized in comparison to the wild type protein. The pH-induced denaturation of the mutant proteins occurs through the formation of a molten globule-like intermediate similar to that of the wild type protein. Thermodynamic parameters for all of the proteins were calculated using the three state model. Stability of equilibrium intermediates at pH ~4.0 was shown to be slightly affected by the mutations. Thus, all of the above substitutions influence the stability of the native state of these proteins. The cooperativity of conformational transitions and the exposed to solvent protein surface were also changed, but not for the substitution at Val17.

DOI: 10.1134/S0006297908060102

Key words: apomyoglobin, conserved residues, protein stability, protein unfolding, intermediate state

During the last 15 years, an increased understanding of protein folding has been achieved, especially for small proteins and protein domains, which fold via a two-state process [1]. However, for the majority of proteins the folding process involves the formation of one or several intermediate states. To understand the folding mechanism of such proteins, it is useful to study the intermediate and transition states formed in the process of folding. It should be mentioned that intermediate states are metastable; they accumulate in the course of folding and can be studied directly by a number of spectroscopic methods. Transition states correspond to the barriers with maximal free energy on protein folding pathways; they are unstable and can be studied only indirectly through the effect produced by amino acid residue substitutions on the protein folding/unfolding rate.

A number of models for the mechanism of protein folding have been proposed, including nonspecific collapse [2, 3], framework or hierarchical models [4-7], diffusion–collision [8, 9], and a nucleation-growth mechanism, which involves the formation of a transition state [10-12]. The latter is based on two models of the transi-

Abbreviations: apoMb) apomyoglobin; 3D structure) three-dimensional structure; N, I, U, MG) native, intermediate, unfolded, and molten globule states, respectively; [Θ]) molar ellipticity; WT) wild type apomyoglobin.

* To whom correspondence should be addressed.

tion state—a nonspecific one, demanding a certain number of native-like contacts [12-14], and a specific one, depending on formation of a specific subset of the native structure (a folding nucleus) [10, 15, 16].

Several theoretical approaches have been developed to predict the folding nucleus. One of these shows that there is a distinct folding nucleus in various designed sequences with low energy displayed by their "native structure" [10, 17]. In 1996, Shakhnovich et al. [18] suggested a sequence-alignment method for determining the folding nucleus of a protein with known structure. Application to chymotrypsin inhibitor CI2 allowed a blind prediction of the residues in the folding nucleus. A correlation was observed between the evolutionary conservation of the residues in aligned real sequences homologous to CI2 and involvement of these positions in the folding nucleus as predicted from sequence design simulations [18]. Then a detailed analysis was made of the conservation in the primary sequences of cytochromes [19] and globins [20]. The positions in the primary and 3D (three-dimensional) structures occupied by identical or similar residues were identified. The conserved residues in these proteins can be divided into two sets—those involved in the protein active site (functional) and others (nonfunctional conserved residues). Ptitsyn suggested [20] that the nonfunctional conserved residues form the specific folding nucleus in the corresponding protein. A similar analysis was also carried out for the families of three structurally related proteins—ubiquitin, raf-kinase, and ferredoxin [21]. A detailed description of these and other theoretical approaches is given in a recent review [22].

To test the hypothesis [20] that conserved nonfunctional residues are involved in the folding nucleus, a series of mutations in apomyoglobin have been made; the equilibrium and kinetic properties of wild type (WT) apoMb (apomyoglobin) were characterized in detail previously [23-43]. Our experimental approach includes the investigation of equilibrium and kinetic properties of mutant proteins along with calculations of parameters characterizing the folding nucleus, assuming that the rate-limiting step in protein folding is the formation of a specific transition state with native-like contacts between the residues in the folding nucleus [10, 18].

Under equilibrium conditions, apoMb undergoes transitions from the native (N) to completely unfolded (U) state upon addition of urea or guanidine, or to substantially unfolded state at low pH. Similarity of the unfolded state at pH 2.0 to that obtained by urea was shown by NMR spectroscopy [36]. Equilibrium pH-induced denaturation of apoMb proceeds from N to U via a stable molten globule (MG) intermediate state. Following rapid dilution of completely unfolded protein in about 6 M urea by the native buffer (where the protein has the native conformation) the protein folds via an early kinetic intermediate state [25] that corresponds to the detectable equilibrium intermediate MG state [24]. This makes apoMb an excellent model for the understanding of folding mechanism of proteins whose folding process follows via an intermediate state.

