The unfolding enthalpy of the pH 4 molten globule of apomyoglobin measured by isothermal titration calorimetry

MARC JAMIN,1,4 MARIAN ANTALIK,2,5 STEWART N. LOH,1,3 D. WAYNE BOLEN,2 AND ROBERT L. BALDWIN1
1Department of Biochemistry, Beckman Center, Stanford University School of Medicine, Stanford, California 94305-5307
2Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas 77555-1052
3Department of Biochemistry and Molecular Biology, SUNY Upstate Medical University, Syracuse, New York 13210

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Abstract
The unfolding enthalpy of the pH 4 molten globule from sperm whale apomyoglobin has been measured by isothermal titration calorimetry, using titration to acid pH. The unfolding enthalpy is close to zero at 20°C, in contrast both to the positive values expected for peptide helices and the negative values reported for holomyoglobin and native apomyoglobin. At 20°C, the hydrophobic interaction should make only a small contribution to the unfolding enthalpy according to the liquid hydrocarbon model. Our result indicates that some factor present in the unfolding enthalpies of native proteins makes the unfolding enthalpy of the pH 4 molten globule less positive than expected from data for peptide helices.

Keywords: apomyoglobin; molten globule; titration calorimetry; unfolding enthalpy

The folding thermodynamics of molten globule intermediates are obscure, largely for technical reasons. Because the molten globule folding intermediate of sperm whale apomyoglobin is stable at pH 4 in low salt but is unfolded by titration to pH 2, we are able to use isothermal titration calorimetry (ITC) to determine its unfolding enthalpy. Our aim is to determine if its unfolding enthalpy is negative at 20°C, like holomyoglobin (Makhatadze & Privalov, 1993) and native apomyoglobin (Griko et al., 1988), or if it is positive, like calorimetrically measured peptide helices (Scholtz et al., 1991; Richardson et al., 1999; Taylor et al., 1999). The unfolding enthalpy of native holoMb at 20°C is −10 kcal/mol while the unfolding enthalpies of peptide helices range from 1 to 0.6 kcal/mol [helical peptide unit], depending on sequence. There are about 60 helical residues in the pH 4 molten globule (Hughson et al., 1990; Eliezer et al., 1998), which would give 60–36 kcal/mol of unfolding enthalpy as peptide helices. The underlying question is how close the unfolding thermodynamics of molten globules lie either to native proteins or to isolated peptide helices. At 20°C, the enthalpy change for dissolving a liquid hydrocarbon in water is close to zero (Gill et al., 1976) and the contribution of the hydrophobic interaction to the unfolding enthalpy is small, according to the liquid hydrocarbon model (Baldwin, 1986), so that this major factor should be minor at 20°C. In contrast to the hydrophobic interaction, the unfolding enthalpy of a peptide helix is relatively temperature independent and arises chiefly from breaking peptide hydrogen bonds and exposing the CO and NH groups to water.

As a first step, it is essential to determine if unfolding of the molten globule is a two-state reaction. Care is needed in considering this problem because two different forms of the molten globule (Ia, Ib) have been detected in ordinary conditions (Jamin & Baldwin, 1998); they coexist in a pH-dependent and [urea]-dependent equilibrium that also is affected by stabilizing anions such as sulfate or citrate. In both urea- and acid-induced unfolding, Ib is converted to Ia before Ia unfolds. The cooperativity of unfolding has been studied chiefly for urea-induced unfolding at pH 4.2, 4°C, where only Ia is present above 1 M urea (Jamin & Baldwin, 1998). Three different approaches have shown that urea unfolding is highly cooperative. The unfolding transition is judged cooperative by two tests: the unfolding/refolding kinetics (Jamin & Baldwin, 1996; Jamin et al., 1999) and a superposition test, comparison of the urea unfolding curves monitored by CD and Trp
fluorescence (FL) (Kay & Baldwin, 1996). Also, helix destabilizing mutations (substitution of Gly or Pro) affect the stability of the entire molten globule and not only the helix in which the substitution is made (Luo et al., 1997), indicating that the helices are stabilized by cooperative interactions. In acid induced unfolding, Ib is converted to Ia below pH 3.3 (Jamin & Baldwin, 1998) and acid unfolding is judged cooperative by the test that the unfolding transition curve is substantially sharper than that which would result from Asp and Glu residues titrating independently with a single pK_a value (Kay & Baldwin, 1998).

