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pH-dependent stability of sperm whale myoglobin in water-guanidine hydrochloride solutions

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Abstract An experimental-theoretical approach for the elucidation of protein stability is proposed. The theoretical prediction of pH-dependent protein stability is based on the macroscopic electrostatic model for calculation of the pH-dependent electrostatic free energy of proteins. As a test of the method we have considered the pH-dependent stability of sperm whale metmyoglobin. Two theoretical methods for evaluation of the electrostatic free energy and pK values are applied: the finite-difference Poisson-Boltzmann method and the semiempirical approach based on the modified Tanford-Kirkwood theory. The theoretical results for electrostatic free energy of unfolding are compared with the experimental data for guanidine hydrochloride unfolding under equilibrium conditions over a wide pH range. Using the optical parameters of the Soret absorbance to monitor conformational equilibrium and Tanford's method to estimate the resulting data, it was found that the conformational free energy of unfolding of metmyoglobin is 16.3 kcal · mol^{-1} at neutral pH values. The total unfolding free energies were calculated on the basis of the theoretically predicted electrostatic unfolding free energies and the experimentally measured midpoints ($pH_{1/2}$) of acidic and alkaline denaturation transitions. Experimental data for alkaline denaturation were used for the first time in theoretical analysis of the pH-dependent unfolding of myoglobin. The present results demonstrate that the simultaneous application of appropriate theoretical and experimental methods permits a more complete analysis of the pH-dependent and pH-independent properties and stability of globular proteins.

Keywords Sperm whale myoglobin · pH-dependent stability · Guanidine hydrochloride unfolding · Protein electrostatic methods

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Introduction

The stability of a protein, as a natural polyelectrolyte, is generally dependent on pH. The pH dependence reflects the contribution of electrostatic interactions which are essential for the conformational stability of globular proteins (Tanford 1970). There is a fundamental interest in developing theoretical models for the correct evaluation of electrostatic interactions involving conformational changes (You and Bashford 1995; Alexov and Gunner 1997; Rabenstein and Knapp 2001) and their contribution to protein stability (Warshel 1991; Yang and Honig 1993, 1994; Antoziewicz et al. 1994; Schaefer et al. 1997). The presence of a large amount of experimental data for the folding and unfolding processes of myoglobin (Mb) (Irace et al. 1981; Fink 1995) makes it a good candidate to examine the structure and the effect of pH on the stability of the holo- and apoprotein.

Apomyoglobin adopts thermodynamically stable intermediates, referred to as "molten globule" states, upon decrease of the pH, and has been the focus of many thermodynamic and kinetic investigations (Irace et al. 1981; Fink 1995 and references therein; Wang and Tang 1996; Elieser et al. 1998; Kay and Baldwin 1998), as well as theoretical studies (Cocco et al. 1992; Tirado-Rives and Jorgensen 1993; Yang and Honig 1994). However, there are not many experimental data concerning holomyoglobin stability, in particular in the alkaline range. An acid-induced intermediate of horse holomyoglobin has been observed at very low ionic strength and at pH values below pH 3 (Goto and Fink 1994). According to

Puett (1973), the acidic denaturation (pH range 3.5–7.5) of horse and sperm whale holomyoglobin is accompanied by an uptake of six protons by the unfolded protein. This agrees with earlier acidic denaturation studies on sperm whale holomyoglobin in the pH range from 3.5 to 5.25 (Acampora and Hermans 1967).

The electrostatic properties of apomyoglobin and holomyoglobin have been extensively studied. The experimentally measured hydrogen ion equilibrium of the holoprotein has been compared (Shire et al. 1974a, 1974b) with that theoretically calculated by the modified Tanford-Kirkwood (MTK) method (Tanford and Kirkwood 1957; Tanford and Roxby 1972). The pK values of histidines in apomyoglobin and metmyoglobin (a liganded form of holomyoglobin, in which Fe^{3+}-porphyrin binds H_2O) were experimentally determined by NMR spectroscopy (Cocco et al. 1992). It was found that some masked histidines in Mb ionized in the pH range 5–9 are of particular interest for the conformational changes of the denaturation (Breslow et al. 1965; Shire et al. 1974a, 1974b). The protonation behavior of the Mb titratable groups computed by the widely applied finite difference Poisson-Boltzmann method (FDPB) (Gilson et al. 1987) for apomyoglobin (Yang and Honig 1994) and for three different liganded forms of holomyoglobin (Bashford et al. 1993; Rabenstein and Knapp 2001) have been reported.

A detailed theoretical analysis of the forces responsible for apomyoglobin acidic denaturation considering the pH-dependent stability in the pH range 1–8 has been proposed (Yang and Honig 1994). As far as we know, the pH-dependent properties of holomyoglobin have been theoretically examined only by the MTK theory (Matthew and Gurd 1986) in pH range 3.5–11.

Although the native conformation of apomyoglobin is compact and contains a secondary and tertiary structure similar to that of holomyoglobin (Griko et al. 1988; Stigter and Dill 1990; Jorgensen et al. 1993; Barrick et al. 1994), the folding of Mb is completed only upon binding of heme to form the holoprotein. The binding of heme results in further stabilization of the secondary and tertiary structures of the molecule (Goto and Fink 1994). It has been shown that apomyoglobin retains an extended hydrophobic core (Griko et al. 1988; Cocco and Lecomte 1990), contains approximately 20% less helical structure (Acampora and Hermans 1967; Griko and Privalov 1994), is less compact (Griko et al. 1988) and less stable (Griko et al. 1988; Griko and Privalov 1994), with lower heat capacity (Puett 1973) and unfolds less cooperatively (Griko and Privalov 1994) relative to holomyoglobin. Moreover, in contrast to less cooperative apomyoglobin unfolding, metmyoglobin unfolding is highly cooperative and fits in a simple two-state model in which only the native and unfolded forms are populated (Acampora and Hermans 1967; Kherinashvili and Atanasov 1971; Atanasov et al. 1972; Puett 1973; Pace and Vanderburg 1979; Privalov et al. 1986). As a consequence, we consider that holomyoglobin is more appropriate for a detailed elucidation of Mb conformational stability.