ApoMb is a member of the large globin family whose amino acid sequences are diverse but whose overall folding patterns are similar. It has been suggested that the structural similarity of folding patterns is due to a few key nonfunctional residues that ensure fast and correct protein folding [18-20, 44]. There are only six nonfunctional conserved positions in all 728 globin sequences [20]: Val10 and Trp14 located on the A helix (positions A8 and A12), Ile111 and Leu115 on the G helix (positions G12 and G16), and Met131 and Leu135 on the H helix (positions H8 and H12). These residues are shown on the 3D structure of myoglobin in Fig. 1, together with the non-

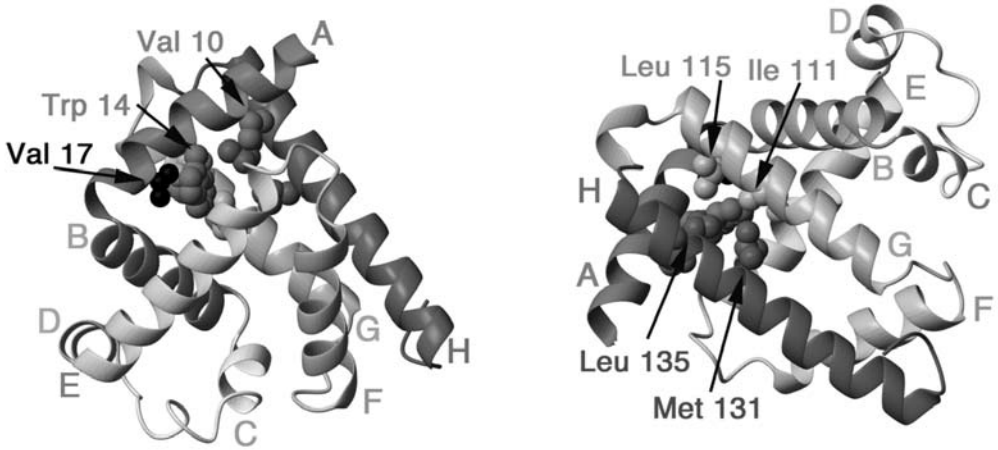

Fig. 1. 3D structure of apomyoglobin showing the location of the conserved core residues: Val10 and Trp14 on A the helix, Ile111 and Leu115 on the G helix, and Met131 and Leu135 on the H helix. All of these residues except Val10 form part of the apomyoglobin hydrophobic core. The non-conserved Val17 is also shown in the left view.

conserved Val17 residue located at position A15 of the A helix; this residue was chosen for comparison with the conserved, nonfunctional residues as it is present in the hydrophobic core of apoMb but is not conserved. Each of the residues was substituted by Ala, and in addition, for Trp14, an aromatic residue was substituted by an aliphatic or by a smaller aromatic one, and for Met131, an aliphatic residue by an aromatic one.

In this paper we report the far UV circular dichroism (CD), Trp fluorescence, differential scanning microcalorimetry, and fluorescence anisotropy studies designed to elucidate the equilibrium properties of the Trp14Phe, Trp14Met, Trp14Ala, Met131Ala, Met131Trp, and Val17Ala as results of the first step of our investigations. It was shown that substitutions of the conserved residues Trp14 and Met131 influenced protein stability, while substitution of the non-conserved Val17 with Ala gave a strong decrease in protein stability. However, the effect of all these substitutions on the intermediate state stability was not so strong. The Φ_I parameter showing a portion of contacts formed by a residue in the MG state has been estimated as 0.3-0.4 for conserved residues and as zero for Val17. The next step of the study that concerns folding kinetics of these proteins is currently in progress.

MATERIALS AND METHODS

Preparation of mutant proteins. Mutant myoglobin genes were prepared by PCR-mediated site-directed mutagenesis [45], starting from a plasmid containing the sperm whale wild type gene. Plasmids obtained for mutant proteins were expressed in *Escherichia coli* BL21(DE3) cells containing the DnaY coexpression plasmid at 37°C. Cells were lysed using lysozyme, and proteins were purified from the inclusion bodies by reverse-phase HPLC as previously described [46]. Protein yields per liter of medium were between 80 and 130 mg. The lyophilized protein was checked for purity by SDS gel electrophoresis and mass spectrometry. Protein concentrations were determined spectrophotometrically using the known extinction coefficient of the WT apoMb (15,900 $M^{-1}\cdot cm^{-1}$ at 280 nm) [47]. The extinction coefficients of the mutant proteins were determined using absorption spectroscopy, measuring protein concentration by nitrogen content determination [48]. For proteins with substitutions of Trp14 by Met, Phe, and Ala the extinction coefficient was found to be the same within experimental error and equal to 10,840 $M^{-1}\cdot cm^{-1}$ and for the mutant protein with substitution of Met131 by Trp the extinction coefficient was 21,680 $M^{-1}\cdot cm^{-1}$. The extinction coefficient of other mutant proteins was the same as that of WT apoMb.

Circular dichroism. Equilibrium CD experiments were performed with the Aviv 60 DS spectropolarimeter equipped with thermoelectric temperature control unit. An automatic titrator was used for some of the experiments. Far UV CD spectra were measured using a 0.1 cm cuvette at 25°C. Protein concentration was 35-40 μM (0.63-0.72 mg/ml). For pH-induced titrations, the detection wavelength was 222 nm and a 5-min time-average was performed for each titration point. All ellipticity values were baseline corrected.

Total tryptophan fluorescence. Fluorescence measurements were made using an Aviv 60 DS spectropolarimeter. The excitation wavelength was 278 nm and the emission spectra were detected in the 320-550 nm region using optical cutoff filter S3S21.

