Role of the Heme Propionates in the Interaction of Heme with Apomyoglobin and Apocytochrome b_5[†]

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ABSTRACT: The heme propionate groups of both myoglobin (Mb) and cytochrome b_5 form hydrogen bonds with nearby surface amino acids residues that are believed to stabilize the heme−protein complex. To evaluate the magnitude of this stabilization, the kinetics of heme dissociation from variants of horse heart Mb and cytochrome b_5 in which these hydrogen bonding interactions have been systematically eliminated were studied by the method of Hargrove and colleagues (1994), and their thermal stability was assessed. Elimination of each hydrogen bond was found to decrease the thermal stability of the proteins and increase the rate constant for heme dissociation in a progressive fashion. For the Mb derivatives, ^{1}H-NMR studies indicate that the elimination of individual hydrogen bonds also affects the rate at which the heme orientational equilibrium is achieved. In both types of kinetics experiment, the effects of decreasing the number of potential hydrogen bonding interactions are found to be cumulative. Despite their kinetic effects, elimination of these hydrogen bonding interactions had no influence on the initial distribution of heme orientational isomers immediately following reconstitution or on the equilibrium constant of heme orientational disorder. The interactions between the heme propionates and nearby protein residues play a partial role in the stabilization of the heme−protein complex and are a major factor in the kinetic "trapping" of the minor heme orientation. Comparisons of the various rate constants determined for the mechanism of heme binding and reorientation suggests that the intramolecular reorientation mechanism is slightly favored over the intermolecular mechanism.

Since the pioneering work of Hill and Holden (1926) in which native apohemoglobin (Hb) was first reconstituted with ferriprotoporphyrin IX, the kinetics and structural consequences of heme binding to apomyoglobin (apo-Mb) and apohemoglobin (apo-Hb) have been the subjects of continuing investigations [e.g., Gibson and Antonini (1960, 1963), Gibson (1964), Breslow (1964), Waks et al. (1973), Adams (1976), Chu and Bucci (1979), Craik et al. (1980), Rose and Olson (1983), Kawamura-Konishi et al. (1988), Benesch and Kwong (1990), and Hargrove et al. (1994)]. One fundamental feature of the interaction of heme with apo-Mb and apocytochrome b_5 that was not appreciated until the application of ^{1}H-NMR spectroscopy to the characterization of these proteins (La Mar et al., 1978; Keller et al., 1976) is that both proteins exhibit two populations that differ in the orientation of heme binding. Specifically, the orientation of heme binding in these two populations differs by a 180° rotation of the heme about the α/γ-*meso* heme axis (Figure 1A)(La Mar et al., 1978; Keller & Wüthrich, 1980). For sperm whale and horse heart metMb, 92% of the protein binds heme in the "major" orientation and 8% binds heme in the "minor" orientation (La Mar et al., 1983), and for beef liver cytochrome b_5, ∼90% of the protein binds heme in the "major" orientation and ∼10% of the protein binds heme in the "minor" orientation (Keller et al., 1976; La Mar et al., 1981). Interestingly, upon initial addition of protoheme IX to apo-Mb or apocytochrome b_5, the two orientational isomers are formed in equal abundance, and with time an equilibrium is established in which one orientational isomer dominates (Jue et al., 1983; La Mar et al., 1981).

The present study evaluates the manner in which hydrogen bonds formed by the heme propionate groups with both apoproteins stabilizes heme binding to these proteins. The strategy used in this work differs from that of previous work in that we have studied the kinetics of heme binding dynamics to variants of Mb and cytochrome b_5 that have been designed to interrupt these hydrogen bonds through specific amino acid substitutions. The hydrogen bonding interactions of the heme propionate groups of horse heart Mb and bovine liver cytochrome b_5 that have been identified by X-ray crystallographic analysis (Evans & Brayer, 1990; Durley & Mathews, 1996) are illustrated in Figure 1B and 1C.

In the case of Mb, a hydrogen bonding lattice is formed by Leu89, Ser92, His93, His97, and the heme 7-propionate that presumably stabilizes the interaction of the heme with

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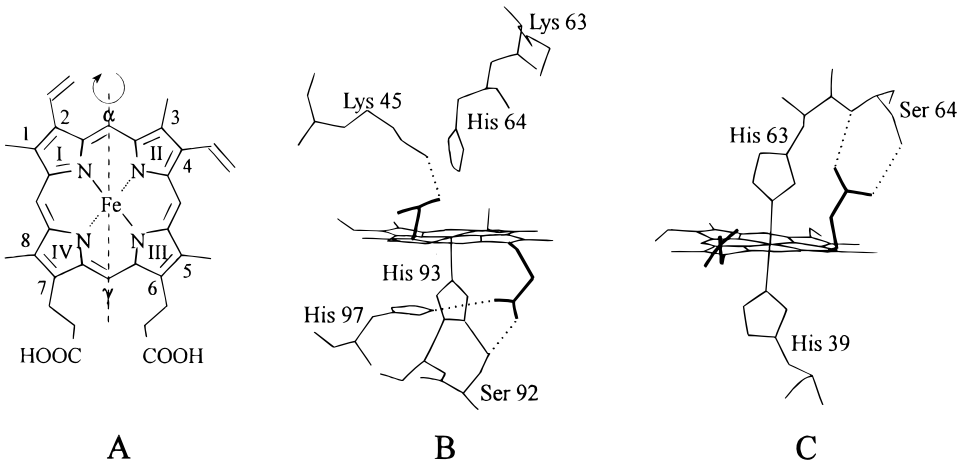

FIGURE 1: (A) Structure of protoheme IX with the nomenclature used in this report indicated and the hydrogen bonds (dotted lines) formed by the heme propionate groups with amino acid residues of (B) horse heart Mb and (C) bovine liver cytochrome b_5.

the apoprotein. In addition, Lys45 forms a hydrogen bond with the 6-propionate group (Evans & Brayer, 1990). In sperm whale Mb, residue 45 is an arginine, which forms a hydrogen bond with the distal His64 through a water molecule and a hydrogen bond with heme 6-propionate (Takano, 1977). Consequently, variant forms of Mb with substitutions at these positions have been included in the current study along with the wild-type protein. Mb reconstituted with dimethylester−protoheme IX (DME−heme) in which both propionate groups have been esterified to reduce hydrogen bonding interactions with the propionates has also been evaluated.

