Putative Interhelix Ion Pairs Involved in the Stability of Myoglobin†

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ABSTRACT: An earlier theoretical study predicted that specific ion pair interactions between neighboring helices should be important in stabilizing myoglobin. To measure these interactions in sperm whale myoglobin, single mutations were made to disrupt them. To obtain reliable ΔG values, conditions were found in which the urea induced unfolding of holomyoglobin is reversible and two-state. The cyanomet form of myoglobin satisfies this condition at pH 5, 25 °C. The unfolding curves monitored by far-UV CD and Soret absorbance are superimposable and reversible. None of the putative ion pairs studied here makes a large contribution to the stability of native myoglobin. The protein stability does decrease somewhat between 0 and 0.1 M NaCl, however, indicating that electrostatic interactions contribute favorably to myoglobin stability at pH 5.0. A previous mutational study indicated that the net positive charge of the A[B]GH subdomain of myoglobin is an important factor affecting the stability of the pH 4 folding intermediate and potential ion pairs within the subdomain do not contribute significantly to its stability. One of the assumptions made in that study is tested here: replacement of either positively or negatively charged residues outside the A[B]GH subdomain has no significant effect on the stability of the pH 4 molten globule.

Pairs of oppositely charged residues on adjacent helices are predicted to make strong electrostatic interactions in sperm whale Mb (1). These interactions are predicted to be screened by counterions although, if they are present as salt bridges (hydrogen-bonded ion pairs), these are not found to be very sensitive to counterion screening, in peptide helices (2, 3). In either case, if there are any strong pairwise interactions between oppositely charged residues on adjacent helices, they are likely to be important in determining the folding pathway of apoMb. We undertook to measure them, to find out if any changes in stability are indeed caused by pairwise interactions, and to determine how the stability of Mb is affected by counterion screening.

Our procedure is to substitute by alanine at least one member of each potential ion pair and to determine the resulting change (ΔG) in its free energy of unfolding (ΔG unfold). If replacing one residue gives a significant change in ΔG unfold, then the other residue of the pair is substituted with alanine and ΔG is measured to find out if the second mutation causes an equally large change, as expected if there is a pairwise interaction. The test for a pairwise interaction is essential when mutation of one member of a pair gives a large change in stability because other effects, such as steric, hydrophobic, or helix propensity effects, may be responsible for the observed change. To obtain reliable ΔG values, the folding transition must be reversible and two-state. We searched for conditions in which the urea induced unfolding of holoMb satisfies this condition. GdmCl is unsatisfactory for this purpose because high Cl− concentrations stabilize a folding intermediate. We found that the cyanomet form of holoMb gives reversible, two-state, urea-induced unfolding at pH 5.0, 25 °C. Urea unfolding of native apoMb at pH 7.8, 4 °C, has been used earlier (5) to study the effects of mutations on the stability of native apoMb, but for mutants it is often necessary to use a three-state analysis, which gives less accurate results than the new two-state procedure introduced here. For technical reasons, the new procedure is limited to pH values close to 5.0 (see below).

The contribution of electrostatic free energy (ΔGel) to ΔG unfold is predicted to be large and favorable for Mb folding at pH 7, where there are similar numbers of positively and negatively charged groups, using either the accessibility-modified Tanford–Kirkwood model (1) or the finite difference Poisson–Boltzmann (FDPB) procedure (4). Both models also predict that ΔGel should become large and unfavorable at pH 2, where the net positive charge is large. At pH 7.0, ionic strength 0.01, ΔGel for Mb is predicted to be near −18 kcal/mol. There are few experimental tests in the literature of the size of ΔGel as it affects protein stability. Hollecker and Creighton (6) made one such test by reversing the basic charges on amino groups through succinylation. They found only small effects of net charge on stability, measured by urea-gradient gel electrophoresis, for β-lactoglobulins A and B, cytochrome c, and ribonuclease A, although succinylation of a single unreactive amino group of cytochrome c did produce a substantial decrease in stability. They used an acidic pH (4.0) in determining the

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1 Abbreviations: Mb, myoglobin; CD, circular dichroism; I, pH 4 folding intermediate; N, native; Cm, urea concentration where the protein is 50% folded; ΔG unfold, free energy of unfolding; ΔGe, electrostatic free energy; Tm, the temperature midpoint of a thermal unfolding curve; GdmCl, guanidinium chloride.
Table 1: Predicted Electrostatic Interactions between Pairs of Mb Residues Studied in this Paper