Differential scanning microcalorimetry. Microcalorimetric measurements were made on a SCAL-1 differential scanning microcalorimeter (Scal Co. Ltd, Russia), equipped with 0.3 ml glass cells. The heating rate was 1 K/min. The protein concentration was 0.8-2.0 mg/ml. All solutions were equilibrated overnight with a corresponding buffer by dialysis. Enthalpy of heat denaturation, ΔH_{cal}, was calculated according to the method of Privalov and Potekhin [49].

Calculations of energy parameters. The changes in free energy of pH-induced unfolding in water ΔG upon transition from N to I (ΔG_{N-I}) and from I to U (ΔG_{I-U}) can be calculated using equations based on the three-states model [50, 51]. The ΔG value was extrapolated to the ΔG value at pH 4.2 ($\Delta G^{4.2}$) similarly distant from both transitions to avoid a long extrapolation to pH 6.2 leading to considerable inaccuracy. The slope of the ΔG versus pH dependences is determined by the m value [52-54] from the three-states equation, which reflects cooperativity of the respective transition and is proportional to an increase in the degree of exposure of the protein surface to solvent upon denaturation:

$$\Delta G_i = \Delta G_i^o - m_i [C],$$

where i is the respective transition, (N–I) or (I–U), and C is denaturant concentration.

Fersht's parameter for the equilibrium intermediate state (Φ_I), calculated in much the same way as Φ for the transition state [55, 56], was determined as the ratio of changes in stabilities of the protein intermediate state ($\Delta\Delta G_{I-U}$) and its native state ($\Delta\Delta G_{N-U}$) relative to the denatured state after the corresponding mutation:

$$\Phi_I = \Delta\Delta G_{I-U}/\Delta\Delta G_{N-U},$$

where

$$\Delta\Delta G_{I-U} = \Delta G_{I-U}(\text{mutant}) - \Delta G_{I-U}(\text{WT}),$$

$$\Delta\Delta G_{N-U} = \Delta G_{N-U}(\text{mutant}) - \Delta G_{N-U}(\text{WT}),$$

and

$$\Delta G_{N-U} = \Delta G_{I-U} + \Delta G_{N-I}.$$

This parameter gave information on the influence of the corresponding substitution on energetic parameters of the intermediate state compared to the native and completely unfolded states under equilibrium conditions. Using this value, it is possible to estimate the portion of contacts formed by the corresponding residue in the intermediate state.

RESULTS

Effects of mutations on protein stability. To test the effects of nonfunctional conserved residues on apoMb folding and stability, substitutions of various amino acids were made for the conserved residues Trp14 and Met131 and for the non-conserved Val17. Equilibrium properties of the mutants were studied at 25°C and compared with those of the WT protein and also used to estimate conditions for kinetic measurements. The stability of the mutant proteins towards increasing temperature was measured by scanning differential microcalorimetry. The Trp14Ala mutant protein was strongly destabilized at pH 5.0, used by Griko et al. [23], and even under conditions where the WT protein was native, did not show the melting peak. The pH-dependence of thermal melting for this protein was studied to find conditions similar to the behavior of the WT protein (data not shown). Only at pH 6.2, the melting peak for Trp14Ala mutant protein was comparable to that of the WT protein, which proved important for subsequent investigation of protein unfolding/folding kinetics. Thus, this pH 6.2 value was selected as a condition under which stabilities of all apomyoglobins were studied and compared. The results of microcalorimetric studies at pH 6.2 are shown in Table 1. A decrease of the denaturation temperature T_d relative to the WT protein was observed for all mutant proteins except Met131Trp. Heat-induced denaturation of all of the mutants was a cooperative process, as well as for the WT protein, where transition from the native to temperature denatured state with substantial secondary but without rigid tertiary structure was observed [23]. However, the calorimetric enthalpy for all mutants is smaller than that of the WT protein, indicating, together with decreased melting temperature, a decrease in their thermodynamic stability compared to the WT protein. The most significant decrease in T_d was observed for the Trp14Ala mutant, which also had the lowest value of ΔH_{cal} (Table 1). As shown by Griko et al. [23], thermal melting of the apoMb intermediate at pH 4.2 is accompanied by linearly increasing heat capacity without a heat absorption peak. It is the absence of this peak that is evidence for the absence of rigid tertiary structure. The presence of the heat absorption peaks for all of the mutants indicated the presence of their tertiary structures equivalent to that of the WT protein. It should be mentioned that thermal denaturation of mutant proteins is not completely reversible, as in the case of WT apoMb [23]. This may be connected with its ability to associate at increasing pH, whereas its cold denaturation is quite reversible [23]. However, this fact still allows determining the heat melting temperature as a temperature at which the heat absorption peak occurs.

Table 1. Thermodynamic parameters of heat denaturation of apomyoglobin mutants with substitutions in the hydrophobic core at pH 6.2

Protein	T_d* (°C)	ΔH_{cal}** (kcal/mol)
WT	62.4	72
Trp14Phe	56.2	49
Trp14Met	57.1	70
Trp14Ala	45.4	48
Met131Trp	64.2	50
Met131Ala	52.8	53
Val17Ala	59.5	67

* Experimental error of T_d determination was 0.3°C.
** Calorimetric enthalpy of protein denaturation, standard error is 7%.