Thermal unfolding of the pH 4 MG shows a shallow transition curve from which it is difficult to extract either a van’t Hoff or calorimetric heat of unfolding (Griko & Privalov, 1994). Thus, it is attractive to try isothermal titration calorimetry, because the pH 4 molten globule can be unfolded by acid in low salt. The enthalpy of protonating the aspartate and glutamate residues during acid titration is small enough to be estimated reliably from model compound data, and this is the main factor that must be taken into account.

The following considerations dictate the choice of conditions. First, the starting material at the initial pH should be entirely in the form of the molten globule. The reason is that the native protein (N) has one histidine residue (His24) that is entirely unprotonated in N and becomes protonated as N is converted to I (Geierstanger et al., 1998); there are also minor amounts of other unprotonated His residues in N. Histidine has a large heat of protonation (about −7 kcal/mol). This problem requires that N be eliminated at the initial pH. We satisfy this condition by introducing a sensitive kinetic assay for N and showing that N is absent in our conditions. Earlier work has shown that some anion stabilization of the molten globule is necessary for cooperative unfolding (see Luo et al., 1997), while conditions must be chosen carefully to avoid inducing dimers and oligomers (Jamin & Baldwin, 1998). We chose 30 mM NaCl with these considerations in mind. The 2 mM citrate used in our earlier work, which is a strong anion stabilizer, is omitted here because it interferes with the calorimetric titration. The protein concentration is set at 50 micromolar; higher concentrations cause significant dimer formation.

Unfolding of I is monitored by two optical probes, CD and FL, to compare the calorimetric curve of heat release with the curve for unfolding, and thus determine if unfolding is responsible for the heat release. These two unfolding curves also provide a test for cooperative unfolding of I to U below pH 3.3 because CD monitors secondary structure while FL monitors tertiary structure in the following sense. The increased fluorescence intensity of I compared to U arises from partial burial of the two Trp residues, W7 and W14 (Jamin & Baldwin, 1998), presumably in the interface region formed by the A, G, and H helices. Superposition of unfolding curves measured by probes of secondary and tertiary structure gives a sensitive test for any folding intermediates in the case of native proteins and also is useful for molten globules (see Kay & Baldwin, 1996; Luo et al., 1997; Luo & Baldwin, 1999). The pH titration curve of the Glu and Asp residues is measured potentiometrically in order to compare it with the calorimetric curve of heat release versus pH.

Results

Kinetic test for the presence of N near pH 4

The formation of N is monitored sensitively by Trp fluorescence and is well resolved kinetically from the formation of the preceding intermediate Ib (Jamin & Baldwin, 1998; Jamin et al., 1999). Consequently, a kinetic assay for the presence of N near pH 4 may be made in a stopped-flow experiment, starting from conditions where N is absent (starting either from the acid-unfolded form U or from the intermediate Ia). Figure 1 shows this assay: in the presence of 30 mM NaCl (Fig. 1A), curve 1 shows that no detectable N is formed at pH 4.2 (between 0.1 and 1 s) while curve 2 shows the formation of N as the sole final species present at pH 5.5. The formation of Ib from Ia (below 0.05 s) is also seen in both curves 1 and 2. In the absence of 30 mM NaCl, a significant amount of N is formed at pH 4.2 (curve 1, Fig. 1B). The kinetic curves in Figure 1B are fitted with three exponentials: the first is the formation of Ib from Ia, the second is the formation of N from Ib, and the last is the formation of N from dimeric Ib as these dimers dissociate (Jamin & Baldwin, 1998). The ratio of kinetic amplitudes at pH 4.2 and 5.5 in Figure 1B indicates that about 30% N is formed at pH 4.2, 0 M NaCl, while less than 1% N is formed at pH 4.2 in 30 mM NaCl (Fig. 1A). The experiment was repeated.