<table>
<thead>
<tr>
<th>paired residues (i–j)</th>
<th>adjacent helices</th>
<th>ΔΔGΔ, B</th>
<th>rij Å</th>
<th>SA (i, j)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu04–Lys79</td>
<td>A–EF</td>
<td>−1.49</td>
<td>2.73</td>
<td>0.20, 0.50</td>
</tr>
<tr>
<td>Glu06–Lys133</td>
<td>A–H</td>
<td>−1.15</td>
<td>2.83</td>
<td>0.50, 0.45</td>
</tr>
<tr>
<td>Lys16–Asp122</td>
<td>A–GH</td>
<td>−1.21</td>
<td>3.38</td>
<td>0.05, 0.60</td>
</tr>
<tr>
<td>Glu18–Lys77</td>
<td>A–E</td>
<td>−1.21</td>
<td>2.97</td>
<td>0.30, 0.55</td>
</tr>
<tr>
<td>Asp20–Arg118</td>
<td>B–G</td>
<td>−1.17</td>
<td>3.28</td>
<td>0.50, 0.25</td>
</tr>
<tr>
<td>Asp44–Asp47</td>
<td>CD–CD</td>
<td>−0.93</td>
<td>3.24</td>
<td>0.45, 0.55</td>
</tr>
<tr>
<td>Arg45–Asp60</td>
<td>CD–E</td>
<td>−1.72</td>
<td>3.05</td>
<td>0.15, 0.15</td>
</tr>
<tr>
<td>Glu52–Lys56</td>
<td>D–D</td>
<td>−1.26</td>
<td>2.98</td>
<td>0.45, 0.35</td>
</tr>
<tr>
<td>Glu105–Arg139</td>
<td>G–H</td>
<td>−0.78</td>
<td>4.52</td>
<td>0.30, 0.40</td>
</tr>
</tbody>
</table>

The data are from Garcia-Moreno et al. (1); this table is similar to Table 4 in that paper. Position of the residues in Mb: helix (one letter) or turn (two letters). Free energy values are given in kcal/mol at pH 7.0 and 0.01 M ionic strength (I). r is the distance between atoms in Å. SA is fractional static solvent accessibility of the charged atom (varies from 0.05 for totally buried to 0.95 for completely solvent-exposed) (I). Average of the intraelemental values for the two residues (from Table 1 of ref 1).

The effect of charge reversal on the stability of cytochrome c and ribonuclease A; because pH 4.0 is close to the pH range where the predicted values of ΔΔGΔ change from negative to positive, they may have found small effects only for this reason. Meeker et al. (7) replaced each of the ionizable residues of staphylococcal nuclease and found only small effects on stability in general, although a few mutations did cause a large decrease in stability, about 3 kcal/mol. Previous mutational studies of specific ion pairs in proteins are discussed below.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification. Mutations were constructed using a cloned sperm whale Mb cDNA (8). The expression and purification of recombinant Mb were as described in Loh et al. (9). ApoMb was prepared as described in Rossi-Fanelli et al. (10) and its concentration determined at pH 4.0 was carried out by diluting the protein in 8 M urea. Experiments with the pH 4 folding intermediate were made in 4 mM citrate, pH 4.2, 0.5 mM KCN, at 25 °C. CyanoMb unfolding reached equilibrium within 1 h after dilution into denaturant.

Data Analyses. Urea-induced unfolding curves were fitted to a two-state equation as described by Santoro and Bolen (17). The curves were fitted with KaleidaGraph (Abelbeck software). All analyses of cyanoMb wild-type or mutant curves were made using the m value obtained for the wild type at pH 5.0 in the absence of NaCl. For the pH 4 folding intermediate, the m value and native baseline of the wild-type apoMb intermediate were used to fit the mutant fluorescence data, since the baselines for many mutants could not be determined accurately.

RESULTS

Urea-Induced Unfolding of cyanoMb and apoMb. Figure 1 shows urea-induced unfolding of wild-type cyanoMb followed by two different probes, CD at 222 nm and absorbance at the Soret band (423 nm). The raw data are shown in Figure 1a, and the fraction folded is shown in Figure 1b. Urea-induced unfolding by both CD or by absorbance at the Soret band shows identical transition curves characteristic of two-state unfolding. Unfolding under these conditions is reversible (Figure 1b). The unfolding curves of cyanoMb and apoMb at pH 5.0 are shown in Figure 2. ApoMb unfolds at a lower urea concentration than cyanoMb in the same conditions.