Another technique to check the stability of proteins is circular dichroism. The far UV CD spectra of all mutants at 25°C are presented in Fig. 2, together with the spectrum of WT protein. The characteristic shape and intensity of the spectra show that all of these mutant proteins contain a high content of helical structure but generally less than that of the WT protein. The largest changes in molar ellipticity [Θ] at 222 and 208 nm are seen in the Trp14Ala and Trp14Met mutants; the Trp14Phe mutant is affected to a lesser extent.

This effect is probably due to destabilization of the A helix by the substitution of a large hydrophobic group with that having smaller side chains. A similar observation was made when destabilizing mutations (substitutions by Gly) were introduced into the apoMb H helix [40]. Alternatively, the ellipticity decrease upon substitution of Trp14 may be due to the disruption of stabilizing contacts of this residue with other helices, which can lead to loosening of tertiary structure that is important for formation of the A[B]GH helical complex in the intermediate and native states. This effect is too large to be explained by the influence of aromatic groups on far UV CD spectra due to a decrease in the content of tryptophans [57].

Influence of mutations on pH-induced unfolding. Despite some decrease of helical content in the native state at pH 6.2, the mutant proteins all undergo pH-induced transitions from N to I and from I to U in a manner similar to the WT protein. Unlike thermal denaturation, pH-induced unfolding is completely reversible. These transitions, monitored by both molar ellipticity at

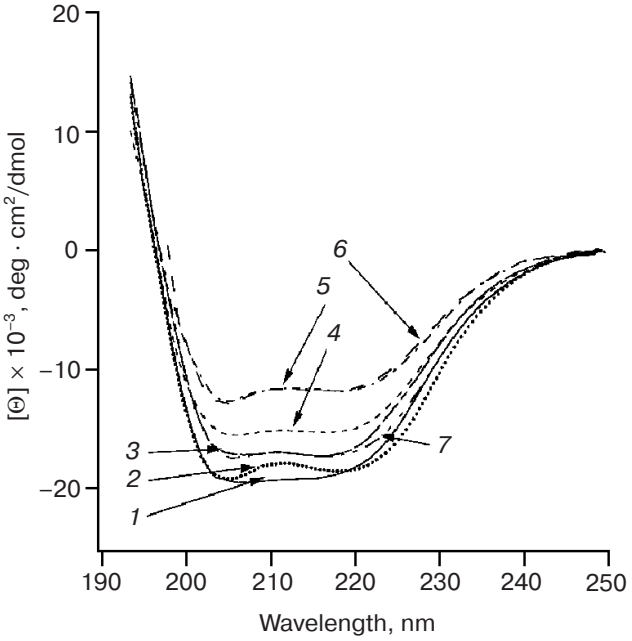

Fig. 2. Far UV CD spectra of the wild type and mutant apoMbs under native conditions (10 mM sodium acetate buffer, pH 6.2, 25°C). Protein concentration was 35-40 μM, cell pathlength 0.1 cm. Curves: *1*) WT; *2*) Met131Trp; *3*) Met131Ala; *4*) Trp14Phe; *5*) Trp14Ala; *6*) Trp14Met; *7*) Val17Ala.

222 nm and fluorescence, at 25°C are presented in Figs. 3-5 (a and b). Calculations of the intermediate state portion are also presented (Figs. 3c-5c). The appearance of maxima in the fluorescence data (Figs. 3b-5b) indicates directly the formation of an intermediate state in pH-induced denaturation. This fact is connected with the increase of Trp fluorescence (strongly quenched by surrounding residues in the native state) upon protein unfolding due to a change in distance between Trp and quenchers of its fluorescence and in their mutual orientation. The position of each fluorescence maximum corresponds to the inflection point in the $[\Theta]_{222}$-versus-pH plot (Figs. 3a-5a) for each mutant protein. The intensity maximum is the highest for the mutant Met131Trp (Fig. 4) due to the presence of the additional Trp residue, as well as a contribution from the absence of Met131 itself (see the experimental curve for Met131Ala), which is a major source of fluorescence quenching for Trp14 at pH below 7.0 [58]. The compactness of the MG state was assessed for each of the mutant proteins by fluorescence anisotropy measurements (data not shown).