In the present work we studied the pH dependence of the stability of sperm whale metmyoglobin (metMb) over a wide pH range (pH 3.5–12.0). The pH-dependent conformational stability was estimated experimentally by means of chemical unfolding in guanidine hydrochloride solutions. Our results do not differ significantly from the data on Mb stability in the acidic region or at single pH values available in the literature (Acampora and Hermans 1967; Puett 1973; Pace and Vanderburg 1979). We calculated the pH-dependent free energies of the native and the denatured Mb structure, following the approach proposed by Yang and Honig (1993), in order to compare the experimentally obtained with the theoretically calculated pH dependence of Mb stability both in acidic and alkaline ranges. We used two different approaches to determinate all electrostatic energy terms: the FDPB and the semiempirical approach (Spassov et al. 1989) based on the MTK method. The theoretical simulations of Mb stability allow a detail analysis of the experimental results. A reasonable agreement has been achieved between the calculated and experimentally measured pH-dependent stability of Mb. The simultaneous theoretical and experimental elucidation gives the opportunity to distinguish between the pH-dependent electrostatic and neutral contributions to the protein stability. The pH-dependent stability term, involving mainly hydrophobic interaction contributions, has been evaluated taking into account the alkaline pH values of the denaturation.

Materials and methods

Materials

Sperm whale skeletal muscle Mb was prepared according to Rothgeb and Gurd (1978). Myoglobin used for these studies was the major (IV) component obtained by CM-Sephadex chromatography. The homogeneity of the preparations was tested by isoelectric focusing on PEG plates. Guanidine hydrochloride (GdnHCl) was purchased from Merck. All of the buffer and other reagents were of analytical grade. The solutions used for the measurements were prepared from deionized water.

Spectral measurements

The absorption values in the ultraviolet and visible spectral ranges were recorded on a Karl Zeiss double beam recording spectrophotometer (Specord UV-VIS). In all cases, 1 cm cuvettes were used. Protein concentrations were determined using $\epsilon_{409} = 171\ mM^{-1}\ cm^{-1}$ in 0.03 M MES-TRIS-Gly cocktail buffer and 0.1 M KCl. The measurements of the unfolding were made at 25 °C on protein solutions of 85 $\mu g\ mL^{-1}$. The change in absorption upon denaturation in the Soret band (near 410 nm) was observed for Mb (Theorell and Ehrenberg 1951), as at native pH values the absorption decreased by a factor of four at 408 nm when the bonds between the protein and the heme group were broken. Thus, this measurement is a sensitive indication of the state of the heme environment of the molecule. The pH of the protein solution was measured with a Radiometer model PH M83 pH meter, and the pH

values of the solutions were adjusted by HCl and KOH of various concentrations.

Denaturation conditions

The GdnHCl unfolding of sperm whale ferrimyoglobin was studied under equilibrium conditions at different pH values of the protein solutions over a wide range of pH values from 3.5 to 13.0. GdnHCl denatured curves were determined by measuring the changes of the Soret absorbance band caused by increases of the concentrations of denaturant. The optical parameters were recorded after an equilibrium was established, which occurred in a few hours for the smaller denaturant concentrations. The GdnHCl concentrations in the samples were checked refractometrically before and after the measurements. The unfolding was reversible for all of the conditions reported here, as checked by the method described in Pace et al. (1989). It was shown previously that GdnHCl denaturation of Mb at neutral pH is reversible but not completely reversible at higher GdnHCl contcentrations after long incubation in these solutions (Puett 1973). In our experiments the spectra were made immediately after the equilibrium had been reached, minimizing the possibility of secondary effects such as globulin aggregation and heme aggregation responsible for some irreversibility (Shen and Hermans 1972).

pH dependence of protein stability

The pH-dependent term for protein stability ($\Delta\Delta G^{el}{}_{ND}$) is determined as the pH-dependent difference between electrostatic free energies of native ($\Delta G^{el}{}_{N}$) and denatured protein states ($\Delta G^{el}{}_{D}$):

$$\Delta\Delta G^{el}_{ND}(\mathrm{pH}) = \Delta G^{el}_{N}(\mathrm{pH}) - \Delta G^{el}_{D}(\mathrm{pH}) \quad (1)$$

In this work the pH-dependent electrostatic energies $\Delta G^{el}(\mathrm{pH})$ (Yang and Honig 1993) in the native, $\Delta G^{el}{}_{N}(\mathrm{pH})$, or denatured state, $\Delta G^{el}{}_{D}(\mathrm{pH})$, are calculated using:

$$\Delta G^{el}(\mathrm{pH}) = -RT\sum_{i=1}^{N}\ln\{1+\exp[-2.3\gamma(i)(\mathrm{pH}-\mathrm{p}K_i(\mathrm{pH}))]\} \quad (2)$$

where $\gamma(i) = -1$ or 1 for an acidic or basic group, respectively; $\mathrm{p}K_i$ is the pK value of ith ionizable group (see below) in the native or denatured state for calculation of the $\Delta G^{el}{}_{N}(\mathrm{pH})$ or $\Delta G^{el}{}_{D}(\mathrm{pH})$ terms, respectively.