Data Analysis. The cyanoMb mutant unfolding curves are analyzed using the m value obtained for the wild type at pH 5.0, 25 °C, in the absence of salt: 1.6 kcal mol−1 M−1. Using this approach, we can relate the unfolding free energies of mutant and wild type by comparing the C_m values: m-value determinations are not very accurate for technical reasons.
In the analysis of the unfolding curves of the pH 4 folding intermediate, using fluorescence data, the same approach is used. The urea unfolding curves of I for mutants are analyzed using the $m$ value obtained for wild type: $1.83 \text{ kcal mol}^{-1} \text{ M}^{-1}$. The wild-type folded baseline is also used to fit the mutant data. These baselines are not determined accurately because of an increase in fluorescence well below the transition zone. For the wild type this effect occurs because of an urea-dependent equilibrium between two forms of the folding intermediate (Ia and Ib) at low urea concentrations ($<1 \text{ M}$) (19).

**Stability of cyanoMb Native State.** Figure 3 shows the raw data (a) and fraction folded (b) for urea-induced unfolding of the wild type and representative charged-site mutants of cyanoMb measured by CD. Wild-type cyanoMb has $C_m = 4.2 \text{ M urea}$ (Table 2). Table 2 gives the urea midpoint and $\Delta AG$ for urea-induced unfolding of wild type and mutants, measured by CD and Soret absorbance. The predicted values of $\Delta AG$ for the potential salt bridge mutants (I) are also given in Table 2. The results obtained by Soret absorbance are in good agreement with the ones obtained by CD (Table 2). One pairwise interaction is found: Asp20–Arg118. Each of the single mutants gives $\Delta AG = -0.5 \text{ kcal mol}^{-1}$. Replacement of Glu18 by Ala gives $\Delta AG = -1.0 \text{ kcal mol}^{-1}$, but replacement of Lys77 by Ala gives only $\Delta AG =$
DISCUSSION

The objective of our work is to determine whether specific ion pair interactions make an important contribution to the stability of Mb, as suggested by the electrostatic predictions of Garcia-Moreno et al. (1), based on the accessibility-modified Tanford—Kirkwood model. To undertake this study, we first found conditions in which the urea-induced unfolding of holoMb is two-state and reversible, namely with cyanoMb at pH 5.0.

**Table 2: Free Energy of Unfolding for Mb Mutants at Charged Sites: Experimental and Predicted Values**

<table>
<thead>
<tr>
<th>Paired residues</th>
<th>$C_m$</th>
<th>$\Delta G$</th>
<th>$\Delta G_{\text{predicted}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu04–Lys79</td>
<td>3.97</td>
<td>-0.35</td>
<td>-1.49</td>
</tr>
<tr>
<td>Glu06–Lys133</td>
<td>4.16</td>
<td>-0.05</td>
<td>-1.15</td>
</tr>
<tr>
<td>Lys16–Asp122</td>
<td>4.13</td>
<td>-0.10</td>
<td>-1.21</td>
</tr>
<tr>
<td>Glu18–Lys77</td>
<td>3.59</td>
<td>-0.95</td>
<td>-1.21</td>
</tr>
<tr>
<td>Glu18–Lys77</td>
<td>4.31</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Asp20–Arg118</td>
<td>3.87</td>
<td>-0.50</td>
<td>-1.17</td>
</tr>
<tr>
<td>Asp20–Arg118</td>
<td>3.78</td>
<td>-0.65</td>
<td>-1.17</td>
</tr>
<tr>
<td>Asp44–Lys47</td>
<td>4.34</td>
<td>0.25</td>
<td>0.93</td>
</tr>
<tr>
<td>Arg45–Asp60</td>
<td>4.13</td>
<td>-0.15</td>
<td>-1.25</td>
</tr>
<tr>
<td>Glu52–Lys56</td>
<td>3.97</td>
<td>-0.35</td>
<td>-1.26</td>
</tr>
<tr>
<td>Glu105–Arg139</td>
<td>3.91</td>
<td>-0.45</td>
<td>-0.78</td>
</tr>
</tbody>
</table>

$C_m$ is the molar urea concentration at 50% unfolded. $\Delta G_{\\text{predicted}}$ is the free energy of unfolding for the wild type. The presence of NaCl causes a loss of the Soret signal and formation of apoMb when H24V/H119F cyanoMb unfolds at pH 4.0.