In the case of bovine liver cytochrome b_5, the heme 7-propionate forms two hydrogen bonds, one with the side chain hydroxyl and one with the main chain amide of Ser64, while the heme 6-propionate forms no additional interactions (Durley & Mathews, 1996). Consequently, the Ser64Ala variant (Funk et al., 1990) and the DME−heme-substituted form of cytochrome b_5 (Reid et al. 1984) were included in this study to evaluate the importance of the propionate interactions in this cytochrome on heme−protein stability.

Two types of kinetics experiments have been employed to evaluate the contributions of these interactions to the stability of heme-apoprotein interaction. In the first type of experiment, we have studied the effects of these modifications on the heme orientational equilibrium and the kinetics by which equilibration is achieved following reconstitution of the apoprotein with heme as described by La Mar et al. (1984). In the second type of experiment, we have studied the kinetics of heme dissociation from these proteins by the method of Hargrove and co-workers (1994) in which the transfer of heme from native protein to a Mb variant with unique optical properties is monitored spectrophotometrically.

EXPERIMENTAL PROCEDURES

Wild-type horse heart Mb (Sigma, lot 112H7120) was purified as described by Tomoda et al., (1981). Variants of horse heart Mb were constructed from a synthetic gene (Guillemette et al., 1991) by oligonucleotide-directed mutagenesis (Zoller & Smith, 1987) and were expressed and purified as described previously (Lloyd & Mauk, 1994). Extinction coefficients of the variant proteins were determined by the pyridine hemochromogen method (De Duve, 1948; Antonini & Brunori, 1971), and apo-Mb was prepared as described by Teale (1959). Reconstitution of wild-type Mb with dimethylester protoheme IX has been described previously (Lim, 1990).

Trypsin-solubilized (Reid & Mauk, 1982) and lipase-solubilized (Funk et al., 1990) bovine liver cytochrome b_5 were prepared by the methods indicated. Trypsin-solubilized bovine liver cytochrome b_5 was reconstituted with dimethylester protoheme IX by the method of Reid et al. (1984).

Analysis of Thermal Stability. Circular dichroism spectra were recorded with a Jasco model J-720 spectropolarimeter that was calibrated with ammonium *d*-camphor-10-sulfonate (Aldrich) and that was equipped with a Neslab model RTE-110 circulating water bath and a Neslab model RS-2 remote sensor. Both the spectropolarimeter and water bath were operated under computer control. Protein samples (10 μM; sodium phosphate buffer (10 mM, pH 7.0)] were placed into a cylindrical, water-jacketed quartz cell (0.1 cm path length), and ellipticity was recorded at 222 nm from 40 to 85 °C with a heating rate of 50 °C/h. The midpoint melting temperature (T_m) was determined from the first derivative of the resulting ellipticity *vs* temperature plot.

Heme Reorientation Experiments. ^{1}H-NMR spectra were recorded with a Bruker model MSL-200 spectrometer at 20 °C in 50 mM deuterated sodium phosphate buffer ("pH" 7.0; pH values of D_2O solutions were not corrected for the isotope effect and are referred to as "pH" values). The measurement of the rate constant for heme reorientation following reconstitution of apo-Mb derivatives with protoheme IX was performed as described by La Mar et al. (1984). As these variants have significantly increased rates of heme reorientation, the KCN quench method was employed (La Mar et al., 1984) to take advantage of the fact that heme reorientation occurs much more slowly in the metMbCN derivative. Hemin (0.9 equiv) dissolved in NaOH (0.2 M) was added to apo-Mb (sodium phosphate buffer (50 mM), "pH" 7.0, 25 °C) to start the reaction; the reconstitution mixture was sampled periodically, quenched with 20 equiv of KCN, and the NMR spectrum was recorded. To determine the initial heme orientation ratio, the first sample was quenched within the first minute after the hemin was added. The equilibrium constant for heme disorder, K_D, can be defined as $k_f/k_b = [M_1]_E/[m_1]_E$, where k_f and k_b are the rate constants for heme dissociation and heme association, respectively, and M_1 and m_1 are the intensities of the 5-methyl or 8-methyl resonance

of the major and minor heme orientations, respectively (La Mar et al., 1984). The methyl resonances, M_1 and m_1, are the resonances shifted furthest downfield in all the Mb variants. As the resonances of the two orientations in some Mb variants were not well resolved, the intensities of the two peaks were determined using the curvefit function of the program Grams/386 (Galactic Industries). A plot of $\ln\{(A_t - A_\infty)/(A_0 - A_\infty)\}$ *vs* time has a slope equal to $-k_{obsd}$, where A_t is $M_1/(M_1 + m_1)$ and A_0 and A_∞ are the initial and equilibrium ratios of the two orientations, respectively. The rate constants for the forward, k_f, and reverse, k_b, reactions can then be calculated from the relationship $k_{obsd} = k_f + k_b$.