The $\Delta\Delta G^{el}{}_{ND}$ term can also be presented following the approach proposed in Yang and Honig (1993):

$$\Delta\Delta G^{el}_{ND}(\mathrm{pH}) = \Delta\Delta G^{el}_{ND}(\mathrm{pH}_1) + \Delta G_{ND}(\mathrm{pH},\mathrm{pH}_1) \quad (3)$$

where $\Delta\Delta G^{el}{}_{ND}(\mathrm{pH}_1)$ is obtained at some initial pH value (pH_1) by use of Eqs. (1) and (2), and $\Delta G_{ND}(\mathrm{pH}, \mathrm{pH}_1)$ is the relative change in the unfolding free energy of the protein at a pH relative to the initial state pH_1. The term $\Delta G_{ND}(\mathrm{pH}, \mathrm{pH}_1)$ is calculated using the integral form of a general expression treating the multiple acid-base equilibrium (Tanford 1970):

$$\Delta G_{ND}(\mathrm{pH},\ \mathrm{pH}_1) = 2.3RT\int_{\mathrm{pH}_1}^{\mathrm{pH}}\Delta Z(\mathrm{pH})\,d\mathrm{pH} \quad (4)$$

where $\Delta Z(\mathrm{pH})$ is the difference between the net charges of the native and denatured protein at a given pH. $Z(\mathrm{pH})$ can be obtained from the pH-dependent charges, q_k, of titratable groups based on calculated pK values (described below), or can be experimentally measured from the potentiometric titration curve of the protein.

The pK values of titratable groups in the denatured protein are assumed to be identical to those of a model compounds composed of *N*-acetyl-L-amino acid amides (Matthew 1985), i.e. we used the "null" model (Bashford et al. 1993; Yang and Honig 1993), which represents a completely unfolded protein. Since our experiments have shown a one-step mechanism for Mb unfolding, and stable intermediate states have not been observed, we consider only the two final states of the native and fully unfolded protein.

Calculations of pK values in native protein

In order to calculate the pK values of titratable groups in native Mb, we follow the well-developed methods described elsewhere (Yang et al. 1993; You and Bashford 1995). The pK values of free residues in solution ($\mathrm{p}K_{mod}$) are modified in the protein environment due to three main factors. The change in solvation energy on transfer of a charge from the highly polar water environment to a protein causes the $\Delta\mathrm{p}K_{Born}$ shift. A second term, $\Delta\mathrm{p}K_{back}$, shifts as a result of the interaction between titratable residues and permanent peptide dipoles. The third energy shift ($\Delta\mathrm{p}K_{tit}$) corresponds to the interaction energy between titratable residues within the protein.

The pK value of ith ionizable group in the protein is determined by:

$$\mathrm{p}K_i(\mathrm{pH}) = \mathrm{p}K_{i,int} + \Delta\mathrm{p}K_{i,tit}(\mathrm{pH}) \quad (5)$$

where $\mathrm{p}K_{i,int}$ is the intrinsic pK value and $\Delta\mathrm{p}K_{i,tit}(\mathrm{pH})$ is the ΔpK shift due to electrostatic interactions between ith and the other ionized groups. The intrinsic pK value ($\Delta\mathrm{p}K_{i,int}$) is defined as:

$$\mathrm{p}K_{i,int} = \mathrm{p}K_{i,mod} + \Delta\mathrm{p}K_{i,Born} + \Delta\mathrm{p}K_{i,back} \quad (6)$$

The term $\Delta\mathrm{p}K_{i,tit}$ is calculated by:

$$\Delta\mathrm{p}K_{i,tit} = -\frac{1}{2.303RT}\sum_{\substack{k=1\\k\neq i}}^{N} q_k(\mathrm{pH})\,\phi_{ik} \quad (7)$$

where $\phi_{ik}(\mathrm{pH})$ is the pH-dependent electrostatic potential generated by the ith group in the location of the kth charged group and q_k are the pH-dependent charges of other titratable groups.

The electrostatic potentials ϕ_{ik} are calculated by the FDPB (Gilson et al. 1987) and MTK (Spassov et al. 1989) methods. An iterative procedure was applied to calculate the values of pK and q_k as a function of pH, according to Spassov et al. (1989).

MTK approach

To calculate $\Delta\mathrm{p}K_{i,tit}$ and $\Delta\mathrm{p}K_{i,back}$ by means of the MTK method, a simple semiempirical potential function for pairwise interactions (Spassov et al. 1989) based on the Kirkwood-Tanford theory (Tanford and Kirkwood 1957) is used:

$$W(r_{ij}) = a_k/(r_{ij})^k \quad (8)$$

where r_{ij} is the distance between the charges i and j. The parameters a_k were optimized such that the titration curve calculated by use of the semiempirical potential function should correspond to the experimental titration curve of the protein (Spassov et al. 1989). A linear relationship between $\Delta\mathrm{p}K_{i,Born}$ and normalized atomic accessibility (AA_i) was previously proposed by Spassov et al. (1989):

$$\Delta\mathrm{p}K_{i,Born} = -\gamma(i)(1-\mathrm{AA}_i) \quad (9)$$

where $\gamma(i) = -1$ for acidic and basic groups.