The objective of our work is to determine whether specific ion pair interactions make an important contribution to the stability of Mb, as suggested by the electrostatic predictions of Garcia-Moreno et al. (1), based on the accessibility-modified Tanford—Kirkwood model. To undertake this study, we first found conditions in which the urea-induced unfolding of holoMb is two-state and reversible, namely with cyanoMb at pH 5.0.

**Figure 4:** Salt dependence of $\Delta G$ for the urea-induced unfolding of cyanoMb, showing how the stability depends on pH and [NaCl]. Dashed lines are used only to link the symbols and have no further meaning.

0.2 kcal mol$^{-1}$, so that Glu18–Lys77 is not a strong pairwise interaction. In the X-ray structure of Mb (20), Glu 18 makes a hydrogen bond to the indole NH of Trp 14, which may explain the destabilizing effect of E18A. Several other mutants give $\Delta G$ values of $\leq 0.2$ kcal mol$^{-1}$ or less (D44A, D60A, D122A, and K133A), and a few mutants give $\Delta G$ values in the range $0.2$ to $0.5$ kcal mol$^{-1}$ (K56A, E04A, and K139A).

**Dependence of Stability on [NaCl].** Figure 4 shows the unfolding free energy of wild-type (pH 5.0 and 4.0) and mutant (pH 5.0 and 4.0) cyanoMb as a function of [NaCl]. There is an increase in stability for wild-type cyanoMb as the pH increases from pH 5.0 to pH 6.0, and there is a decrease in stability for the pH 5 intermediate at [NaCl] greater than 0.5 M. The wild-type stability measurements at pH 4.5 indicate a deviation from two-state behavior (data not shown).

The double mutant H24V/H119F is also studied here (Figure 5) because apoMb remains native at pH 4 with this mutant, unlike the wild type. The stability of H24V/H119F decreases as the pH decreases from 5.0 to 4.0, and there is a decrease in stability with increasing [NaCl] (Figure 5).
creases rapidly as the pH is increased above 5. At pH 6.0, and at pH 7.8 cyanoMb is only partially unfolded in 10 M urea (data not shown). On the other hand, at pH 4.5 unfolding of cyanoMb is no longer two-state (data not shown). These considerations limit our study of wild-type cyanoMb to pH 5.0. The double mutant H24V/H119F, which does not form a folding intermediate at pH 4.0 (21), can be studied at pH 4.0, at 0 M NaCl, where its cyanoMb form shows two-state unfolding (Figure 5).

Hargrove and Olson (22) point out that, at sites where mutations contact the heme group, the stability of holoMb is determined chiefly by the affinity of apoMb for heme. The mutations studied here all involve replacement of a charged residue by alanine, and these sites do not contact the heme. Moreover, the mutations affect the stability of native apoMb and holoMb (cyanoMb) in a similar manner (pH and urea-induced unfolding curves of native apoMb not shown).

Using C_m Values To Measure Relative Stability of Mutants. In Tables 2 and 3 we follow the procedure of Johnson and Fersht (18) in calculating \( \Delta G \) for mutants from their C_m values by using the m value for wild type. As they point out, the C_m values can be measured more precisely than the m values, and if the conventional interpretation of the m value is adopted (m is proportional to the buried surface area exposed on unfolding), then m values are not expected to vary significantly in a series of point mutants unless the amount of buried surface area in the denatured ensemble can be changed by point mutations. Shortle and co-workers (23) have shown that point mutations do affect the apparent m values of staphylococcal nuclease (SNase) mutants, and they attribute the changes to the denatured ensemble. It is controversial, however, whether SNase and these mutants show two-state, or more complex, unfolding behavior (24–27) and whether the mutational changes in m values may in fact reflect deviations from two-state behavior. By size exclusion chromatography, Baskakov and Bolen (27) have shown recently that one of Shortle’s mutants (V66W) deviates from two-state behavior and that the properties of the denatured ensemble of SNase are complex (“variable”), in contrast to those of RNase A whose properties are “fixed” as a denatured ensemble.

Our results do not suggest that there are significant mutant-specific changes in the m value when the stability of holoMb is measured. The urea unfolding curves (Figure 1) satisfy standard tests for two-state folding: the curves are symmetrical and reversible, and the two curves monitored by probes of secondary structure (far-UV CD) and tertiary structure (Soret absorbance) are superimposable. Thus, it is plausible that cyanoMb represents a well-behaved two-state system like RNase A rather than a more complex system like SNase, but future work is needed to decide this point. The results presented here for the pH 4 folding intermediate of apoMb also satisfy a standard test for two-state folding: the unfolding curves monitored by probes of secondary structure (far-UV CD) and tertiary structure (tryptophan fluorescence) are superimposable. However, earlier work showed that severely destabilizing mutations cause a breakdown of two-state folding (28, 29) and this caveat should be remembered. The charged site mutants studied here cause only moderate changes in stability of the intermediate.