The vertical inflection points in the fluorescence curves, which define the half-maximal concentration of N and MG states, occur between pH 4 and 6. A shift of this inflection point to higher pH is an indication that stability of the protein has decreased. A significant increase is observed for Trp14Ala, from pH 4.35 for WT to pH 5.6 for Trp14Ala, but all of the mutants with the exception of

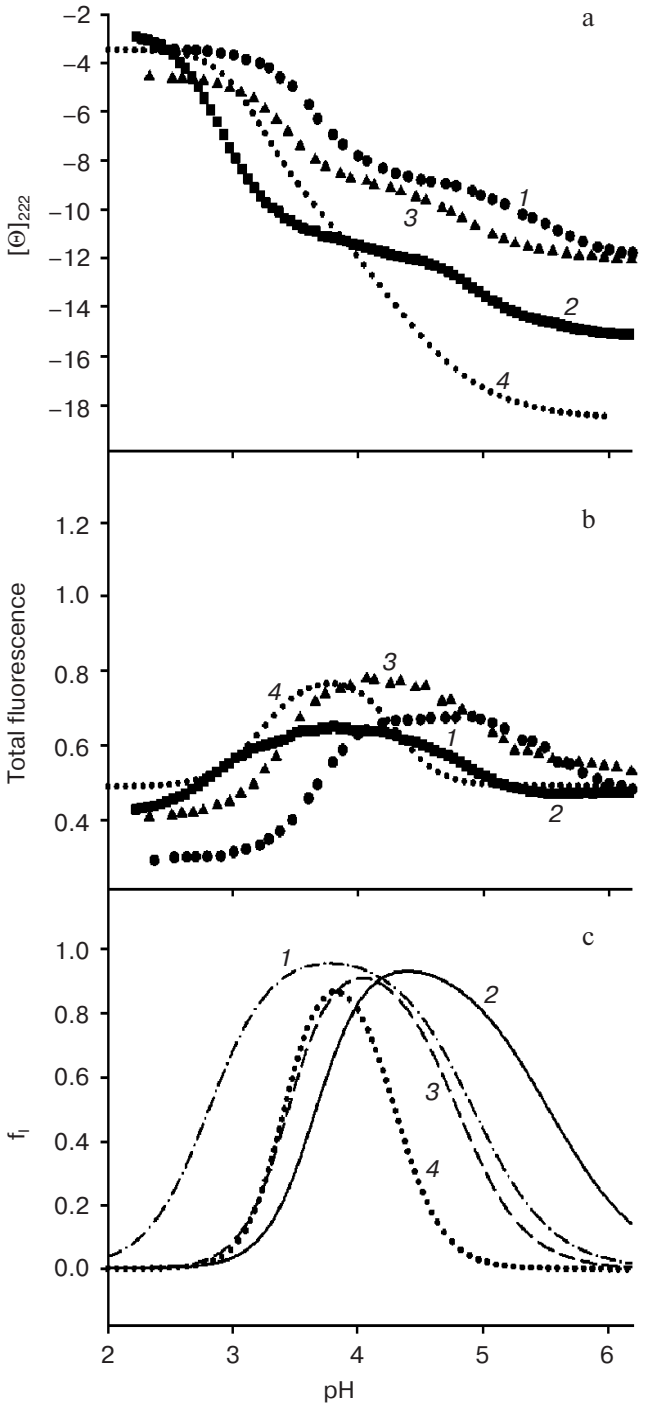

Fig. 3. pH-induced unfolding of the wild type and mutant apoMbs with substitutions in position of Trp14. a) Changes in molar ellipticity $[\Theta]_{222}$ (10^{-3} deg·cm^2/dmol) monitored by far UV CD at 222 nm at 25°C in 10 mM sodium acetate buffer. Protein concentration was 3 μM, cell pathlength 1 cm. b) Changes in the total fluorescence intensity for wild type and mutant apoMbs under the same conditions. The excitation wavelength was 278 nm and the emission was detected at the 320-550 nm using a 320 nm optical cutoff filter. Designations of curves in (a) and (b): *1*) Trp14Ala; *2*) Trp14Phe; *3*) Trp14Met; *4*) WT (data for the WT protein are shown for comparison). c) Calculated portion of the intermediate state f_i: *1*) Trp14Phe; *2*) Trp14Ala; *3*) Trp14Met; *4*) WT.