FDPB approach

Applying the FDPB method, the linearized Poisson-Boltzmann equation was solved in each cube of the space grid with 65×65×65 nodes using the program DelPhi (Gilson et al. 1987). Two DelPhi focusing runs provided a final grid of 0.58 Å. Although a better agreement between calculated and experimental pK values was found by using a higher protein dielectric constant for some small proteins (Antoziewicz et al. 1994), the previously calculated pK values for Mb (Bashford et al. 1993) using the dielectric constant for protein powders of 4 (Takashima and Schwan 1965) have shown a good coincidence with the experimental data. Thus, in this

work, dielectric constants for the protein and solution were chosen to be 4 and 80, respectively.

The partial permanent atomic charges based on the AMBER force-field (Weiner et al. 1984) and atomic radii based on the OPLS parameter set (Jorgensen and Tirado-Rives 1988) were used. Ionizations were determined as the addition of a single proton charge to a single atom in each titratable group. The proton position was taken between the $N^{\delta}H$ and $N^{\epsilon}H$ atoms in the case of histidine residues. The water probe radius was 1.4 Å and the Stern ion exclusion layer was 2.0 Å. Atomic coordinates for the sperm whale Mb were taken from the Protein Data Bank (Bernstein et al. 1977), entry 4MBN (Takano 1984).

Results and discussion

Denaturation by GdnHCl

The variation of the extinction coefficient of the Soret band with GdnHCl concentration is given in Fig. 1. The GdnHCl unfolding curves are shown only at three pH values among all the family of curves obtained over a wide pH range from 3.5 to 13.0 in 0.03 M buffer and 0.1 M KCl. The intensity changes between native and denatured states and the midpoints at neutral pH are quite similar to those resulting from the GdnHCl unfolding of Mb reported earlier (Puett 1973). The initial optical densities are equal for unfolding in the pH range 4–8. At pH 10.2 the extinction coefficient is considerably lower since the optical density of the Soret band at about 409 nm is decreased. The deprotonation of the water molecule in the heme-water complex (transitional methydroxy) occurs in this region and is responsible for this decrease (Acampora and Hermans 1967).

pH dependence of Mb stability

The earlier experimental data on unfolding of holomyoglobin, carried out mainly in the acidic range (Acampora and Hermans 1967; Puett 1973; Privalov et al. 1986), closely approach a two-state mechanism. Our measurements over a wide pH region from pH 3.5 up to pH 13 do not exhibit any stable intermediates. This allows the calculation of the equilibrium constant for a two-state transition, K_D, from the unfolding curves shown in Fig. 1. The free energy of unfolding, ΔG_{ND}, is related to the equilibrium constant (Pace and Vanderburg 1979):

$$\Delta G = -RT \ln K_D = -RT \ln \frac{\varepsilon_N - \varepsilon}{\varepsilon - \varepsilon_D} \quad (10)$$

where ϵ_N and ϵ_D are the millimolar extinction coefficients for the native and the unfolded Mb states, respectively; ϵ is the observed millimolar extinction coefficient.

The unfolding free energy at zero concentration of the denaturant, ΔG_{H2O}, does not depend on the range of concentrations where the conformational transition occurs and is thus a measure of the total protein stability. The conformational protein stability is estimated by extrapolating the ΔG values, measured in the presence of GdnHCl or urea, to zero concentration of the denaturant. Three methods of extrapolation are currently applied to analyze data from the denaturation curves (Pace 1986; Ahmad 1991; Makhatadze 1999): the so-called linear extrapolation method, Tanford's (transfer) model and the binding model. The discussion on the use of these approaches (Pace 1986; Ahmad 1991; Santoro and Bolen 1992; Makhatadze 1999) shows unambiguously that for urea and temperature-induced denaturation curves the liner extrapolation method gives a reasonable free energy estimation. However, in the case of GdnHCl-induced unfolding, the binding and the transfer models lead to the same results and provide a correct estimation of the protein stability. In our study the values of ΔG_{H2O} at different pH values for the Mb molecule are calculated by the transfer model with the basic relationship:

$$\Delta G = \Delta G_{H_2O} + \left(\sum \alpha_i n_i \Delta g^*_{t,i}\right) C \quad (11)$$

where $\Delta g^*_{t,i}$ is the free energy resulting from transfer of an amino acid residue of type i from water to GdnHCl in proteins at 1 M denaturant (Ahmad 1991), n_i is the total number of residues of type i present in the protein molecule, and C is the denaturant concentration. The averaged values of the normalized static accessibility to the solvent (SA_i) for each type of amino acid residue were calculated according to the terms of Lee and Richards (1971). The parameters $\alpha_i = 1 - SA_i$ correspond to those for Tanford's theory (Tanford 1970), assuming a two-state model of unfolding.

In Figure 2 is shown the ΔG_{H2O} change for Mb, determined over the pH range 3.5–13.0 (curve 1). The maximum stability of Mb of about 15.0 kcal mol^{-1} is observed between pH 7 and 8. This is a reasonable agreement with the Mb stability estimates: 13.2 kcal mol^{-1} in the pH range 7–8 from GdnHCl unfolding (Puett 1973) and 14.0 kcal mol^{-1} at pH 9 from thermal denaturation at 25 °C (Acampora and Hermans 1967).

