Electrostatic Modulation of Ligand Binding and Electrochemical Properties of Myoglobin: The Role of Charge Compensation

Emma Lloyd,[1a,b] Bertha C. King,[1c] Fred M. Hawkridge,*[,1c] and A. Grant Mauk*[,1a]

Protein Engineering Network of Centres of Excellence and Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada, and Department of Chemistry, Virginia Commonwealth University, Box 2006, Richmond, Virginia 23284-2006

Received August 25, 1997

The effect of electrostatic substitutions in the heme pocket of myoglobin on the ligand binding and electrochemical properties of three variants of horse heart myoglobin (S92D, V67R, and V68H) have been examined. Introduction of a negative charge on the proximal side of the heme (S92D variant) has no effect on the ability of the protein to bind an exogenous cyanide ligand ($K_d = 0.99(4)$ μM compared to the value for the wild-type protein of 2.31(4) μM (pH 7.0, 20.0(1) °C, $\mu = 0.10$ M)). The formal potential of the cyano-met derivative of this variant, $E^{o\prime}$ vs SHE, is decreased by 27 mV relative to the potential of the wild-type protein (−412 and −385 mV, respectively (pH 7.0, 20(1) °C, $\mu = 0.10$ M)). Introduction of a positive charge on the distal side of the heme resulted in K_d values for cyanide binding of 0.110(9) μM for the V67R variant and 140(20) μM for the V68H variant (pH 7.0, 20.0(1) °C, $\mu = 0.10$ M). The formal potentials for the cyano-met derivatives of the V67R and V68H variants were −392 and −257 mV, respectively (pH 7.0, 20(1) °C, $\mu = 0.10$ M). These results are discussed in terms of charge compensation and alterations in electrostatic potential as a consequence of the mutations.

Introduction

Although the roles of a number of critical residues at the active site of myoglobin (Mb[†]) in determining the functional properties of this protein have been evaluated by site-directed mutagenesis,[2] the influence of these residues on the oxidation−reduction properties of the protein have received relatively little attention. Recent demonstration of direct, unmediated electrochemistry of wild-type Mb[3] has for the first time made detailed electrochemical investigations of myoglobin variants feasible.[4,5a,c] In the present work, electrostatic modifications have been introduced close to the heme iron in horse heart Mb (see Figure 1) through replacement of S92 by aspartate (S92D), V67 by arginine (V67R), and V68 by histidine (V68H), and the effects of these alterations on the reduction potential and the affinity of the protein for cyanide have been assessed.

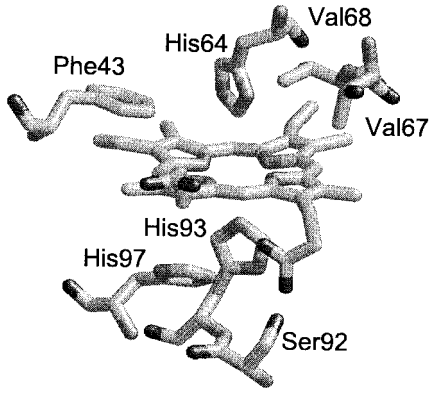

Figure 1. Active site of horse heart myoglobin (coordinates from: Evans, S. V.; Brayer, G. D. *J. Mol. Biol.* **1990**, *213*, 885).

Experimental Section

Mutagenesis and Protein Expression. Expression and purification of the wild-type and variant forms of Mb used in this work have been described elsewhere.[5a−c,6,7] The resulting, purified proteins had absorbance ratios of $A_{408.5}/A_{280} \geq 5.3$ (S92D), $A_{408.5}/A_{280} \geq 5.2$ (V67R) (both pH 6.0, $\mu = 0.10$ M), and $A_{412}/A_{280} \geq 4.8$ (V68H). Cyano-metMb derivatives were obtained by the addition of appropriate amounts of potassium cyanide to met-aquoMb.

Equilibrium Binding Measurements. Buffered solutions of potassium cyanide (sodium phosphate buffer, pH 7.0, $\mu = 0.10$ M) were

[†] Abbreviations: Mb, myoglobin; metMb, oxidized (Fe(III)) met-aquo myoglobin; deoxyMb, reduced (Fe(II)) Mb; cyano-metMb, oxidized cyanide-bound myoglobin.