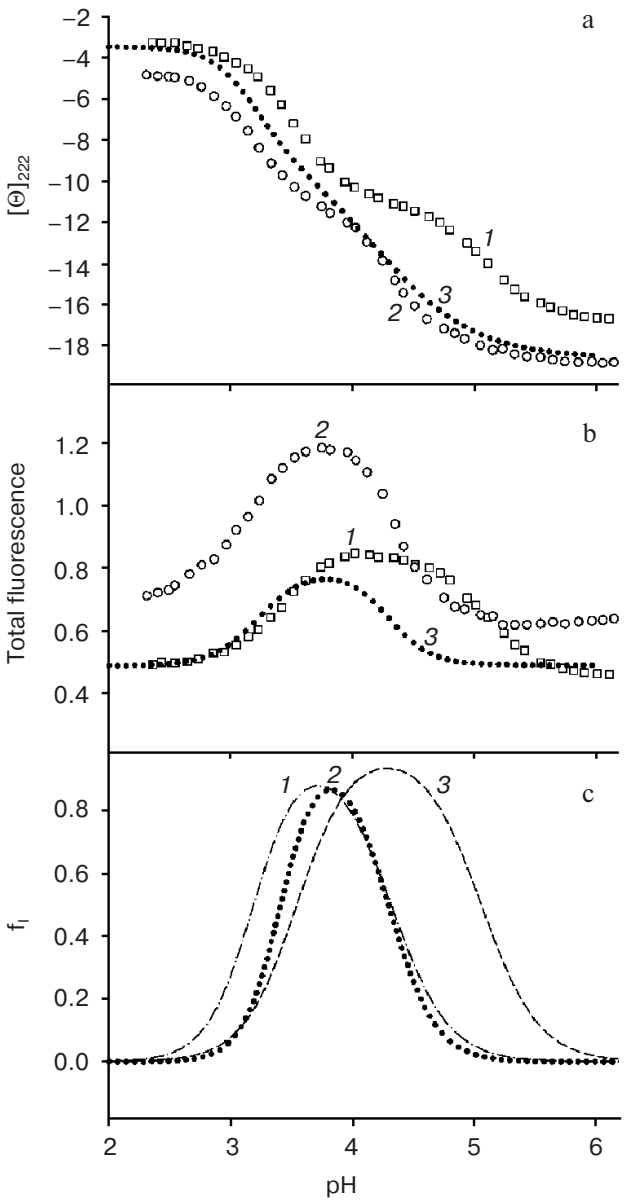

Fig. 4. pH-induced unfolding of the wild type and mutant apoMbs with substitutions in position of Met131. a) Changes in molar ellipticity $[\Theta]_{222}$ (10^{-3} deg·cm^2/dmol) at 222 nm; conditions the same as in Fig. 3. b) Changes in total fluorescence intensity for wild type and mutant apoMbs under the same conditions as in Fig. 3. Designations of curves in (a) and (b): *1*) Met131Ala; *2*) Met131Trp; *3*) WT. c) Calculated portion of the intermediate state f_I: *1*) Met131Trp; *2*) WT; *3*) Met131Ala.

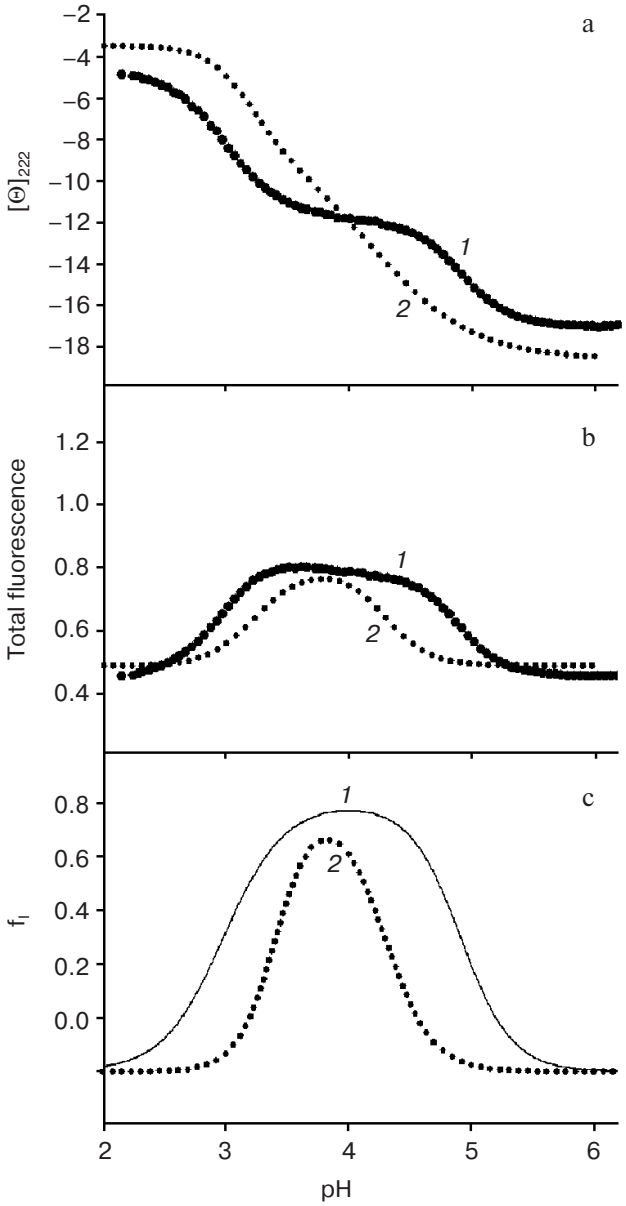

Fig. 5. pH-induced unfolding of the wild type and mutant apoMb with substitution in position of Val17. a) Changes in molar ellipticity $[\Theta]_{222}$ (10^{-3} deg·cm^2/dmol) monitored by far UV CD at 222 nm at 25°C; conditions the same as in Fig. 3. b) Changes in the total fluorescence intensity for wild type and mutant apoMb under the same conditions as in Fig. 3. Designations of curves in (a) and (b): *1*) Val17Ala; *2*) WT. c) Calculated portion of the intermediate state f_I: *1*) Val17Ala; *2*) WT.