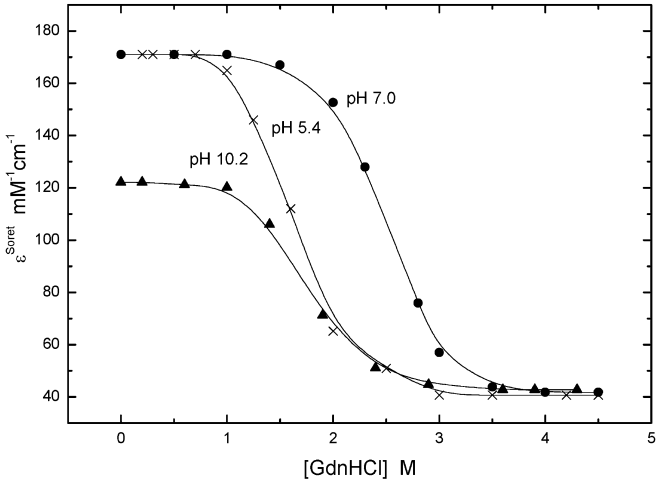

Fig. 1 Dependence of the Soret millimolar extinction coefficient of sperm whale metMb on the concentration of GdnHCl at pH 7.0, 5.4 and 10.2 under equilibrium conditions (from several minutes to a few hours). All solutions contain 0.1 M KCl and 30 mM buffer

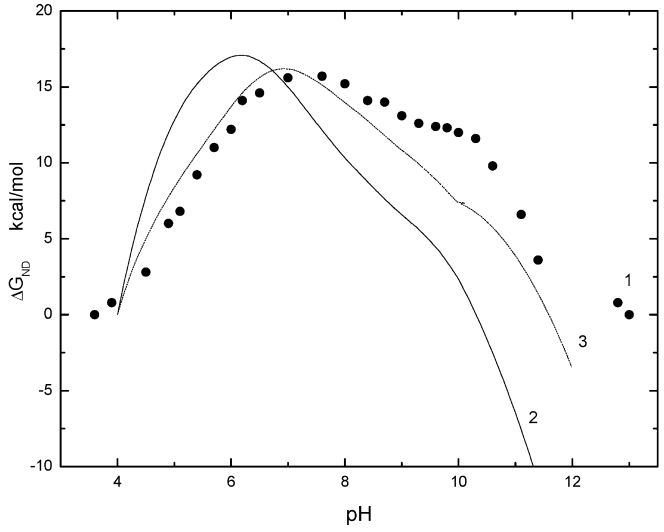

Fig. 2 *Filled circles*: ΔG_{H2O} as a function of pH for the unfolding of metMb at 25 °C. *Curve 2*: relative unfolding free energy of metMb as a function of pH computed from charges of the native state obtained from experimental potentiometric titration (Shire et al. 1974a) and charges of the "null" model denatured state (Eq. 4). *Curve 3*: relative unfolding free energy of metMb as a function of pH computed from charges of the native state obtained from experimental potentiometric titration (Shire et al. 1974a) and charges of the modified "null" model denatured state (Eq. 4)

It is seen that the pH dependence of ΔG_{H2O} in the acidic range practically coincides with the results obtained by Acampora and Hermans (1967). In the alkaline range between pH 8 and 10.5 a small decrease of stability is found that could be explained by some conformational changes around the heme, due to the transitional methydroxy, with a midpoint about 9. In the same pH region the optical density change of the Soret band was also observed, as has been discussed above. The process of alkaline denaturation starts above pH 10.5, demonstrated by a considerable stability decrease.

Using the experimental potentiometric titration data for native Mb (Shire et al. 1974a), we calculated the relative unfolding free energy as a function of pH (Eq. 4). The resulting curves are compared to the plot of the unfolding free energy, ΔG_{H2O} (Fig. 2, curve 1). To facilitate the comparison, the calculated and experimental curves are superimposed at pH 4. The relative unfolding free energy calculated using the "null' model for the denatured state is shown in Fig. 2, curve 2. In the "null" model of the denatured state, all ionizable groups are assumed to be completely exposed to the solvent and not interacting with each other. However, the comparison of the results for the electrostatic contribution to the stability showed significant differences by means of the different models for the unfolded state (Bashford et al. 1993). This indicates the presence of interactions between the ionizable groups in the denatured state. Some experimental studies (Oliverberg et al. 1995; Tan et al. 1995) showed a decrease of the pK values by an average of 0.4 units from those for isolated model compounds in the acidic range (Oliverberg et al. 1995). This is why we used a modified "null " model with pK values for the ionizable groups in the denatured state of 0.4 units lower and higher than those for model compounds for acidic and basic groups, respectively. The relative stability curve estimated by the pK values for the modified "null" denatured state is shown in Fig. 2, curve 3. Obviously the agreement between the experimental curve 1 and curve 3, calculated by the modified "null" model, is significantly improved. Consequently, electrostatic interactions present in the denatured Mb molecule partially stabilize the ionized states of titratable groups in comparison with the completely unfolded extended structure without electrostatic interactions.

Calculation of pK values of histidine residues

A comparison of calculated pK values in this work at ionic strength 0.1 M with other simulations, as well as with experimental values, is given in Table 1. As was seen previously (Bashford et al. 1993), the calculated pK values for some residues can vary strongly, depending on the parameter set used for the partial charges and Born radii. Differences between the computed pK values by the two different electrostatic approaches, FDPB and MTK, could be expected. In our calculations the AMBER charge set with a larger peptide dipole in comparison with the CHARMm parameter set is used, which can lead to larger pK shifts. In addition, we do not take into account the conformational flexibility of titratable residues that can cause errors in the pK calculations. The pK values are estimated for the native "rigid" crystal structure only in order to compare them with the fully unfolded protein.