(1) (a)University of British Columbia. (b) Current address: Department of Chemistry, University of Leicester, University Road, Leicester LE1 7RH, England, U.K. (c) Virginia Commonwealth University.
(2) For a review, see: Springer, B. A.; Sligar, S. G.; Olson, J. S.; Phillips, G. N. *Chem. Rev.* **1994**, *94*, 699.
(3) (a) Ohno, H.; Tsukuda, T. *J. Electroanal. Chem.* **1994**, *367*, 189. (b) Taniguchi, I.; Watanabe, K.; Tominaga, M.; Hawkridge, F. M. *J. Electroanal. Chem.* **1992**, *333*, 331. (c) King, B. C.; Hawkridge, F. M. *J. Electroanal. Chem.* **1987**, *237*, 81. (d) King, B. C.; Hawkridge, F. M. *Talanta* **1989**, *36*, 331. (e) King, B. C.; Hawkridge, F. M.; Hoffman, B. M. *J. Am. Chem. Soc* **1992**, *114*, 10603. (f) Nishiyama, K.; Hawkridge, F. M. *Biochem. Biophys. Res. Commun.* **1994**, *205*, 1724. (g) Rusling, J. F.; Nassar, A.-E. F. *J. Am. Chem. Soc.* **1993**, *115*, 11891. (h) Nassar, A.-E. F.; Bobbitt, J. M.; Stuart, J. D.; Rusling, J. F. *J. Am. Chem. Soc.* **1995**, *117*, 10896. (i) Onuoha, A.; Zu, X.; Rusling, J. E. *J. Am. Chem. Soc*. **1997**, *119*, 3979.
(4) (a) Varadarajan, R.; Zewert, T. E.; Gray, H. B.; Boxer, S. G. *Science* **1989**, *243*, 69. (b) Van Dyke, B. R.; Saltman, P.; Armstrong, F. A. *J. Am. Chem. Soc.* **1996**, *118*, 3490.
(5) (a) Lloyd, E.; Burk, D. L.; Maurus, R.; Ferrer, J. C.; Doran, J.; Carey, P. R.; Brayer, G. D.; Mauk, A. G. *Biochemistry* **1996**, *35*, 11901. (b) Lloyd, E.; Ferrer, J. C.; Maurus, R.; Brayer, G. D.; Mauk, A. G. To be submitted for publication. (c) Lloyd, E.; Hildebrand, D. P. H.; Tu, K. M.; Mauk, A. G. *J. Am. Chem. Soc.* **1995**, *117*, 6434.
(6) Guillemette, J. G.; Matsushima-Hibiya, Y.; Atkinson, T.; Smith, M. *Protein Eng.* **1991**, *4*, 585.
(7) Lloyd, E.; Mauk, A. G. *FEBS Lett.* **1994**, *340*, 281.

S0020-1669(97)01082-3 CCC: $15.00
Published on Web 06/15/1998

prepared daily and stored in well-sealed vessels to avoid loss of HCN. Cyanide solutions were approximately 0.5, 5, 100, and 500 mM in KCN (exact concentrations were determined by weight) and were allowed to equilibrate for approximately 30 min[8] before titration measurements began. All binding experiments were carried out at 20.0(1) °C. Cuvettes were filled with solution and tightly sealed with a stopper to prevent escape of HCN. Protein solutions were prepared in sodium phosphate buffer, pH 7.0, $\mu = 0.10$ M, and were allowed to equilibrate for 30 min after each addition of cyanide before the spectrum was recorded. The volume of cyanide added was small (0.5−2.0 μL) so that sample dilution was minimal. Protein concentrations were determined from molar absorptivities of $\epsilon_{408.5} = 1.54 \times 10^5$ M^{-1} cm^{-1} (S92D), $\epsilon_{408.5} = 1.51 \times 10^5$ M^{-1} cm^{-1} (V67R), and $\epsilon_{412} = 1.34 \times 10^5$ M^{-1} cm^{-1} (V68H).[5a−c] Electronic absorption spectra were recorded with a Cary 219 spectrophotometer interfaced to a microcomputer (OLIS, Bogart, GA) and fitted with a circulating thermostated water bath.