Met131Trp appear to have been affected (Table 2). In addition, the mutations appear to have had less effect on the midpoint of the transitions from MG to U, which all appear to occur with a midpoint around pH 2.8-3.4 (Table 2). The Trp14Ala mutant again appears to be an outlier. The observed movements of the transition midpoints in the N–MG transition and practically unchanged in the MG–U one cause the molten globule state of mutant proteins to be observed over a wider pH range and with a different pH of maximal formation compared to the WT protein (Figs. 3-5, Table 2). These measurements establish a hierarchy of pH stability for the mutant proteins of: WT and Met131Trp > Trp14Phe > Met131Ala and Trp14Met > Trp14Ala.

To estimate the contribution of the studied residues to the intermediate state formation, the parameter Φ_I,

Table 2. Thermodynamic parameters of pH-induced transitions*

Protein	$\Delta G^{4.2}_{N-I}$, kcal/mol	m_{N-I}, kcal/mol·pH unit	pH of the middle transition N–I, where $\Delta G_{N-I} = 0$, pH unit	$\Delta G^{4.2}_{I-U}$, kcal/mol	m_{I-U}, kcal/mol·pH unit	pH of the middle transition I–U, where $\Delta G_{I-U} = 0$, pH unit	$\Delta\Delta G_{N-U}$, kcal/mol	$\Delta\Delta G_{I-U}$, kcal/mol	Φ_I
WT	0.3	–3.0	4.3	–2.9	–3.7	3.4			
Trp14Ala	2.2	–1.5	5.6	–1.8	–2.8	3.6	3.0	1.1	0.4
Trp14Met	1.2	–2.1	4.8	–2.5	–3.1	3.4	1.3	0.4	0.3
Trp14Phe	1.3	–1.8	4.9	–3.1	–2.3	2.8	0.8	–0.2	–0.3
Met131Ala	2.0	–2.4	5.1	–1.8	–2.6	3.5	2.9	1.1	0.4
Met131Trp	0.3	–2.4	4.3	–3.0	–3.0	3.2	–0.1	–0.1	–**
Val17Ala	1.9	–2.7	4.9	–2.8	–2.3	3.0	1.8	0.1	0.1

* Experimental error at pH measurement are ±0.05 pH unit; ΔG, ±0.3 kcal/mol; Φ_I, ±0.1; m, ±0.2.
** Φ_I cannot be determined in this case due to incertitude of 0/0.

showing the portion of contacts formed by this residue in the intermediate state, was calculated (Table 2). Knowledge of Φ_I allows determining the influence of mutations on energetic parameters of the intermediate state and estimating the portion of contacts formed by the corresponding residue in this state. Its advantage in comparison with calculation of solely thermodynamic parameters and population of various conformational states is evident.

As seen from Table 2, Trp14 and Met131 residues have a 30-40% share of contacts in the MG state, while Val17 practically does not form any in this state.

It should be emphasized that despite destabilization of the mutant apoMb structures, all of them can bind the heme group and form appropriate holoproteins (data not shown).

DISCUSSION

The data on pH-induced denaturation show that all mutant proteins undergo a two-stage transition with a highly populated intermediate state. The agreement between CD and fluorescence results suggests simultaneous changes in the protein secondary and tertiary structure formation, which is confirmed by decrease of Trp fluorescence at pH 6.2 due to quenching of Trp fluorescence by the protein environment, indicating the cooperativity of the folding process. Except for the Met131Trp apoMb (which shows behavior very close to that of the WT protein), the folded ("native") structure of all mutant proteins is strongly destabilized relative to that of the WT apoMb, according to the decreased melting temperature (Table 1), decreased UV CD signal at pH 6.2, and the shift of the midpoint of the N–I transition to higher pH values (Figs. 3-5). Destabilization of the intermediate state is less significant, but substitutions of Trp14 in the A helix and Met131 in the H helix lead to a decrease in the helical content relative to that formed in the WT protein in both native and intermediate states (Figs. 3a-5a).

For Trp14Phe and Val17Ala (and to less extent for Met131Trp) apparent stabilization of the intermediate state is observed (pH value of the I–U transitions are slightly shifted towards lower pH; Figs. 3-5, a and b). For these mutant proteins, the range where the intermediate state is observed is substantially wider in comparison to the WT protein. However, due to different slopes of the left branches of the f_I-versus-pH curves for the mutants and the WT protein, different ΔG_{N-I} values were obtained from calculation of changes in ΔG. This resulted in some stabilization of the I-state for Trp14Phe and in slight destabilization for Val17Ala. A final conclusion about the stabilizing role of these residues in the intermediate state formation can be made only after investigation of their unfolding/refolding kinetics, which is in progress.

As shown earlier [40], destabilization of the apoMb H helix by substitution of Asn132 and Glu136 by Gly caused changes in the structure and dynamics of the molten globule state of apoMb at pH 4.1. Both WT and mutant proteins were shown to fold via a compact intermediate state, but the secondary structure content and its location differ significantly between the two intermediates. In our case the introduced substitutions lead to up to 30% decrease of the helical content in the intermediate state (Figs. 3a-5a) and may cause slight disruptions in the hydrophobic packing as well as destabilization of the A or H helix by Trp14 and Met131 substitutions, respectively. It is important to stress that all mutant proteins are fold-

ing-competent despite the substitutions, and after reconstitution with heme they are all able to form the fully-structured holoproteins [61]. This is true even for the Trp14Ala mutant protein.