In some experimental studies on metMb (Puett 1973; Sage et al. 1991) it was suggested that the titration of six histidines buried in the native structure, but exposed in the unfolded state, can play a significant role in the acidic denaturation. Therefore we analyzed the calculated pK values for the histidine residues. Despite the lower pK values for the His residues calculated by the MTK approach in comparison with the experimental ones, it can be seen (Table 1) that in general the pK values are reproduced well. The pK values of the histidine residues obtained by the FDPB method differ significantly from those experimentally measured. Possibly this is due to overestimation of the Born ΔpK shift for histidines 24, 82 and 97 and to charge-charge interactions with the protonated histidines 113 and 119 for His116.

The pK values of the apomyoglobin histidine residues (Yang and Honig 1994) calculated by the FDPB method (see Table 1, PBAPO) also differ from the experimental values. It was experimentally demonstrated (Cocco et al. 1992) that the titration curves of holo- and apomyoglobin histidines are practically identical except for His64 situated in the heme environment. The best pK values calculated previously for the Mb histidines by the FDPB method were presented in Rabenstein and Knapp

Table 1 p*K* values for myoglobin

Residue	MTK (this work)	FDPB (this work)	PBAM[a]	PBAPO[b]	PBMC[c]	Experimental[d]
His12	5.05	7.82	5.57	6.8	7.2	6.39
His24	2.45	7.74	–	5.1	–	< 4.8
His36	5.69	5.39	6.24	6.0	> 7	8.18
His48	5.34	5.62	4.79	6.7	5.09	5.5
His64	0.46	2.81	–2.90	5.0	3.43	–
His81	5.42	5.08	6.87	6.2	7.1	6.7
His82	5.97	9.92	–2.29	6.5	4.04	< 4.8
His93	–0.22	1.89	–	6.5	–	–
His97	5.65	9.50	6.73	6.5	6.32	5.6[e]
His113	3.59	6.53	4.26	5.0	4.36	~5.0
His116	5.55	1.51	6.22	7.0	6.28	6.6
His119	3.84	6.41	3.34	6.8	4.35	6.1–6.2
Asp20	1.99	1.29	1.48	1.7	–	–
Asp27	0.75	0.47	1.40	1.9	–	–
Asp44	2.14	1.20	1.16	2.9	–	–
Asp60	0.30	> 7	1.90	0.7	–	–
Asp122	1.26	> 7	–0.44	1.6	–	–
Asp126	3.92	6.39	3.63	3.6	–	–
Asp141	–1.62	6.55	5.71	3.4	–	–
Glu4	3.54	> 7	1.46	2.8	–	–
Glu6	2.15	1.51	1.63	0.9	–	–
Glu18	2.44	> 7	2.82	2.2	–	–
Glu38	3.38	0.90	3.54	3.3	–	–
Glu41	3.38	0.22	3.73	3.4	–	–
Glu52	2.34	3.52	1.18	1.6	–	–
Glu54	4.17	0.45	3.93	3.3	–	–
Glu59	3.96	0.15	4.44	3.8	–	–
Glu83	4.27	2.30	3.55	4.3	–	–
Glu85	1.49	0.15	2.73	4.3	–	–
Glu105	1.17	4.23	0.88	3.2	–	–
Glu109	3.66	0.41	4.43	3.0	–	–
Glu136	3.19	6.49	3.54	3.3	–	–
Glu148	3.96	0.10	3.14	3.2	–	–
Lys16	11.48	13.46	9.60	11.8	–	–
Lys34	10.74	9.84	11.81	11.3	–	–
Lys42	9.46	5.86	9.09	10.2	–	–
Lys47	12.13	9.56	13.82	11.6	–	–
Lys50	10.26	9.11	10.74	11.1	–	–
Lys56	11.37	11.35	11.24	11.4	–	–
Lys62	11.47	10.55	11.00	11.6	–	–
Lys63	10.70	9.71	11.00	10.3	–	–
Lys77	11.48	8.07	11.99	12.0	–	–
Lys78	12.19	11.26	11.94	10.8	–	–
Lys79	11.40	7.50	13.66	10.2	–	–
Lys87	10.57	6.11	10.57	10.8	–	–
Lys96	10.87	9.00	10.82	10.8	–	–
Lys98	10.05	5.96	10.57	11.2	–	–
Lys102	11.57	10.45	12.47	11.0	–	–
Lys133	11.99	12.12	13.24	11.6	–	–
Lys140	11.70	8.83	11.98	11.8	–	–
Lys145	12.02	8.87	10.88	13.2	–	–
Lys147	10.44	3.04	11.04	11.4	–	–
Arg31	13.07	10.64	12.53	12.8	–	–
Arg45	15.78	> 16	14.69	13.6	–	–
Arg118	14.49	14.54	14.29	> 14	–	–
Arg139	14.65	15.65	11.46	13.8	–	–
N-terminus	7.52	8.77	6.32	8.3	–	–
C-terminus	2.50	7.79	7.33	3.6	–	–
PropA	–0.14	1.42	0.94	–	–	–
PropD	1.29	1.43	2.50	–	–	–

[a]PBAM: PB calculations with Amber parameter set for carboxymyoglobin (Bashford et al. 1993)
[b]PBAPO: PB calculations for apomyoglobin (Yang and Honig 1994)
[c]PBMC: PB calculations with multiple conformations for carboxymyoglobin (Rabenstein and Knapp 2001)
[d]From Cocco et al. (1992)
[e]From Bashford et al. (1993)

(2001) (Table 1, PBMC), where multiple pH-dependent conformations of Mb were supposed.