**Figure 6:** Replacement of negatively charged residues by alanine in the AGH subdomain destabilizes the pH 4 folding intermediate. The normalized fraction folded, measured by CD at 222 nm, is plotted as a function of urea concentration. Unfolding was measured up to 5.6 M urea, but only the results between 0 and 4 M are shown. The measured values are shown by symbols and the fitted curves by lines, and the parameters obtained from the data analysis are given in Table 4.

### Table 3: Free Energy of Unfolding of the pH 4 Folding Intermediate (I) for New Charged Site Mutants Not Studied Previously (See Ref 14)

<table>
<thead>
<tr>
<th>Mutant</th>
<th>C_m(C,D, fluorescence)</th>
<th>ΔG^a(C,D, fluorescence)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>1.58, 1.50</td>
<td>0.0^a</td>
</tr>
<tr>
<td>D44A</td>
<td>1.50, 1.40</td>
<td>−0.15, −0.20</td>
</tr>
<tr>
<td>K56A</td>
<td>1.45, 1.40</td>
<td>−0.25, −0.20</td>
</tr>
<tr>
<td>D60A</td>
<td>1.53, 1.42</td>
<td>−0.10, −0.15</td>
</tr>
<tr>
<td>E18A</td>
<td>1.20, 1.23</td>
<td>−0.70, −0.50</td>
</tr>
<tr>
<td>E04A</td>
<td>1.20, 1.20</td>
<td>−0.70, −0.55</td>
</tr>
<tr>
<td>K77A</td>
<td>1.56, 1.50</td>
<td>−0.05, −0.02</td>
</tr>
</tbody>
</table>

^a_ C_m is the molar urea concentration at 50% folded. ^a_ ΔG = ΔG(wild type) − ΔG(mutant); values given in kcal/mol. ^a_ The free energy of unfolding for the wild type is −2.90 kcal/mol by CD and −2.75 kcal/mol by fluorescence.
stabilize Mb by more than 1 kcal mol$^{-1}$ (Table 1), only one pairwise interaction (Asp20$-$Arg118) was found to stabilize Mb by as much as 0.5 kcal mol$^{-1}$. The residues studied here are chiefly solvent exposed (I), and for that reason, not much change in stability might be expected as a result of mutating them. Nevertheless, they were predicted to be stabilizing (I). The nine potential ion pairs include several interhelix candidates (Table 1) which, if they had been found, might have proven important in determining the folding pathway of apoMb. Two other replacements of a charged residue by alanine (Glu4 and Arg139) gave almost a 0.5 kcal mol$^{-1}$ decrease in stability, and they remain possible candidates for minor pairwise interactions. The stabilizing effects of the mutations made here were tested at pH 5.0 for reasons explained above, but the predicted interhelix interactions were evaluated at pH 7.0 (1). The overall electrostatic interactions of these residues were predicted, however, both at pH 5.0 and at pH 7.0 (see Table 1 of ref I), and the interhelix interactions account for the major part of the overall electrostatic interactions. The predicted overall interactions change only slightly between pH 5.0 and pH 7.0, which suggests that the results found here may also be applicable at pH 7.0.

These results suggest that the accessibility-modified Tanford$-$Kirkwood model overestimates the actual electrostatic interactions in proteins, for a reason that remains to be determined. The current FDPB procedure also overestimates electrostatic effects when used to predict $pK_a$ values in proteins (30). The best agreement with actual $pK_a$ values is obtained by using a fictitious value of 20 for the dielectric constant rather than a more realistic value of 4. This is equivalent to saying that the actual electrostatic interactions in proteins are 5 times weaker than predicted. A possible solution to the puzzle has been offered by Havranek and Harbury (unpublished results). They use a dielectric constant of 4 and allow the rigid side chains of the X-ray structure to unfold by as much as 0.5 kcal mol$^{-1}$, as judged both by the effects on $pK_a$ values and by the effects on $\Delta G_{\text{unf}}$ of mutating either the His or the Asp residue to Asn. Similarly, an Asp$-$Arg interaction makes a major contribution to the stability of barnase (32). On the other hand, Arc repressor contains a complex ion pair interaction involving three partially buried charged side chains and replacement of all three charged residues by nonpolar residues increases its stability (33). Side chain ion pairs, at specific spacings of $i$, $i + 4$, stabilize peptide helices by modest but measurable amounts (3, 4). They are salt bridges (hydrogen-bonded ion pairs) in which the hydrogen bond is a major contributor to stability, as judged both by the effects of protonating carboxylate groups and by attempting to screen the interaction with NaCl. A complex RER salt bridge significantly stabilizes the GCN4 dimeric coiled coil (34), and the RER residues are expected to be solvent exposed. Complex networks of ionized side chains are commonly found on the surfaces of thermophilic proteins, but evaluation of their stabilizing effects is still under discussion (35).