Determination of Heme Dissociation Rate Constants. Kinetic analysis of heme dissociation from wild-type and modified forms of Mb and cytochrome b_5 was performed as described by Hargrove et al. (1994) except that the His64Tyr/Val68Phe double variant of horse heart Mb was used rather than the corresponding variant of sperm whale Mb. Rate data were collected with a Cary model 3 spectrophotometer that was equipped with a water-jacketed cell holder and a Lauda model RC3 circulating, thermostated water bath and interfaced to a microcomputer. For Mb derivatives, heme dissociation was monitored at 410 nm, while heme dissociation from cytochrome b_5 was monitored at 600 nm. Heme dissociation was monitored under pseudo-first-order conditions with a His64Tyr/Val68Phe variant apo-Mb (90 μM) to Mb or cytochrome b_5 (3 μM) ratio of 30:1 so that $k_{obsd} = k_{-H}$, where k_{-H} is the rate constant for hemin dissociation (Hargrove et al., 1994). The resulting data were fitted to a single exponential with the program Scientist (MicroMath, Inc.). Sodium acetate buffer (0.15 M) was used at pH 5.0 and 5.5, and sodium phosphate buffer (0.15 M) was used at pH 7.0. All assay solutions also contained 0.45 M sucrose to stabilize the His64Tyr/Val68Phe apo-Mb variant (Hargrove et al., 1994).

RESULTS

The electronic absorption spectrum of the His64Tyr/Val68Phe double variant of horse heart metMb is essentially identical to that of the corresponding variant of sperm whale Mb (Hargrove et al., 1994) and is consistent with coordination of Tyr64 as the sixth ligand to the heme iron. The electronic absorption spectra of the other Mb variants were also identical to that of the wild-type protein apart from small decreases in the molar absorptivities of the Soret band. Wild-type apo-Mb was found to exhibit a melting temperature (T_m) of 63.2 °C while the corresponding value for the His64Tyr/Val68Phe variant was found to be 63.9 °C.

Kinetics of Heme Reorientation Following Reconstitution of Mb with Protoheme IX. The rate constant for heme reorientation in sperm whale Mb is known to be highly dependent on pH and to exhibit a minimum at neutral pH (La Mar et al., 1984). For this reason, kinetic studies are most conveniently performed at "pH" 7.0. The ^{1}H-NMR spectrum of the metMbCN derivative of the Lys45Glu horse heart Mb variant was monitored as a function of time immediately following reconstitution with heme as described above (Figures 2 and 3). The resonances labeled M_1 and m_1 correspond to the heme methyl peaks previously assigned in the spectrum of sperm whale metMbCN to the major and minor heme orientation, respectively (La Mar et al., 1984), and are identified here by analogy. The ratio of major to minor forms is ~1:1 immediately after reconstitution (Figure 2A). With time, the intensity of M_1, the 5-methyl heme resonance of the major heme orientation, increases at the expense of m_1, the 8-methyl heme resonance of the minor heme orientation, as the minor form converts into the major form. Equilibrium is achieved in ~21 h (Figure 2D). The other resonances that change in intensity with heme reorientation are also indicated in Figure 2.

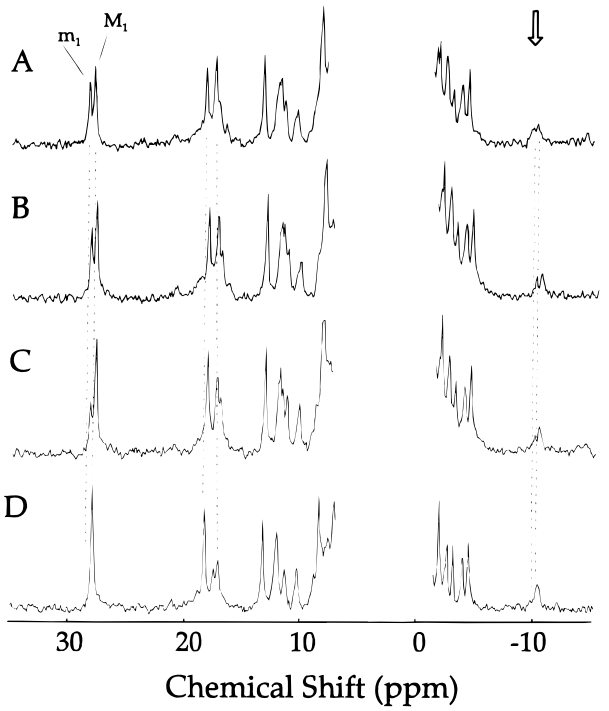

FIGURE 2: ^{1}H-NMR spectra (200 MHz) of the Lys45Glu variant of horse heart metMbCN (0.5 mM) in deuterated sodium phosphate buffer (μ = 0.1 M, "pH" 7.0, 20 °C). The metMb was reconstituted with heme (0.9 equiv) at 25 °C, samples were removed, and the reaction was stopped with KCN at (A) 1.5 min, (B) 31.5 min, (C) 81.5 min and (D) 21 h. Peaks corresponding to the two heme orientational isomers are labeled as follows: M_1 denotes the 5-methyl heme resonance of the major orientation (28.0 ppm), and m_1 denotes the 8-methyl heme resonance of the minor orientation (28.4 ppm).