We have obtained a good agreement with the experimental p*K* values for histidines 48, 64 and 81 by both methods (FDPB and MTK). Obviously, distal His64, closely situated to the heme iron, is unprotonated in the neutral pH range according to our calculations and the experimental data (Cocco et al. 1992). It was suggested (Morikis et al. 1989) that the titration of His64 is influenced by a local conformational change in the heme

pocket near pH 4.4. Our results for the titration of His48 and His81 are reasonable. The MTK approach gives a correct titration behavior for His24 (see below). Neither our calculations by the FDPB and MTK methods nor the theoretical results presented previously (Bashford et al. 1993; Yang and Honig 1994) (see Table 1, PBAM, PBAPO) show the abnormally high pK for His36 as obtained from experiment (Cocco et al. 1992). Low pK values for Glu38 calculated by the FDPB and MTK methods are not enough to explain the abnormally high pK of His36.

The titration behavior of the histidines involved in ion pairs has already been discussed elsewhere (Bashford et al. 1993; Rabenstein and Knapp 2001). Our calculations by the FDPB method show overestimated pK shifts for the ion pairs. The negatively charged PropA of the ion pair His97-PropA, for example, increases strongly the pK value of His97. It is known that the buried His24 is not titrated owing to the closely situated charged His119 partially exposed to the solvent (Cocco et al. 1992). That is why the calculated high pK of His24 strongly coupled with His119 is not correct.

Our calculations by both FDPB and MTK methods do not show six unusual titrations of histidines involved in the denaturation. According to our results from MTK calculations, histidines 24, 64 and 113 do not show normal protonation behavior. The titration of these histidines could be responsible for the initiation of the acidic denaturation. However, the change of the charged state of other ionizable groups, leading to structural changes, has to be also involved in the process of complete protein unfolding.

The calculated isoelectric points of pH 8.0 and 8.15 for Mb by the FDPB and MTK methods, respectively, are in good agreement with the measured isoionic point of pH 8.2 (Shire et al. 1974a). The results for the titration behavior of the ionizable groups in Mb justify the application of both theoretical methods for the elucidation of Mb pH-dependent stability.

Prediction of unfolding free energy of metMb

The total free energy of unfolding, ΔG_{ND}, obtained as the difference in the free energies between the native (N) and the denatured (D) state, is presented by:

$$\Delta G_{\mathrm{ND}}(\mathrm{pH}) = \Delta G^{\mathrm{neut}}_{\mathrm{ND}} + \Delta\Delta G^{\mathrm{el}}_{\mathrm{ND}}(\mathrm{pH}) \qquad (12)$$

where $\Delta G^{\mathrm{neut}}{}_{\mathrm{ND}}$ is the pH-independent denaturation energy of the hypothetical protein with all neutral residues and $\Delta\Delta G^{\mathrm{el}}{}_{\mathrm{ND}}$ is the pH-dependent term for the protein stability. The electrostatic contribution to the unfolding free energy, $\Delta\Delta G^{\mathrm{el}}{}_{\mathrm{ND}}$, of Mb (Eqs. 1 and 2), calculated by the FDPB approach, is given in Fig. 3, curve 1. The calculations were done at an ionic strength of 0.1 and 1.0 M since the results are compared to the GdnHCl unfolding experiment in which a high ionic strength was maintained. Using the MTK method the pH-dependent term $\Delta\Delta G^{\mathrm{el}}{}_{\mathrm{ND}}$ is also calculated at ionic strengths of 0.1 and 1.0 M (Fig. 4, curves 1 and 2). At the extreme pH values, large destabilization electrostatic energies are obtained since in these regions identically charged groups are titrated. Thus, it can be concluded that, as a net effect, electrostatic interactions, including the solvation penalty and charge-charge attractions, stabilize the protein structure only at neutral pH values. In general, the charge-charge interactions in holo- and apomyoglobin are attractive (Matthew and Gurd 1986; Yang and Honig 1994). It is seen from Figs. 3 and 4 that at higher ionic strength the increased electrostatic free energy reduces the protein stability. This is due to the screening of the attractive intramolecular charge-charge interactions by the salt ions in solution.

It is not evident to evaluate correctly the pH-independent term only from theoretical calculations (Yang and Honig 1993; Antoziewicz et al. 1994) or the experimental measurements. However, it is possible to calculate it using the values of the electrostatic unfolding energies at the experimentally determined denaturation midpoints $\mathrm{pH}_{1/2}$ in the acidic and the alkaline regions. Since Mb unfolding is a two-state process, at the midpoint of the transition the value of ΔG_{ND} is zero and $\Delta G^{\mathrm{neut}} = -\Delta\Delta G^{\mathrm{el}}{}_{\mathrm{ND}}$. The zero experimental points for ΔG_{H2O} (Fig. 2, curve 1) are observed at pH 3.6 and 13.0 for the acidic and the alkaline denaturations, respectively. To determine the ΔG^{neut} term, we take the average of the calculated $\Delta\Delta G^{\mathrm{el}}{}_{\mathrm{ND}}$ values at pH 3.6 and 13. Since the experimentally measured pH-dependent stability of Mb is obtained by unfolding at high GdnHCl concentrations, we present the total pH-dependent free energy of unfolding, ΔG_{ND}, at an ionic strength $I = 1.0$ M. The total unfolding free energy obtained by adding the pH-dependent ΔG^{neut} and electrostatic terms (Eq. 12) computed by the FDPB and the MTK methods