Equilibrium dissociation constants, K_d, for wild-type, S92D, and V67R myoglobins were calculated by fitting the spectrophotometric titration data to eq 1.[9] $\bar{Y}$, the fractional saturation, is calculated from

$$\bar{Y} = [CN^-]_{free}/(K_d + [CN^-]_{free}) \quad (1)$$

the change in absorbance divided by the total change in absorbance (eq 2). A_i and A_f are the initial and final absorbance values, and A_t is

$$\bar{Y} = (A_i - A_t)/(A_i - A_f) = [MbCN]/[Mb]_{tot} \quad (2)$$

the absorbance value at each point in the titration. This analysis assumes that $[CN^-]_{tot} = [CN^-] + [HCN]$.[8−13] The free cyanide concentration, $[CN^-]_{free}$, is defined by eq 3. The V68H variant exhibited

$$[CN^-]_{free} = [CN^-]_{tot} - [MbCN] \quad (3)$$

a significantly reduced affinity for cyanide, so in this case an alternative expression was derived (eq 4). Fitting the titration data to this equation

$$Abs = (K_d A_i + [CN^-]_{tot} A_f)/(K_d + [CN^-]_{tot}) \quad (4)$$

eliminates the need to achieve complete saturation.[9b] An average of two to four measurements of K_d derived from fitting data collected for different protein samples to eq 1 or 4 was used to determine each of the values reported below.

Electrochemistry. Protein samples were prepared for electrochemistry by exchanging into Tris/cacodylate buffer (Sigma), pH 7.0, $\mu =$ 0.10 M. The cacodylic acid was twice recrystallized from 2-propanol. Myoglobin concentrations were determined spectrophotometrically ($\epsilon_{409} = 1.64 \times 10^5$ M^{-1} cm^{-1} at pH 6.2) prior to addition of cyanide. Spectra were collected using a Hewlett-Packard (HP8452A) diode array spectrophotometer interfaced to a microcomputer.[14] Electrochemical experiments were carried out in a Lucite cell that had been modified to accommodate small (<1 mL) sample volumes. The working electrode was a tin-doped indium oxide film deposited on glass (Donnelly Corp.) which was connected to the circuit by means of a brass shim.[15] An Ag/AgCl reference electrode, in 1 M KCl, and a platinum auxiliary electrode completed the cell. The reference electrode was calibrated against saturated quinhydrone[16] using the same buffer employed for the protein measurements. The working electrode was prepared by sonication in detergent, in ethanol, and twice in water.[17] A new working electrode was used for each set of determinations. Data were collected with a computer interfaced to a 12-bit analog-to-digital and digital-to-analog board (Data Translation DT2801A) programmed with software from Data Translation, Marlboro, MA. Electrochemical instrumentation included a potentiostat constructed in-house and a triangle wave generator. All electrochemical data were background-subtracted. Formal potentials, $E^{\circ\prime}$ vs SHE, were averaged values obtained from the two slowest scan rates (20 and 50 mV/s) on the same sample.

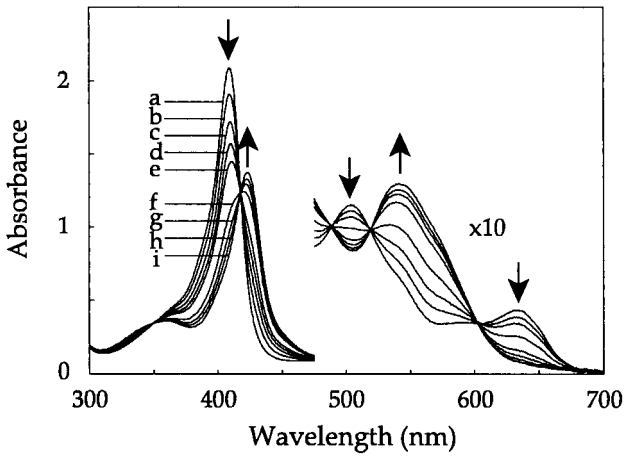

Figure 2. Electronic absorption spectra of S92D metMb in the presence of (a) 0 μM, (b) 2.086 μM, (c) 4.327 μM, (d) 6.306 μM, (e) 8.105 μM, (f) 15.16 μM, (g) 21.56 μM, (h) 40.35 μM, and (i) 505.3 μM cyanide ($[S92D\ Mb]_{initial} = 13.56$ μM, sodium phosphate buffer, pH 7.0, 20.0(1) °C, $\mu = 0.10$ M).