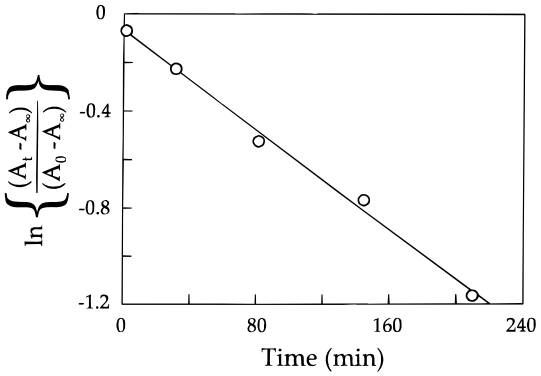

FIGURE 3: Kinetics of equilibration of the Lys45Glu variant of horse heart Mb with native heme at 25 °C, "pH" 7.0 as monitored by ^{1}H-NMR spectroscopy.

Comparison of the ^{1}H-NMR spectra obtained at equilibrium ("pH" 7) for the metMbCN derivatives of all the variants indicates that the ratios of the major and minor heme orientational isomers, estimated from the intensity of the methyl resonances, M_1 and m_1, are similar to that previously observed for sperm whale Mb (La Mar et al., 1984). The

Table 1: Rate Constants for Heme Reorientation in Wild-Type and Variant Forms of metMb[a]

protein	k_f ($\times 10^2$ h^{-1})	k_b ($\times 10^2$ h^{-1})
sperm whale Mb		
wild-type	1.9 ± 0.2[b]	
horse heart Mb		
wild-type	3.7 ± 0.4	0.31 ± 0.02
Lys45Glu	51 ± 2	4.2 ± 0.1
Lys45Glu/Lys63Glu	66 ± 6	6.0 ± 0.6
His97Leu	42 ± 6	3.4 ± 0.4
Ser92Asp	150 ± 10	12.6 ± 0.6
Lys45Glu/His97Leu	450 ± 30	37 ± 2
Lys45Glu/Ser92Asp	1440 ± 120	120 ± 6
Lys45Glu/Lys63Glu/ His97Leu/Ser92Asp	4200 ± 600	350 ± 40
His64Tyr/Val68Phe	9.0 ± 0.6	0.78 ± 0.06

[a] "pH" 7.0, 25 °C. [b] pH 6.4, 25 °C (La Mar et al., 1984).

equilibrium constant for the heme disorder equilibrium (K_D) was found to be ~12 for all of the Mb variants included in this study. Furthermore, addition of KCN to each of the freshly reconstituted protoheme IX/apo-Mb mixtures establishes, in each case, that equal amounts of the two orientational isomers are present immediately after addition of heme, as was also found for the wild-type sperm whale protein (La Mar et al., 1984).

The rate constants for heme reorientation determined from these experiments are shown in Table 1. Wild-type recombinant horse Mb exhibits a value for k_f that is slightly greater than that reported previously following reconstitution of sperm whale Mb with protoheme IX at pH 6.4 (La Mar et al., 1984). For those variants in which amino acid substitution was expected to eliminate one of the hydrogen bonds formed by the heme propionate groups (Lys45Glu, Lys45Glu/Lys63Glu, and His97Leu), both k_f and k_b increased 11−17-fold. The fact that the Lys63Glu substitution does not exhibit any significant influence on the kinetics of heme reorientation indicates that nonspecific electrostatic effects are not a factor in this process; Lys63 is not involved in hydrogen bonding interactions with the heme, so substitutions at this position are not expected to influence this process. On the other hand, the single variant Ser92Asp exhibits a rate constant for heme reorientation that is 3−4 fold greater than that of the other single variants. The somewhat greater influence of this substitution may result from the additional effect of disrupting the hydrogen bond normally formed by Ser92 with the proximal His93 ligand.

Elimination of two of the hydrogen bonds in the Lys45Glu/His97Leu double variant increases the rate constant for heme reorientation ~120-fold over that observed for the wild-type protein, while the elimination of two hydrogen bonds in the Lys45Glu/Ser92Asp double variant results in corresponding increases of ~390-fold. The greater kinetic effect exhibited by the Lys45Glu/Ser92Asp again reflects the additional structural consequences of replacing Ser92 discussed above. For the quadruple variant, where all hydrogen bonding interactions between the heme propionate groups and the apoprotein are eliminated, the reorientation rate is ~1100-fold greater than that of the wild-type protein. To a first approximation, therefore, it appears that the effects of individual hydrogen bonds on these rate constants are multiplicative. In other words, for each hydrogen bond that is eliminated, the rate constant for heme reorientation increases approximately 10-fold. Finally, we note that the

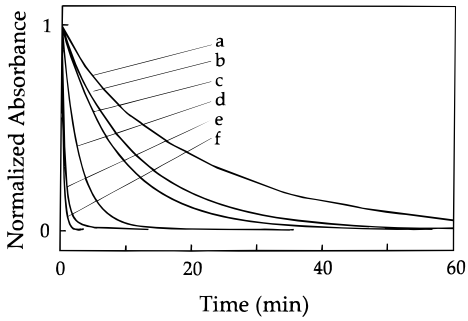

FIGURE 4: Absorbance changes (normalized) at 410 nm observed upon mixing Mb variants (3 μM) with the His64Tyr/Val68Phe variant apoprotein (90 μM) [sodium acetate buffer (0.15 M), 0.45 M sucrose, pH 5.0, 37 °C]: (a) wild-type; (b) Lys45Glu; (c) His97Leu; (d) Lys45Glu/His97Leu; (e) DME−Mb; (f) Lys45Glu/Lys63Glu/His97Leu/Ser92Ala.