Fig. 1. Kinetic refolding assay for the presence of N. The refolding kinetics at pH 4.2 (curve 1) and at pH 5.5 (curve 2), monitored by tryptophan fluorescence, are shown in the (A) presence and (B) absence of 30 mM NaCl. Final conditions: 2 mM Na acetate, 4.5 °C; initial conditions: same buffer, pH 3.4 (molten globule species Ia is present initially). The formation of N occurs between 0.1 and 1.3 s, while the formation of Ib occurs below 0.05 s.
at 20 °C with the same result: no N is formed at pH 4.2 in the presence of 30 mM NaCl. The 4.5 °C experiment is shown in Figure 1 because most of the earlier folding kinetics were made at 4.5 °C and the kinetic phases are already assigned at this temperature.

**pH-induced unfolding transitions monitored by CD and fluorescence**

Figure 2 shows first the unfolding by acid of N (pH 5.8–4.2), then the conversion of Ib to Ia below pH 4.2, followed by the acid unfolding of Ia. The process is monitored both by CD (222 nm) and by Trp fluorescence at 320 nm, and the two scales are adjusted so that the unfolding of Ia to U follows a common curve between pH 3.8 and 2.2. Ia is known to be the sole intermediate present below pH 3.3 in the similar conditions studied by Jamin & Baldwin (1998). They found that Ib has a lower specific fluorescence than Ia but a similar specific CD; thus, the partial formation of Ib from Ia above pH 3.8 is observed here by a decrease in fluorescence. The ability to superimpose the unfolding curves of Ia monitored by CD and FL below pH 3.8 means that the superposition test for cooperative unfolding is satisfied.

**Potentiometric titration of ionizing groups**

Figure 3 shows potentiometric titration curves of a 50 μM (0.87 mg/mL) apoMb solution in 30 mM NaCl. To test for reversibility, the protein was subjected to retitration and the retitration data are superimposed on the first titration data. Because pH cannot be measured directly in the ITC cell, the potentiometric data are needed to determine the pH of the solution from the added moles of HCl titrant. The pH titration curve of the ionizing Asp and Glu residues is needed also to correct the calorimetric curve for the heat effect of titrating these residues. The potentiometric data show that approximately 18 groups are protonated between pH 4.2 and 2.5, in reasonable agreement with the 22 carboxylate groups present in the sequence of apoMb: 14 Glu, 7 Asp, and 1 C-terminal. As the repeated titration curves indicate, the reproducibility becomes poor below pH 2.8 so that the complete titration curve cannot be measured.

**Calorimetric pH titration**

Acid-induced unfolding of apoMb at 20 °C occurs with a release of heat (Fig. 4), both between pH 5.2 and 4.2, where unfolding of N to Ia, Ib goes to completion, and between pH 3.8 and 2.5, where unfolding of Ia to U occurs. As measured by CD and FL (Fig. 2), both unfolding transitions are sigmoid, or cooperative, in shape. On the other hand, while the heat release for the N to I reaction follows the last part of a sigmoid curve, the heat release for the Ia to U reaction is nearly linear. Figure 4A shows the heat of dilution of the 0.097 M HCl used, compared with titrating the protein. After correction for the heat of dilution, ~17 kcal/mol is released during calorimetric titration of apoMb from pH 4.2 to 2.2.

The heat effect caused by protonating the 22 carboxylate groups of apoMb is simulated in Figure 4B for two cases. The first case (upper curve) uses the ΔH and ΔCp values for ionization of Asp and Glu residues determined in model compounds (peptides) but uses an estimate of the average pKₐ (3.5) of these groups in the apoMb molten globule. This corresponds to the estimated average pKₐ found from the potentiometric curve in Figure 3. Moreover, by studying apoMb with ¹³C-labeled aspartate residues and performing chemical shift titrations, Geierstanger et al. (1998) determined the average pKₐ of the aspartate residues to be 3.2 (35 °C, 0.02 M citrate, D₂O). The dispersion in aspartate pKₐ values was not large. In model compounds, Glu has a higher pKₐ than Asp, and Geierstanger’s result agrees fairly well with the average pKₐ of 3.5 used in Figure 4B. The molten globule unfolds in the pH range where the aspartate residues titrate, so that both U and Ia are present in a pH-dependent equilibrium when the aspartate residues are titrated. The second case shown in Figure 4B (lower curve) shows the heat effect expected for protonating the 22 carboxylate groups if they have the pKₐ values found in model peptides. This case is discussed in the following section.
Correction for the heat effect of protonating the carboxylate groups