Results

Equilibrium Binding Measurements. The effects of the mutations on the ability of the variant proteins to bind cyanide were assessed by equilibrium binding measurements. A family of spectra (sodium phosphate buffer, pH 7.0, 20.0 °C, $\mu = 0.10$ M) collected during titration of a sample of S92D metMb (13.56 μM) with various concentrations (0−505.3 μM) of cyanide is shown in Figure 2. The spectra are consistent with a 1:1 binding stoichiometry and show well-defined isosbestic points, indicating the presence of only two absorbing species (metMb and cyano-metMb). The corresponding change in absorbance at 408.5 nm is consistent with eq 1 (Figure 3), and the equilibrium dissociation constant, K_d, calculated from a fit of these data to eq 1 was 0.99(4) μM. The corresponding K_d value for the V67R variant was 0.110(9) μM. Titration of wild-type metMb with cyanide yielded a value for K_d of 2.31(4) μM, in close agreement with values reported for sperm whale Mb (4.4 μM[18] and 5 μM[3e,19,20]). The V68H variant exhibited a significantly lowered binding affinity, and an alternative expression for fitting of these data was therefore derived, eq 4. A typical titration curve for this variant is shown in Figure 4, from which a K_d value of 140(20) μM was obtained.

Electrochemistry. Cyclic voltammetry of the cyano-metMb variants was similar to that previously observed for the wild-type protein. A typical series of voltammograms is shown in

(8) Kassner, R. J.; Kykta, J. G.; Cusanovich, M. A. *Biochim. Biophys. Acta* **1974**, *831*, 155.
(9) (a) Ikeda-Saito, M.; Hori, H.; Andersson, L. A.; Prince, R. C.; Pickering, I. J.; George, G. N.; Sanders, C. R.; Lutz, R. S.; McKelvey, E. J.; Mattera, R. *J. Biol. Chem.* **1992**, *267*, 22843. (b) Bogumil, R.; Hunter, C. L.; Maurus, R.; Tang, H.-L.; Lee, H.; Lloyd, E.; Brayer, G. D.; Smith, M.; Mauk, A. G. *Biochemistry* **1994**, *33*, 7600.
(10) Mintorovitch, J.; Satterlee, J. D. *Biochemistry* **1988**, *27*, 8045.
(11) Erman, J. E. *Biochemistry* **1974**, *13*, 39.
(12) Ellis, W. D.; Dunford, H. B. *Biochemistry* **1968**, *7*, 2054.
(13) George, P.; Tsou, C. L. *Biochem. J.* **1952**, *50*, 440.
(14) Hapner, K. D.; Bradshaw, R. A.; Hartzell, C. R.; Gurd, F. R. N. *J. Biol. Chem.* **1968**, *243*, 683.
(15) Armstrong, N. R.; Lin, A. W.; Fujihara, M.; Kuwara, T. *Anal. Chem.* **1976**, *48*, 741.
(16) Hill, R. *Modern Methods of Plant Analysis*; Peach, K., Tracy, M. V., Eds.; Springer-Verlag: New York, 1956; Vol. 1, p 393.
(17) Bowden, E. F.; Hawkridge, F. M.; Blount, H. N. *Bioelectrochem. Bioenerg.* **1980**, *7*, 447.
(18) Keilin, D.; Hartree, E. F. *Biochem. J.* **1968**, *61*, 153.
(19) Blanck, J.; Graf, W.; Scheler, W. *Acta Biol. Med. Ger.* **1961**, *7*, 323.
(20) Ver Ploeg, D. A.; Alberty, R. A. *J. Biol. Chem.* **1968**, *243*, 435.

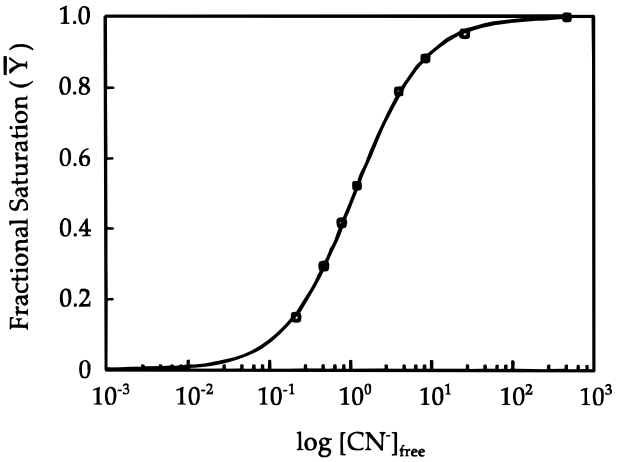

Figure 3. Titration of the S92D metMb variant with cyanide monitored at 408.5 nm and the fit of these data to eq 1. Conditions are the same as those for Figure 2.