Table 2: Rate Constants for Heme Dissociation from Wild-Type, Variant, and Heme-Substituted Forms of metMb[a]

	k_{-H} (h^{-1})		
protein	pH 5.0	pH 5.5	pH 7.0
sperm whale Mb			
wild-type[b]	0.7		0.01
Arg45Glu[b]	5.5		0.2
horse heart			
wild-type	2.2 ± 0.1	0.37 ± 0.08	0.06 ± 0.01
wild-type[c]	2.5		
Lys45Glu	6.2 ± 0.2	0.48 ± 0.01	0.22 ± 0.03
Lys45Glu/Lys63Glu	6.8 ± 0.3	0.44 ± 0.01	0.14 ± 0.02
His97Leu	6.6 ± 0.2	0.75 ± 0.04	0.32 ± 0.07
Ser92Asp	14.5 ± 03	0.75 ± 0.04	0.18 ± 0.01
Lys45Glu/His97Leu	21.70 ± 0.05	3.60 ± 0.03	1.64 ± 0.01
Lys45Glu/Ser92Asp	45.2 ± 0.3	4.7 ± 0.1	1.5 ± 0.2
Lys45Glu/Lys63Glu/ His97Leu/Ser92Ala	146 ± 3	22.7 ± 0.5	17.0 ± 0.5
DME−heme Mb	90 ± 10	7.1 ± 0.1	1.1 ± 0.1

[a] 37 °C. [b] Hargrove et al., 1994. [c] Hargrove et al., 1996b.

reorientation rate constant for the His64Tyr/Val68Phe double variant used to monitor heme dissociation kinetics (*vide infra*) is just ~2-fold faster than that of the wild-type protein. Similar measurements were not attempted with the cytochrome b_5 derivatives owing to the faster rate of heme reorientation (Singh & Wilson, 1990) in this protein and to limited amounts of material.

Kinetics of Heme Dissociation from Horse Heart Mb. The molar absorptivities of the Mb variants and derivatives studied in this work were greater than that of the His64Tyr/Val68Phe variant, which made it possible for heme dissociation to be monitored at the Soret maximum of the variant or derivative considered. The absorbance changes observed (pH 5.0) for the dissociation of heme from some of the Mb variants studied in this work are shown in Figure 4. In each case, the data were adequately described by a single exponential function. The rate constants for heme dissociation determined in this manner at pH 5.0, 5.5, and 7.0 are set out in Table 2.

At all values of pH, the rate constants for heme dissociation from the variant Mbs are greater than that observed for the wild-type protein though the differences are more apparent at pH 5 than at pH 7 (Table 2). The rate constants for heme dissociation increase at low pH as the result of protonation of the proximal histidine and disruption of the Fe−histidine bond (Giacometti et al., 1977; Coletta et al., 1985). The uncertainty in determining rate constants for

Table 3: Rate Constants for Heme Dissociation from Wild-Type, Variant, and Heme-Substituted Forms of Cytochrome b_5[a]

protein	k_{-H} (h^{-1}) pH 5.0	pH 5.5	pH 7.0
bovine liver			
lipase-solubilized, wild-type[b]	4.2 ± 0.5	1.4 ± 0.2	0.3 ± 0.1
lipase-solubilized, Ser64Ala[b]	43 ± 3	28.6 ± 0.3	13 ± 1
trypsin-solubilized, native[c]	6.8 ± 0.5	1.9 ± 0.1	0.23 ± 0.02
trypsin-solubilized, DME−heme[d]	37 ± 1	10.4 ± 0.3	1.9 ± 0.1

[a] 37 °C. [b] Recombinant (Funk et al., 1990). [c] Native cytochrome isolated from bovine liver. [d] Native cytochrome reconstituted with DME−protoheme IX.

heme dissociation at the higher values of pH, however, is greater owing to the difficulty in precise measurements of such slow processes. In particular, the extended exposure of the apoprotein to elevated temperature (37 °C) during the long assay times required for such measurements could result in partial denaturation of the apoprotein. The effect of pH on the behavior of the variants is difficult to interpret in detail owing to the electrostatic nature of the substitutions involved and the potentially complex electrostatic consequences that each of these substitutions could exhibit. As a result, our discussion of these rate constants is restricted to those obtained at pH 5 where the differences in rate constants are most apparent.

At pH 5.0, wild-type horse heart Mb exhibits a 3-fold greater rate constant for heme dissociation than reported for sperm whale Mb under the same conditions (Hargrove et al., 1994). For the horse heart protein, elimination of a single hydrogen bond formed by heme propionate groups results in ~3-fold increase in the rate constant for heme dissociation as demonstrated by the results obtained for the Lys45Glu, Lys45Glu/Lys63Glu, and His97Leu variants. As observed above for the heme reorientation kinetics, these effects are specific for hydrogen bonding interactions insofar as the additional replacement of Lys63Glu in the Lys45Glu/Lys63Glu double variant does not produce any kinetic consequences. The Ser92Asp variant exhibits a rate constant for heme dissociation that is 7-fold greater than that of the wild-type protein and that is, therefore, twice as great as that of the other single variants. Again, the greater perturbation introduced by replacement of this residue may be related to the elimination of the hydrogen bond formed by Ser92 with the proximal His93 ligand to the heme iron.

As observed for the heme reorientation kinetics, the effects of removing individual heme propionate−apoprotein hydrogen bonds on the rate constant for heme dissociation appear to be multiplicative. This relationship is most clearly illustrated by the observation that the rate constant for heme dissociation from the Lys45Glu/His97Leu double variant is ~3-fold greater than that of the single variants, Lys45Glu and His97Leu, the rate constants for which are both, in turn, ~3-fold greater than that of the wild-type protein. Similarly, the Lys45Glu/Ser92Asp variant exhibits a heme dissociation rate constant that is ~3-fold greater than that of the Ser92Asp variant, the rate constant for which is, in turn, 6.6-fold greater than that of wild-type Mb. The quadruple variant exhibits a 66-fold increase in heme dissociation rate constant relative to the wild-type protein, which is consistent with the 3-fold rate enhancement expected to result from incorporation of the Lys45Glu and His97Leu substitutions (for a total 9-fold increase in rate constant) and the Ser92Ala substitutions (6.6-fold increase) into a single protein.