Accurate pK_a values for aspartic and glutamic acids have been measured by potentiometric measurements over a range of temperature between 0 and 100 °C (Smith & Smith, 1942; Christensen et al., 1976). For these two model compounds, the enthalpy of ionization (ΔH_ion), the entropy of ionization (ΔS_ion), and the heat capacity change for ionization (ΔC_p) have been calculated from the temperature dependence of their dissociation constants (Edsall & Wyman, 1958). The ΔH_ion of the aspartic acid side chain at 20 °C measured by calorimetry is within 10% of the value obtained from the temperature dependence of potentiometric data (Ref. Ca in Christensen et al., 1976).

Figure 5 shows the expected heat effect for protonating 22 carboxylate groups, with pK_a, ΔH_ion, and ΔC_p values appropriate for Asp and Glu in model compounds (see Fig. 5 caption; Table 1).

There are some noteworthy points. First, although the heat effect of protonating carboxylate groups in proteins is sometimes neglected because the enthalpy change is small, the ΔC_p of carboxylate protonation is not small. Second, the magnitude and sign of the heat effect depend critically on the temperature. Third, the predicted heat effect at 20 °C has the same sign and about the same value (17 kcal/mol released between pH 4.2 and 2.2) as the observed results for the apoMb molten globule (Fig. 4).

Discussion

Unfolding enthalpy of the apoMb molten globule

Our results indicate that, at 20 °C, the unfolding enthalpy of the apoMb molten globule is zero within error because the observed heat release from pH 4.2 to 2.2 equals the heat effect predicted for protonating the 22 carboxylate residues. Moreover, in this pH range both the calorimetric pH titration curve (Fig. 4) and the potentiometric curve (Fig. 3) are fairly linear. If the heat release were caused by cooperative unfolding, the curve in Figure 4 should have a sigmoid shape like the unfolding curves monitored by CD and FL (Fig. 2).

Griko and Privalov (1994) found that thermal unfolding of the pH 4 molten globule monitored by differential scanning calorimetry (DSC) gives no observable cooperative unfolding and no measurable unfolding enthalpy. Nishii et al. (1995) made DSC measurements of the thermal unfolding of an apoMb MG from horse heart in 0.25 M NaCl, pH 2, and they concluded that a

| Table 1. Thermodynamic parameters for the ionization of the side chains of aspartate and glutamate

<table>
<thead>
<tr>
<th></th>
<th>Aspartic acid</th>
<th>Glutamic acid</th>
</tr>
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<tbody>
<tr>
<td>pK_a (25 °C)</td>
<td>3.90</td>
<td>4.27</td>
</tr>
<tr>
<td>ΔH_ion (25 °C) (cal/mol)</td>
<td>1,100</td>
<td>370</td>
</tr>
<tr>
<td>ΔS_ion (25 °C) (cal/mol/K)</td>
<td>-14.2</td>
<td>-18.3</td>
</tr>
<tr>
<td>ΔC_p (25 °C) (cal/mol/K)</td>
<td>-34.5</td>
<td>-62.9</td>
</tr>
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*Data for aspartic acid are from Smith and Smith (1942), and those for glutamic acid are from Christensen et al. (1976).
shallow unfolding curve is observable. By CD spectroscopy they found that their protein shows both heat and cold denaturation, and this explains the shallow nature of the thermal unfolding curve. Anion-induced folding of the horse heart molten globule at pH 2 was used by Hamada et al. (1995) to measure the folding enthalpy by ITC. Both NaClO₄ and Na₂SO₄ were used to induce folding. However, the observed enthalpy depends somewhat on the anion used, indicating that anion binding contributes to the heat effect. Although the authors made a baseline correction, there is evidence that the nature of the molten globule changes with the anion used to induce folding. The protection factors of the pH 4 molten globule increase 40-fold in the presence of 20 mM Na trichloroacetate and the midpoint of the urea-induced unfolding transition increases from 1.5 to 3.9 M (Loh et al., 1995). The observed enthalpy of anion-induced folding of the horse heart MG at 20°C is zero (Hamada et al., 1995), in good agreement with our result.