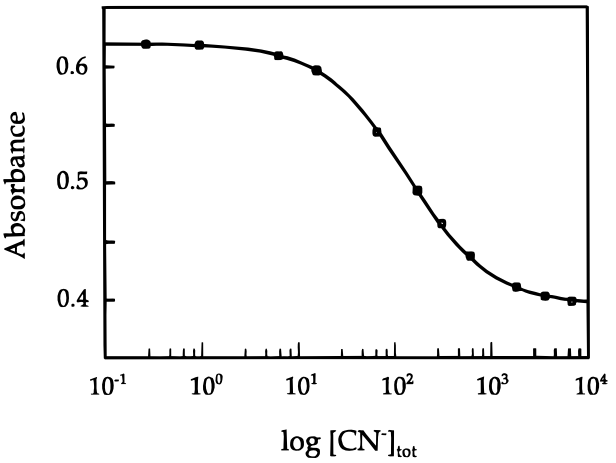

Figure 4. Titration of the V68H metMb variant with cyanide monitored at the Soret absorption maximum and the fit of these data to eq 4 ($[V68H\ Mb]_{initial} = 4.870\ \mu M$, sodium phosphate buffer, pH 7.0, 20.0(1) °C, $\mu = 0.10$ M).

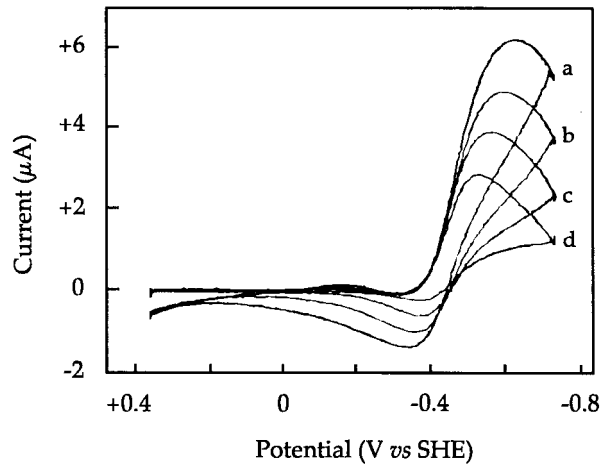

Figure 5. Background-subtracted cyclic voltammograms of the V67R Mb variant in the presence of excess cyanide at (a) 200 mV/s, (b) 100 mV/s, (c) 50 mV/s, and (d) 20 mV/s ([V67R Mb] = 128 μM, [V67R Mb]:[CN^-] = 1:300, Tris/cacodylate buffer, pH 7.0, 20(1) °C, μ = 0.20 M).

Figure 5 for the V67R variant with an excess of cyanide. The formal potential, $E^{\circ\prime}$ (vs SHE), for this variant determined from these data was −392 mV (Table 1). The corresponding values for the S92D and V68H variants were −412 and −257 mV, respectively (Table 1). Knowing the potentials of the wild-type and variant proteins in the presence and absence of cyanide and the dissociation constants for cyanide binding to the oxidized proteins, it is possible to calculate the dissociation constants for cyanide binding to the reduced proteins (Table 1). These values mimic the affinity of cyanide for the oxidized proteins and are not considered further.

Table 1. Formal Potentials, $E^{\circ\prime}$, and Heterogeneous Rate Constants for Electron Transfer, $k^{\circ\prime}$, Obtained from Cyclic Voltammetry Experiments on Wild-Type, V67R, S92D, and V68H Cyano-metMb (Tris/Cacodylate Buffer, pH 7.0, 20(1) °C, $\mu = 0.20$ M)[a]

protein	$E^{\circ\prime}$ vs SHE (mV)	$k^{\circ\prime}$ (10^5 cm/s)	K_d (M)
WT	−385[b]	60[c]	0.63
V67R	−392	33	7.8×10^{-3}
S92D	−412		0.56
V68H	−257	33	2.5×10

[a] The dissociation constants for cyanide binding to the reduced proteins that can be calculated from the formal potential in the presence and absence (from spectroelectrochemistry) of cyanide by spectroelectrochemistry and the dissociation constant for cyanide binding to the oxidized proteins are provided in the column designated K_d. [b] Reference 3e. [c] Reference 5c.