Evidence is growing that native-like tertiary and secondary structures formed during the folding process are important features for fast and correct protein folding [59]. Quench-flow NMR experiments on apoMb mutants are also consistent with this: destabilization of secondary structure in the intermediate [60, 61] or stabilization of hydrophobic interactions in the core and hence secondary structure [62] lead to changes in the apoMb folding pathway. Instead of the formation of a folding intermediate consisting of the ABGH helical complex characteristic of the WT protein, destabilization of the H helix changes the structure of the intermediate to an ABG complex, while the stabilizing mutation His64Phe promotes formation of an intermediate state containing the ABEGH helices. Recent data on mutations in the B helix [60] show that these substitutions not only destabilize this helix in the intermediate state formation but also induce interactions between B and E helices that are absent from the native protein. These results emphasize the importance of the primary and tertiary structure and the native topology in formation of a stable structure of the intermediate molten globule state.

Leghemoglobin (Lb) (a myoglobin analog found in plants) provides an even more interesting case. The A and B helices of Lb are strongly destabilized in comparison to those of sperm whale apoMb [63, 64]. Although Lb folds via a kinetic burst phase intermediate, the structure of the intermediate, which contains the E, G, and H helices, is different from that of apoMb, with A[B]GH. Major differences in primary structure between the two proteins include substitution of Trp14 in the A12 position of the A helix of apoMb for Phe in Lb, Trp7 for Asp, and Leu11 and Val13 for Ser. Similar destabilizing changes occur in the B helix sequence, but there are stabilizing replacements in the H helix of Lb (Ala127 is substituted for Leu, Met131 for Trp, Lys133 for Val). Some of these positions that are substituted in Lb correspond to our mutant proteins. All three mutations at Trp14 cause destabilization of the protein secondary and tertiary structure; it is quite possible that the structure of their intermediates might be similar to that of Lb, with its naturally destabilized A helix. So, it would be interesting to investigate the kinetic behavior of these mutants (appropriate measurements are in progress) and to compare their behavior with that of Lb [64].

Another member of the globin family, the apomyoglobin from *Aplysia limacina*, shows behavior different from that of apoLb or mammalian apoMb. The H helix of *Aplysia* Mb contains a tryptophan at position 130 (analogous to the Met at position 131 that we have mutated to tryptophan in the present study). Trp130 in *Aplysia* apoMb forms four out of six conserved contacts that are formed by Met131 in mammalian apoMb to residues in the A and G helices (identified by Ptitsyn and Ting [20]). It was recently shown [65] that substitution of Trp130 by Tyr in *Aplysia* apomyoglobin destabilizes both the native and acid-induced intermediate, but has almost no effect on the thermally stable core that is specific to the *Aplysia* protein. The results obtained by Musto et al. [65] clearly show that Trp130 is involved in the stabilization of the main intermediate in both *Aplysia* and sperm whale AGH complex. The authors emphasize that compact helical structure of intermediates in the folding of apoMb is tuned by local secondary structure and by tertiary interactions. They mention that Trp130 plays a role that is evolutionarily conserved in the globin family, and that the AGH complex is important in the folding pathway of different globins.

However, additional experimental work is required to elucidate the role of these residues in protein kinetics. It is apparent that pH-induced denaturation of all the studied mutant proteins with substitutions at positions Trp14 and Met131 manifested destabilization of the sperm whale apoMb structure, except Met131Trp where it is similar to the WT protein. The pH range of the MG state becomes significantly wider, and the exposed to solvent protein surface increases for all of the mutants, while cooperativity of the pH-induced transitions decreases, although to a lower extent for Val17. Our results for substitutions at the nonfunctional conserved positions of Met131 and Trp14 in sperm whale apoMb by various amino acid residues emphasize the importance of these positions for folding/unfolding of apomyoglobin and its stability, and correlate with a growing amount of experimental data for other globins.

This work is dedicated to the memory of Prof. Oleg B. Ptitsyn, who initiated this study and inspired it at all initial stages. The authors are indebted to Prof. P. E. Wright for kind cooperation, for the possibility of the short fellowship (for AED and ENS) and the preparation of all plasmids in his lab (for DAD). We are grateful to H. J. Dyson and A. V. Finkelstein for critical reading of the manuscript and fruitful discussion. The authors thank Linda Tennant and Nelly B. Ilyina for excellent technical assistance in protein preparation and in the determination of the extinction coefficients of mutant proteins (NBI).

This research was supported in part by awards RN1-405 and RB2-2022 from the U. S. Civilian Research and Development Foundation for the Independent States of the Former Soviet Union (CRDF), by INTAS grant (05-1000004-7747), by the Program on "Molecular and Cellular Biology" of the Russian Academy of Sciences, and by award (55005607) from the Howard Hughes Medical Institute to A. V. Finkelstein (ENS, VAB, VEB).

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