The unfolding transitions of the molten globules formed by two other proteins have also been studied by calorimetry. The pH 2 molten globule of cytochrome c shows a highly cooperative thermal unfolding transition when stabilized by sulfate, and there is good agreement between the calorimetrically measured and van’t Hoff values of the enthalpy change (Hamada et al., 1994). These authors find agreement between the enthalpy changes measured by DSC and by ITC for anion-induced folding. The α-lactalbumin molten globule does not show cooperative thermal unfolding, and different interpretations have been given for its unfolding curve monitored by DSC (Yutani et al., 1992; Griko et al., 1994).

**Nature of the unfolding enthalpy of the apoMb molten globule**

Our results show that the unfolding enthalpy of the pH 4 molten globule is approximately zero at 20°C, in agreement with the study by Hamada et al. (1995) of anion-induced folding of the horse heart molten globule at pH 2. In contrast to peptide helices, whose unfolding enthalpies depend chiefly on breaking peptide H-bonds and exposing the peptide CO and NH groups to water, the unfolding enthalpies of native proteins are more mysterious. Besides the well-understood dependence on exposing buried nonpolar surface to water, their unfolding enthalpies depend also on other factors in a complex and not yet well-understood manner. One factor is the unusually strong van der Waals interactions expected in native proteins which are more tightly packed than close-packed spheres (Knapp, 1971) and are comparable to organic crystals (Richards, 1977). Breaking these tight van der Waals interactions should contribute a positive enthalpy term (whose size is not yet known accurately) to the unfolding enthalpies of native proteins. The relative lack of chemical shift dispersion in the proton NMR spectra of the apoMb molten globule (Loh et al., 1995; Eliezer et al., 1998) indicates that its side chains are not close packed, unlike those of native apoMb, and a positive contribution to its unfolding enthalpy is not expected from this source. A second factor is the negative enthalpy of interaction of water with polar groups (especially the peptide C=O and NH groups) buried in the interior of native proteins and exposed to water on unfolding (Ben-Naim, 1991; Yang et al., 1992; Makhatadze & Privalov, 1993; F. Avbelj, P. Luo, & R.L. Baldwin, unpubl. data). This negative term is expected to be reduced in size compared to native proteins, but nevertheless present in the unfolding enthalpies of molten globules. Its presence may compensate the positive contribution expected from unfolding the helices of the apoMb molten globule and explain why the observed unfolding enthalpy is close to zero. Repeating our experiment at different temperatures should produce a value for ΔCp, which could be compared with the value for the pH 2, high salt, molten globule of horse heart apoMb (Hamada et al., 1994; Nishii et al., 1995), and compared also with estimates based on models with varying amounts of buried nonpolar and polar surface area. As Figure 5 shows, the temperature range 20–50°C would be a favorable range for making this experiment, as regards the uncertainty contributed by the heat of protonating carboxylate residues.

A main conclusion from our study is that, as regards its unfolding enthalpy, the pH 4 apoMb molten globule is intermediate in its behavior between nativeMb (either holo or apoMb) and a set of peptide helices. A similar conclusion can be drawn from the work of Nishii et al. (1995): they found that the anion-stabilized pH 2 molten globule from horse heart undergoes cold denaturation as well as heat denaturation, like holoMb and native apoMb, and unlike peptide helices that do not undergo cold denaturation (although cold denaturation of certain peptide helices in fluorooalcohol–water mixtures has been reported by Andersen et al., 1999). Our result supports the proposal by Kay and Baldwin (1996) and Kay et al. (1999) that the pH 4 molten globule of apoMb resembles more an embryonic form of the native protein than a set of weakly interacting helices.