**Decrease of Mb Stability with increasing [NaCl] and at Acid pH.** The results shown in Figure 4, in which $\Delta G_{\text{unf}}$ decreases significantly as [NaCl] increases from 0 to 0.1 M, demonstrate directly that favorable electrostatic interactions contribute to the stability of Mb at these pHs. The decrease in $\Delta G_{\text{unf}}$ is not large (about 2 kcal mol$^{-1}$), but it is significant in comparison with the value of $\Delta G_{\text{unf}}$ itself, which varies from about 3 to 9 kcal mol$^{-1}$ in Figure 4, depending on the pH and the mutant studied as well as on [NaCl], pH 5 should not be far from the pH where the net charge is zero and $\Delta G_{\text{unf}}$ changes sign, from being unfavorable at low pH, where only positively charged groups are present, to being favorable at neutral pH, where both positive and negative charges are present. Consequently, if similar experiments could be made at higher pHs, larger effects of [NaCl] might well be found. Unfortunately, cyanoMb is too stable to permit urea unfolding experiments at higher pHs. The crossover pH where the net charge is zero must be measured, not calculated, because it depends on anion binding as well as on the distribution of acidic and basic residues in the protein; it can be measured by electrophoresis.

A strong decrease in $\Delta G_{\text{unf}}$ at acid pH is predicted by electrostatic calculations and is observed for almost all proteins; usually the decrease in stability is observed as $T_m$ versus pH, where $T_m$ is the temperature midpoint of a thermal unfolding curve. Direct measurements of $\Delta G_{\text{unf}}$ versus pH by urea or GdmCl unfolding have been made for a few proteins such as RNase A (36, 37), and they confirm that there is a large decrease in $\Delta G_{\text{unf}}$ at acid pH. The $pK_a$ values of all the carboxylate residues of T4 lysozyme have been measured by NMR and used to predict the pH dependence of $\Delta G_{\text{unf}}$. At pH 1 the predicted value of the pH-dependent contribution is very large, about 17 kcal/mol (38). These results are consistent with large unfavorable values of $\Delta G_{\text{el}}$ at acid pH, resulting from mutual repulsion between positively charged groups; however, some of the abnormally low $pK_a$ values might arise from other causes, such as hydrogen bonding. His 24 in native apoMb is buried and H-bonded to His 119, and it is not detectably protonated in native apoMb, even at pH 4.0 (21). Its $pK_a$ is below 3.0, but it is predicted to be 5.1 by a FDPB calculation (4).

**Effects of Charged-Site Mutations on the Stability of the pH 4 Folding Intermediate.** The partly folded form of apoMb found at pH 4 (I), which contains stable A, G, and H helices and a partly stable B helix, is unfolded by acid below pH 4, in a pH range where only Asp and Glu residues are protonated. An earlier study (14) considered two models for the acid-induced unfolding of I: a net charge mechanism, in which I unfolds when the electrostatic repulsion between positively charged residues exceeds a critical value as Asp and Glu residues become protonated, and a salt bridge mechanism, in which I unfolds when the salt bridges that stabilize I are broken as Asp and/or Glu residues become protonated. The two mechanisms have been tested by mutating the basic residues in the putative salt bridges to alanine. According to the salt bridge mechanism, these mutations should destabilize I. According to the net charge mechanism, they should stabilize I. Six basic residues (Lys,
for discussion and suggestions. We also thank Dr. Jose ACKNOWLEDGMENT effect, which is destabilizing. lower helix propensity than alanine, the helix stabilizing of charge

procedure for experimentally quantitating the contributions may produce a smaller effect for this reason. Earlier, mutation to alanine may have compensated the net charge effect. Earlier results strongly support the net charge mechanism. Putative Ion Pairs of Myoglobin

REFERENCES