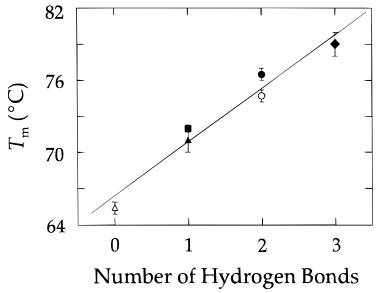

FIGURE 5: Dependence of melting temperature (T_m) on the number of potential hydrogen bonding interactions between the apoprotein and the heme propionate groups: (◆) wild-type Mb; (●) the His97Leu variant; (○) the Lys45Glu variant; (■) the His97Leu/Lys45Glu variant; (▲) the Ser92Asp/Lys45Glu variant; and (△) the Lys45Glu/Lys63Glu/His97Leu/Ser92Ala variant.

Although the esterification of the heme propionate groups in the DME−Mb derivative perturbs the electrostatics of the solvent-exposed heme edge significantly, esterification does not rule out the possible formation of a hydrogen bond with an adjacent amino acid residue. In the absence of structural information, therefore, it is difficult to predict what hydrogen bonding interactions may occur in this derivative. From the apparent quantitative relationship between heme propionate hydrogen bonding interactions and heme dissociation rate constants outlined above, however, it seems reasonable to infer that the ~41-fold greater rate constant observed for DME−Mb relative to the native protein results from destabilization of heme binding by the loss of an equivalent of three hydrogen bonds.

Kinetics of Heme Dissociation from Cytochrome b_5. The molar absorptivity of cytochrome b_5 in the Soret region is relatively low compared to that of the His64Tyr/Val68Phe double variant with heme bound, so the dissociation of heme from cytochrome b_5 was more conveniently determined by monitoring the change in absorbance at 600 nm. The rate constants for heme dissociation from various forms of cytochrome b_5 are uniformly greater than those observed for metMb. For example, the rate constant for heme dissociation from lipase-solubilized cytochrome b_5 was ~2-fold greater than that of wild-type horse heart Mb at pH 5 and 6-fold greater at pH 7. Replacement of Ser64 with an alanine residue increases the rate constant of heme dissociation ~10-fold over wild-type lipase-solubilized cytochrome b_5. On the basis of the observations made for the Mb variants, the magnitude of this increase suggests that neither of the hydrogen bonds normally formed by the propionate are formed in the variant. The rate constant for heme dissociation from DME−heme IX-substituted trypsin-solubilized cytochrome b_5 was increased ~6-fold over that of wild-type trypsin-solubilized cytochrome b_5. In this case, the increase in rate constant is intermediate between the increase expected from the elimination of one or two hydrogen bonds, based on the increases observed for Mb.

Thermal Stability. Of the Mb derivatives included in the present study, wild-type Mb exhibited the greatest thermal stability. Upon successive elimination of potential hydrogen bonding interactions with the heme propionates, an incremental decrease in thermal stability was observed (Figure 5). The T_m values observed for bovine cytochrome b_5 also decreased from the values observed for the lipase-solubilized

(66 ± 1 °C) and trypsin-solubilized (63 ± 2 °C) solubilized proteins by slightly more than 10 °C upon replacement of Ser64 with Ala (52 ± 1 °C) or upon esterification of the heme propionate groups in the trypsin-solubilized cytochrome (54 ± 1 °C).

DISCUSSION

The present study has evaluated the influence of hydrogen bonding interactions involving the heme propionate groups and surface amino acid residues on the dynamics of heme−protein interaction for horse heart Mb and cytochrome b_5. Although our work provides detailed insight into the contribution of heme propionates to the mechanism of heme−protein interaction, a comprehensive and detailed structural and mechanistic rationale for the dynamics of heme association, dissociation, and reorientation requires similarly detailed studies of the other stabilizing interactions noted previously. In comparing the results from the two types of experiment used in this work our most general observation is that the kinetics of heme reorientation are affected to a greater extent by the hydrogen bonding interactions of the heme propionate groups than are the kinetics of heme dissociation. For each variant at pH 7.0, the increase in reorientation rate constant that results from elimination of a hydrogen bond is ~5−10-fold greater than the corresponding increase in rate constant for heme dissociation.

We also note that some risk is involved in interpretation of relatively small changes in rate constants as observed in effects of single amino acid substitutions on the kinetics of heme dissociation owing to the possibility that the experiments were fortuitously conducted at or near the isokinetic temperature for this series of proteins. However, further studies concerning correlation of the number of possible heme propionate−protein hydrogen bonds with the activation energies for heme dissociation from these proteins and the energies required to dissociate these heme−apoprotein complexes during electrospray ionization (Hunter et al., 1997) provide additional evidence in support of the interpretation of the results provided in this report. Furthermore, the dependence of the stability of these proteins to thermal denaturation on the number of hydrogen bonds formed by the heme propionates provides additional evidence concerning the legitimacy of this correlation.

As indicated above, our studies complement and extend previous reports concerning the influence of heme substituent groups on the interaction of heme with apo-Mb and apocytochrome b_5 that employed structural analogues of protoheme IX. Through use of modified heme derivatives reconstituted into apo-Mb and apocytochrome b_5, the heme vinyl groups (La Mar et al., 1981, 1984, 1986; Keller et al., 1976), the heme propionate groups (La Mar et al., 1985, 1986, 1989; Hauksson et al., 1990; Santucci et al., 1993), and axial ligands (La Mar et al., 1989; Yee & Peyton, 1991) have been suggested to be of particular importance to the dynamics of heme binding to both apo-Mb and apocytochrome b_5.