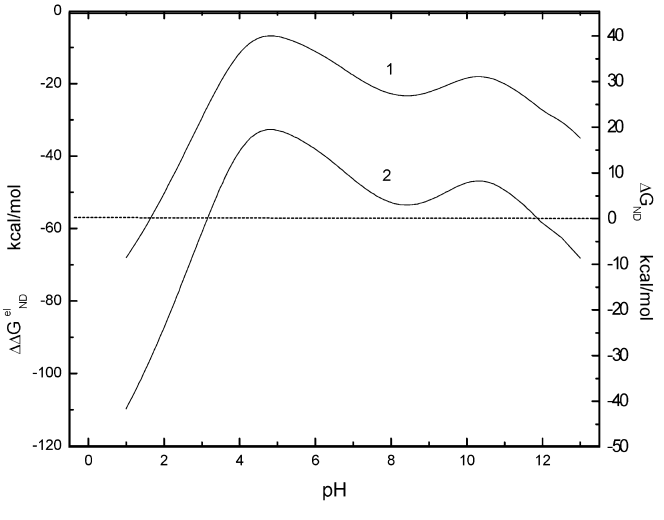

Fig. 3 Electrostatic free energy (*curve 1*) and unfolding free energy (*curve* 2) of metMb as a function of pH at 1.0 M ionic strength computed by the FDPB method (Eqs. 1 and 2)

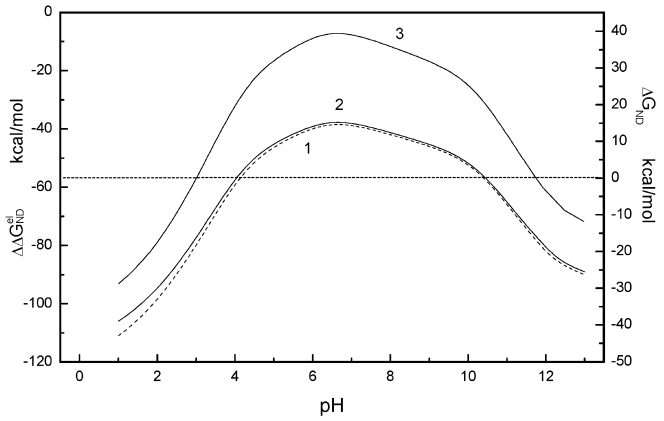

Fig. 4 Electrostatic free energy at ionic strength 0.1 M (*curve 1*) and 1.0 M (*curve 2*) and unfolding free energy at ionic strength 1.0 M (*curve 3*) of metMb as a function of pH computed by the MTK approach (Eqs. 1 and 2)

are shown in Fig. 3 (curve 2) and Fig. 4 (curve 3), respectively. These mixed theoretical/experimental stability curves (Figs. 3 and 4) are comparable to the ΔG_{H2O} curve. The curves in Figs. 3 and 4 reproduce well the overall form of the experimental stability curve (Fig. 2, curve 1). The observed deviation could be due to the additional specific interactions between the denaturant and the protein molecules not taken into account in the theoretical calculations. The overall shape of the stability curve obtained based on the MTK method (Fig. 4) shows better correspondence to the experiment than the one computed by the FDPB method (Fig. 3).

A good quantitative correspondence with the experimentally obtained stability (Fig. 2, curve 1) is achieved by the FDPB method (Fig. 3). By this approach we calculated the maximal stability of 16.3 kcal mol^{-1} at pH 5, which is close to our experimental value of 15.7 kcal mol^{-1} at pH 7 as well as to that estimated by Puett (1973) of 13.2 kcal mol^{-1}. The pH-independent term ΔG^{neut}, evaluated from the electrostatic unfolding contribution by the FDPB method (Fig. 3) at $I = 1.0$ M, is 26.3 kcal mol^{-1}. This is a reasonable result, taking into account the obtained neutral contribution of 18 kcal mol^{-1} to the apomyoglobin stability (Sage et al. 1991). However, a significant difference between the experiment (Fig. 2, curve 1) and the theory (Fig. 3) in the pH range 7–9 is obtained. The disagreement could be due to the conformational changes accompanying the transitional met-hydroxy (Breslow et al. 1965), which are not taken into account in the theoretical model. Another reason for this deviation could be unreal pK values for some histidines calculated by the FDPB method. For example, it is known that histidines 82 and 97 are not protonated in this pH range and small errors in the charge states can cause a substantial effect on the stability estimation (Antoziewicz et al. 1994).

The present study of the pH-dependent stability of Mb in the acidic range confirms a process of denaturation with a midpoint at pH 3.6 without any observable intermediate. Apparently the transitional met-hydroxy with a pK_a of 8.9 influences the protein stability. This process can be seen by the pH-dependent change of the optical density of the Soret band. Above this pH region the alkaline denaturation occurs. It was found that a good qualitative description of pH-dependent stability of Mb results from the use of the semiempirical electrostatic approach, while the FDPB method yields a more correct quantitative evaluation for Mb stability in the neutral pH range.

Despite some simplifications of the applied theoretical methods, this work shows a good agreement between the predicted and experimentally determined pH-dependent stability of myoglobin. The pH-independent stability term is calculated by computed electrostatic unfolding energies and experimental pH midpoints for acidic and alkaline denaturations. The present results justify the proposed methodology for determination of the total pH-dependent free energies of unfolding. They reveal that the pH-dependent and pH-independent contributions to the protein stability can be successfully evaluated by simultaneous use of theoretical and experimental approaches.

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