The heterogeneous electron-transfer rate constant, $k^{\circ\prime}$, estimated according to Nicholson[21] for a quasi-reversible system, was calculated for the cyano-met derivatives of the V67R and V68H variants from the peak separations for the lowest two scan rates. For both these cyano-metMb derivatives, no significant effect on the electron-transfer rate constant was observed, and a value of $k^{\circ\prime} = 3.3 \times 10^{-4}$ cm/s was obtained for both variants. This value is close to that reported earlier for the wild-type protein ($k^{\circ\prime} = 5.4 \times 10^{-4}$ cm/s, Table 1[3e]). In the case of the S92D variant, the peak separations for the cyclic voltammograms were too large for this method to be applicable. However, calculation of $k^{\circ\prime}$ using the method for a totally irreversible system shows that the rate constant for this variant falls in the quasi-reversible range but is too low to be determined by the Nicholson method. Single potential step chronoabsorptometry could have been used to determine the relevant rate constant for this variant but was not attempted.

Discussion

Effect of Charged Amino Acid Residues on Cyanide Binding Affinity. The dissociation constant determined for cyanide binding to the wild-type protein ($K_d = 2.31(4)\ \mu$M) corresponds well with those previously reported for the sperm whale protein ($K_d = 4.4\ \mu$M and $K_d = 5\ \mu$M).[3e,18−20] The S92D variant behaved in a similar manner ($K_d = 0.99(4)\ \mu$M, Figure 2). Introduction of a negatively charged group close to the proximal histidine ligand evidently has very little effect on the binding of exogenous anions on the distal side. This observation is consistent with the finding that the water molecule coordinated to the iron in the metMb derivative of this variant exhibits a pK_a identical to that of the wild-type protein.[5a] The existence of two equal and opposite effects could explain this behavior. On one hand, cyanide binding is favored relative to hydroxide formation by the decreased imidazolate character of the proximal histidyl residue that results from elimination of the hydrogen bond between residue 92 and the proximal ligand in the S92D variant.[5a] On the other hand, cyanide binding is disfavored relative to hydroxide formation by the introduction of a potentially charged group in the proximal side of the heme binding pocket.

(21) Nicholson, R. S. *Anal. Chem.* **1965**, *37*, 1351.

Introduction of a positive charge on the distal side of the heme in the V67R variant increased the affinity for cyanide 20-fold ($K_d = 0.11(1)$ μM). This increase represents stabilization of cyanide ligation to the variant of 1.8 kcal/mol. Somewhat smaller stabilizing effects have been reported for binding of azide to the V67R variant.[9b] As shown above for the S92D variant, this increase in binding affinity correlates well with the observed pH-dependent titration behavior of the V67R protein.[5b] In this case, the V67R variant shows a lowered pK_a for titration of the distal water molecule that corresponds to a 1.1 kcal/mol stabilization of the hydroxide-bound form.[5b] This increase in stability for anion-bound derivatives can be interpreted in terms of electrostatic compensation of the bound anion by a positively charged group on the distal side. Indeed, it has been shown for *Aplysia* Mb[22] and a double variant of sperm whale Mb (H64V/T67R)[23] that the distal arginine swings into the pocket on binding of the anion to hydrogen-bond to the bound ligand. Although a similar mechanism is implicated in the V67R variant in the present study, we are currently unable to define the orientation of this arginine group.[5b]

Formation of a hexacoordinate heme iron coordination environment in both the oxidized and reduced states of V68H Mb[5c] made interpretation of data for this variant more complex and partially precludes independent assessment of the effect of charge modification on the ligand-binding properties. The V68H variant behaved much differently from the other variants in that the binding of cyanide is disfavored by a factor of 100 ($K_d = 140(20)$ μM), as expected for a variant in which the distal ligand is provided by a protein residue.