The mechanism for the dynamics of heme association with apo-Mb proposed by La Mar and colleagues is depicted in Figure 6 [adapted from La Mar et al. (1984)]. This mechanism is presumably applicable to cytochrome b_5 and many other proteins that possess non-covalently bound heme prosthetic groups. This mechanism consists of two processes. The first process involves the initial binding of heme

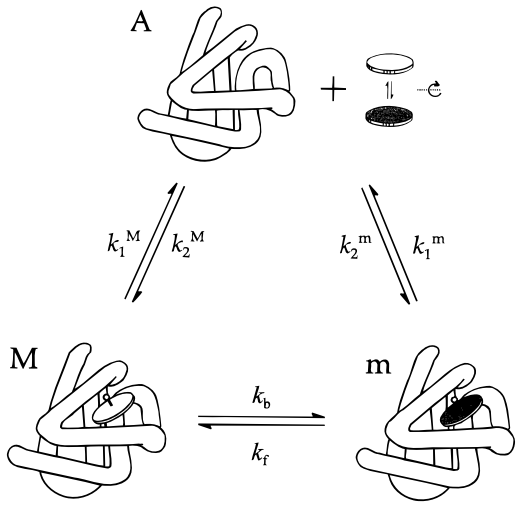

FIGURE 6: Schematic representation of the reaction of apo-Mb with heme. The major and minor orientations are represented by the unshaded and shaded circles, respectively, and differ by 180° rotation about the α/γ-*meso* heme axis.

to the apoprotein in one of two orientations that differ in rotation of the heme about the α/γ-*meso* carbon axis. For both Mb and cytochrome b_5, these two orientational isomers are formed in equal amounts initially, and the relative amounts of these two forms subsequently equilibrate (La Mar et al., 1981; Jue et al., 1983). In the present study, we have investigated the kinetics of heme dissociation from several forms of Mb and cytochrome b_5 after their equilibration as a means of characterizing the first of these two processes, and we have investigated the heme reorientation kinetics of the Mb variants as a means of characterizing the second process. The much greater rate of heme reorientation following reconstitution of apocytochrome b_5 with heme combined with limited quantities of these proteins prevented us from studying this process for the cytochrome.

Kinetics of Heme Reorientation. For all of the Mb variants studied here, the ^{1}H-NMR spectra obtained following addition of cyanide immediately after reconstitution with heme indicate formation of equal amounts of the two heme orientational isomers [i.e., the initial ratio of major (M) to minor (m) orientational forms was ~1:1]. The rate constants for the binding of heme in both orientations, k_2^M and k_2^m, must, therefore, be equal for each of the Mb variants investigated. La Mar and co-workers (1984) also found these rate constants to be independent of the nature of the heme substituents at positions 2 and 4. In addition, the ^{1}H-NMR spectra of the metMbCN derivative for each of the Mb variants at equilibrium showed the equilibrium ratio (K_D) of major to minor orientation to be unchanged from that of wild-type horse and sperm whale Mb (La Mar et al., 1983). As pyrroles III and IV are symmetrical about the α/γ-*meso* axis, the binding of heme in these two orientations does not change the positions of the two heme propionates relative to the surface residues of the protein with which they normally interact. As a result, the initial and equilibrium heme disorder ratios are unchanged.

Nevertheless, substitution of the horse heart Mb residues 45, 92, and 97, all of which form hydrogen bonds with the heme propionates does have a significant effect on the heme reorientation rate. As each hydrogen bond is removed, the heme is able to equilibrate much more rapidly within the

heme binding pocket, and significant increases in the rate constants of heme reorientation (k_f and k_b) are observed. Interaction of the heme propionates with these residues, therefore, appears to be critical in the kinetic "trapping" of the minor orientation.

The contributions of hydrogen bonding by heme propionate groups in the dynamics of heme binding to Mb have been studied previously through the use of synthetic hemes with modified substituent groups. For example, sperm whale Mb reconstituted with hemins lacking the 6- or 7-propionate (6-methyl-6-despropionate hemin and 7-methyl-7-despropionate hemin) exhibited the same heme orientational equilibrium as Mb possessing protoheme IX (Takano, 1977; La Mar et al., 1986, 1989). However, the initial complex formed upon reconstitution with either propionate-deficient heme in excess CN^- resulted in preferential placement of the remaining propionate near Arg45 (La Mar et al., 1989). Similar results were subsequently reported for interaction of the same heme derivatives with horse heart Mb as monitored by circular dichroism spectroscopy (Santucci et al., 1993). These results indicate that removal of individual heme propionate groups does not affect the heme orientational equilibrium but does influence the relative amounts of the two orientational isomers formed immediately following reconstitution of the apoprotein.

Kinetics of Heme Dissociation. The interactions between the propionates and the protein surface residues were also found to have an effect on the rate constant for heme dissociation (k_{-H}) [k_{-H} is dominated by k_1^M (Figure 6) as only 8% of the protein binds heme in the minor orientation at equilibrium]. Inspection of the results in Table 2 reveals that as individual hydrogen bonding interactions involving the heme propionates are eliminated, the resulting increases in k_{-H} are multiplicative (Table 2). Similar increases in heme dissociation rate constant were also observed in related forms of cytochrome b_5 in which the hydrogen bonds involving the propionates were eliminated and suggest that this functional role of heme propionate groups is not restricted to Mb.