**Materials and methods**

**Protein expression and purification**

A synthetic gene for sperm whale myoglobin was expressed in Escherichia coli BL21(DE3) (Novagen, Madison, Wisconsin), and the protein was purified as described previously (Loh et al., 1995). The heme was removed by acid-acetone precipitation (Rossi-Fanelli et al., 1958). Protein concentrations were determined by absorbance in 6.0 M GdmCl (20 mM sodium phosphate, pH 6.5, 20°C) as described by Edelhoch (1967), using ε₂₈₀nm = 15,200 M⁻¹ cm⁻¹ and ε₂₈₈nm = 10,800 M⁻¹ cm⁻¹.

**Equilibrium pH titrations monitored by Trp fluorescence and CD**

Circular dichroism at 222 nm (CD) was measured at 20°C on an AVIV 62A DS spectropolarimeter, using a 1 mm pathlength cuvette. Fluorescence emission spectra (FL) were measured at 20°C on a SLM AB2 spectrofluorimeter (SLM-Aminco, Rochester, New York) using a 0.4 × 1.0 cm cuvette. The excitation was at 288 nm, and the emission was recorded from 320 to 380 nm with a scan rate of 1 nm/s and bandpasses for excitation and emission of 1 and 8 nm, respectively.

Titrations of 50 μM apoMb solutions containing 30 mM NaCl were performed in a thermostatted vessel by adding small aliquots of 0.4 or 1.0 M HCl. The pH was continuously monitored in the vessel using a 6030-M3 pH electrode from Mettler (Highstown, New Jersey). After each injection, samples were taken for FL and CD measurements. Corrections were made to account for protein dilution and volume change.

**Potentiometric titrations**

Potentiometric titrations were carried out using equipment described previously (Santoro & Bolen, 1988; Huang & Bolen, 1993;
Yao & Bolen, 1995). Continuous stirring with a Teflon-coated stir bar was maintained during calibrations and titrations. The titration vessel was thermostatted at 20.0 ± 0.1 °C and continuously purged with nitrogen gas. The nitrogen gas was purified by successive passages through three scrubbers containing 0.1 M alkaline BaCl$_2$, 2 M phosphoric acid, and a solution identical in composition to the one being titrated, respectively. The electrode and pH meter were calibrated at 20°C immediately before the experiments by using a set of standard buffer solutions at pH 4.000 ± 0.002 and 7.000 ± 0.002.

HCl titrants were prepared in 30 mM NaCl. The exact concentration of HCl titrants was determined using a freshly prepared CO$_2$-free solution of Trizma base in 30 mM NaCl as a primary standard. Several determinations of the equivalent point were made using Gran’s plot (Rossotti & Rossotti, 1965).

An unbuffered stock solution of apoMb was dialyzed overnight at 4°C against distilled water. Solutions of 50 μM apoMb (0.87 mg/mL) were prepared by diluting the stock solution with a CO$_2$-free NaCl stock solution to obtain a final NaCl concentration of 0.030 M. ApoMb solutions were continuously titrated from about pH 6.0 to 2.2 by adding 1, 3, or 10 μL aliquots of HCl titrant at 15 s intervals. The pH readings and added volumes of HCl titrant were recorded.

The reversibility of titration was verified by performing a second titration with the same sample, described as follows. After reaching the final acidic pH in the first titration, the pH of the solution was raised back to above pH 6.0 using CO$_2$-free NaOH, and the solution was titrated a second time. Control experiments were carried out by titrating a 30 mM NaCl solution (blank titration).

The number of moles of proton bound to 1 mol of protein (ν) at each pH is given by the difference between the number of moles of HCl used for each point of the protein titration and the number of moles of HCl used to reach the same pH in the absence of protein (blank titration). The blank titration was calculated at each pH value by measuring the ratios between the volume of HCl titrant used for titrating the blank solution from a reference pH to any

\[ \Delta \text{mol HCl} = \text{vol HCl} \times \text{conc HCl} \]

at a given pH. The number of moles of proton bound per mole of protein was determined at each pH by subtracting the moles of HCl titrant needed to titrate the blank solution to this particular pH from the moles of HCl titrant needed to titrate the protein solution to the same pH. The number of moles of proton bound per mole of protein is obtained by dividing this difference by the total number of moles of protein. Because we did not titrate the protein over the full pH range, the absolute protonation state of the protein is unknown at any pH. As a point of reference, the titration curves shown in Figure 3 were adjusted so that the value of ν at pH 4.2 is set to zero.