For both Mb and cytochrome b_5, the stability of the heme−protein complex is dependent on the characteristics of the histidine residues that coordinate to the heme iron atom. In Fe^{2+}−Mb, the Fe^{2+}−His93 bond is effectively covalent in nature, consistent with slow heme dissociation (Hargrove et al., 1994) and heme reorientation (Jue et al., 1983). In Fe^{3+}−Mb, the proximal histidine protonates as the pH is decreased below pH 5.0, the Fe^{3+}−histidine bond is disrupted, and the rate constant for heme dissociation increases. Cytochrome b_5 exhibits a pH dependence of heme dissociation kinetics that is similar to and has the same origin as that of Mb. Substitution of Ser92 in Mb removes one of the hydrogen bonds to the proximal histidine as well removing the one to the heme propionate group. For this reason, the effect of this substitution on both heme reorientation and dissociation is somewhat greater than might be expected otherwise. The inability to observe electron density for the Asp92 in the electron density map of Ser92Asp Mb crystals (Lloyd et al., 1996) is consistent with mobility of this residue that would prevent or at least compromise the hydrogen bonding interaction of this residue with the proximal histidine residue of Mb. Therefore, the additional destabilization of the heme−protein complex resulting from the Ser92Asp or Ser92Ala substitutions in Mb could be attributed to decreased imidazolate character of the proximal histidine and a weaker iron−histidine bond (Goodin & McRee, 1993) or to increased solvent accessibility of the proximal pocket (Smerdon et al., 1993).

The Mechanism of Heme Reorientation. The manner in which the heme prosthetic group bound to apo-Mb undergoes the rotation required for interconversion of heme orientational isomers is a matter of some speculation. Conversion from the minor (m) form to the major (M) form could occur through one of two limiting mechanisms: an intramolecular rearrangement (m $\rightleftarrows$ M) or an intermolecular pathway (m $\rightleftarrows$ A $\rightleftarrows$ M) (Figure 6). An intramolecular mechanism must involve a considerable degree of unfolding of the protein to allow the large, planar heme group to rotate 180° within the heme binding pocket. An intermolecular mechanism requires complete dissociation of the heme prosthetic group from the heme pocket. One means of evaluating the likelihood of these two mechanisms is through examination of the rate constants for the two opposing mechanisms.

The rate constants for heme dissociation (k_{-H} or k_1^M) from two Mb variants (0.3 h^{-1} for Lys45Glu/His97Leu and 0.12 h^{-1} for Lys45Glu/Ser92Asp) were determined at 25 °C and pH 7.0 for comparison with the reorientation rate constants. These variants were chosen for this purpose because they exhibit relatively large values for k_{-H} and are, therefore, more amenable to accurate kinetic measurements under these solution conditions. Because the heme disorder equilibrium constants ($K_D = k_b/k_f$) exhibited by these variants are the same as that of the wild-type protein and because the heme association rate constants for both orientations are the same for all the variants ($k_2^M = k_2^m$), the ratios of the rate constants for heme dissociation from both orientational isomers must also remain the same. Therefore, the rate constants for heme dissociation from the minor orientation (k_1^m) can be calculated to be 3.6 and 1.4 h^{-1} for Lys45Glu/His97Leu and Lys45Glu/Ser92Asp, respectively. Comparison of these values to those of the forward reorientation rate constants (k_f) displayed in Table 1 reveals that the dissociation of heme from the heme cavity is slightly slower than the forward reorientation rate constant. These rate constants of the variants can be compared to corresponding values reported for wild-type Mb. The value of k_1^M determined for sperm whale Mb (Smith et al., 1982) yields a $k_1^m = 7.8 \times 10^{-3}$ h^{-1}, which is also only slightly lower than the forward reorientation rate constant (1.9×10^{-2} h^{-1}; La Mar et al., 1984). Previous experiments by La Mar et al. (1984) indicate that heme reorientation occurs more rapidly than heme displacement which suggests that heme reorientation does not involve exchange of heme with the bulk solvent and implies that it occurs through an intramolecular mechanism. This observation is apparently in conflict with the observation in the present study of a small increase in the rate constant of heme reorientation, k_f, relative to that for heme dissociation, k_1^m. However, preliminary kinetic simulations show that although k_f is only slightly greater than k_1^m, the intramolecular mechanism is the dominant pathway. In other words, the reaction mechanism for this process is sufficiently complex that simply comparing the magnitudes of the rate constants does not provide an accurate picture of the reorientation process. Therefore, our results are not in disagreement with previous results of La Mar et al. (1984).

While this manuscript was under review, Olson and colleagues reported studies of heme binding to a wide range

of myoglobin variants that complement two aspects of the current study (Hargrove et al., 1996a,b; Hargrove & Olson, 1996). First, the rate constant for association of CO−heme with apo-Mb was found to be relatively insensitive to substitutions in the heme binding pocket. This finding indicates that the equilibrium constant for heme binding is determined primarily by the rate constant for heme dissociation (Hargrove et al., 1996a). Second, Hargrove and Olson (1996) observed that the resistance of sperm whale Mb to denaturation is a function of heme binding affinity. This observation is consistent with the correlation observed here between the number of hydrogen bonding interactions of the heme with the apoprotein and the thermal stability of the variant. Finally, we note that our previous observation that ∼30% of the total activation energy required for heme dissociation from horse heart myoglobin (Hunter et al., 1997) correlates with the conclusion by Hargrove et al. (1996a) that ∼25% of the the affinity of sperm whale apo-Mb for heme results from specific interactions of the protein and the heme (e.g., hydrogen bonding interactions) and that the remaining stabilization is provided by hydrophobic interactions of the apolar heme pocket and the heme (∼50%) and by coordination of the heme iron by the proximal His93 ligand (∼25%).

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