Isotermal titration calorimetry

Stepwise titration was performed using an isothermal titration calorimeter from MicroCal (Northampton, Massachusetts). Fifty micromolar apoMb solutions at pH 5.5 containing 30 mM NaCl were prepared by mixing aliquots of the stock solution with solutions of CO$_2$-free NaCl. The solutions were loaded in the ITC sample cell, and the titration was performed with injections of 1, 3, or 10 μL each of an HCl titrant with a syringe of 100 μL at 20°C. Before each experiment, the ITC cell was washed several times with the blank solution and once with the apoMb solution.

Control experiments were performed in which an identical solution but without protein was titrated throughout the same pH range. These data were used to correct for contributions from the enthalpy of HCl dilution and the enthalpy of formation of water.

The pH in the ITC cell was calculated from the added amount of HCl titrant, using calibration curves for the blank and protein solution established from the potentiometric titrations. A polynomial equation was used to fit the plot of moles of added HCl vs. pH and the pH in the ITC cell was calculated from this equation. The handling of the unbuffered samples with glass syringes causes a small variation of pH (corrections of the order of 0.07 pH units). A calibration curve was established as a function of pH and corrections of the calculated pH values were made accordingly. At the end of the titrations, the solutions were extracted from the ITC cell and the pH of these solutions was found to be within ±0.03 pH units of our estimated values.

The enthalpy change for each injection was calculated by integrating the area under the peaks of recorded time course of change of heat. The cumulative enthalpy of successive injections was calculated setting the zero point at pH 5.5.

Simulation of the heat effect for protonating apomyoglobin

The enthalpy for protonating the carboxylic groups (aspartates, glutamates, and C-terminal group) of apomyoglobin during the acid titration is estimated from the thermodynamic parameters of ionization of model compounds. Values of Δ$H_{\text{ion}}$, Δ$S_{\text{ion}}$, and Δ$C_{\text{p,ion}}$ have been obtained for the ionization of aspartate and glutamate side chains from the temperature dependence of their dissociation constants. The heat effect for titrating all carboxylic groups of apomyoglobin is calculated with the assumption that each carboxylic group titrates independently from the others. The enthalpy of protonation (−Δ$H_{\text{ion}}$) is obtained as the sum of the individual heats of titration of each group to a given pH value according to Equation 1:

\[
-\Delta H_{\text{ion}}(\text{pH}) = \sum_i \alpha_i(\text{pH})(-\Delta H_{\text{ion}}) 
\]

where $\alpha_i(\text{pH})$ is the protonated fraction of the carboxylic group $i$ at a given pH. $\alpha_i$ is calculated for each group by using the Henderson-Hasselbalch equation (Equation 2):

\[
\alpha_i(\text{pH}) = \frac{10^{pK_{a,i}-\text{pH}}}{1 + 10^{pK_{a,i}-\text{pH}}}. 
\]

For simplification, in our simulation the C-terminal group is treated as an aspartate residue and all aspartates and glutamates are assigned generic $pK_a$ values. The heat of protonation as a function of pH is calculated at varying temperatures, by using values of $pK_a$, Δ$H_{\text{ion}}$, and Δ$S_{\text{ion}}$ calculated at these temperatures from the reference values at 25°C (Table 1) and the values of Δ$C_{\text{p,ion}}$ for aspartate (Δ$C_{\text{p,ion}} = -34.5$ cal/mol/K) and glutamate (Δ$C_{\text{p,ion}} = -63.0$ cal/mol/K) according to Equations 3A–3C:
\[ \Delta H_{\text{ion}}(T) = \Delta H_{\text{ion}}(T_{\text{ref}}) + \Delta C_{\text{p,ion}}(T - T_{\text{ref}}) \] (3A)

\[ \Delta S_{\text{ion}}(T) = \Delta S_{\text{ion}}(T_{\text{ref}}) + \Delta C_{\text{p,ion}} \ln \left( \frac{T}{T_{\text{ref}}} \right) \] (3B)

\[ pK_a(T) = \frac{\Delta H_{\text{ion}}(T) - T \Delta S_{\text{ion}}(T)}{2.303RT} . \] (3C)

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