Effect of Charged Amino Acid Residues on Electrochemical Properties. Replacement of S92 with a negative aspartate residue has a slight effect on the formal potential of the cyano-met derivative (Table 1). This 27 mV decrease in the potential is interesting when compared with the reduction potential determined in the absence of cyanide by spectroelectrochemistry,[5a] which shows an 8 mV increase relative to the potential of the wild-type protein (72(2) mV for S92D compared to 64.0(1) mV for wild-type, both vs SHE at 25.0 °C, $\mu = 0.10$ M, pH 6.0) and the absence of any effect on cyanide-binding affinity. Introduction of a negatively charged group on the proximal side of the heme might be expected to alter the electrostatic potential and, hence, the reduction potential of the iron. In this context, it has been postulated that solvent accessibility to the heme, the pK_a of H97, the orientation of the axial ligands and the heme, and the hydrogen-bonding character of the proximal ligand may all influence the potential of Mb.[5a] The close proximity of H97 to the heme has led to the suggestion that titration of this residue affects the electronic properties of the heme as detected by ^{1}H NMR spectroscopy,[24] so the protonation state of this residue may influence the oxidation−reduction equilibrium of the heme iron. In the present work, the pK_a of H97 is unaffected by cyanide binding to the heme iron, so any pH linkage of this residue to the reduction potential is the same for both forms of the protein. For the S92D cyano-metMb derivative, the heme orientation equilibrium and the pK_a of H97 are the same as those for the wild-type protein.[5a,25] A slight reorientation of the proximal ligand has been detected in the S92D variant,[5a] although it is difficult to assess the possible effect of this change on the reduction potential. Significantly, stabilization of a neutral heme core appears to be favored in both cases. Hence, in the absence of cyanide, the reduced form (with a neutral heme core: iron, +2; porphyrin, −2) is stabilized relative to wild-type Mb. In the presence of cyanide, a neutral heme core is also stabilized, but this time as the oxidized derivative (counting iron +3, porphyrin −2, and cyanide −1). Perhaps the introduction of the negatively charged Asp group therefore affects the charge distribution around the heme, and a thermodynamic driving force for maintenance of electroneutrality exists. Absence of any noticeable effect on the cyanide-binding capability of this variant can, therefore, be explained by the distance of the Asp group from the distal ligand-binding site (compared to the distance from the heme itself) and the unaltered proximal ligand bond strength.[5a]

Introduction of a positively charged group on the distal side has a small effect on the formal potential of the cyano-met derivative of the V67R variant and a substantial effect on the affinity of the protein for cyanide. The reduction potential for the V67R metMb, determined by spectroelectrochemistry[5b] is 106.0(9) mV vs SHE (25.0 °C, $\mu = 0.10$ M, pH 6.0), an increase of 42 mV over the potential of the wild-type protein. The principle of charge compensation is again apparent: neutralization of the positive charge in the cyano-met derivative gives a potential which is closer to the wild-type value ($\Delta E^{\circ\prime} = -7$ mV, Table 1) than in the absence of cyanide ($\Delta E^{\circ\prime} = 42$ mV), where the influence of the positive charge is clearly more substantial. The importance of charge neutrality at the active site was also recently emphasized for the binuclear iron-storage protein bacterioferritin.[26] In the present case, this interpretation implies an intimate electrostatic interaction between the heme iron and the R67 group. The exact position of R67 is under investigation and will be reported separately.[5b]

The formal potential of the V68H cyano-met derivative is −257 mV vs SHE. The potential observed in the absence of cyanide by spectroelectrochemical analysis was −110.0(1) mV (25.0 °C, $\mu = 0.10$ M, pH 7.0).[5c] Comparisons of the two electrochemical measurements of this variant are complicated by the more substantial changes in coordination environment. However, displacement of the distal histidine ligand followed by replacement with cyanide increases ($\Delta E^{\circ\prime} = 128$ mV, Table 1) the formal potential. The presence of a partially charged histidine residue close to the heme in the distal heme pocket would account satisfactorily for the stabilization of the reduced form of this cyano-met derivative (assuming a pK_a of 6.0 for this histidine[27]). Unfortunately, analysis of the influence of this unbound histidine, through examination of the pH dependence of the reduction potential, was not possible because of low expression yields and an increased susceptibility of the protein to denaturation at acidic pH.

From the results presented here, it is apparent that the changes in ligand-binding affinity produced by electrostatic modification of the active site of myoglobin can on the whole be explained on electrostatic grounds. The related effects of such substitutions on the electrochemical properties of the protein are more difficult to explain with simple electrostatic arguments, par-

(22) (a) Bolognesi, M.; Coda, A.; Frigerio, F.; Gatti, G.; Ascenzi, P.; Brunori, M. *J. Mol. Biol.* **1990**, *213*, 621. (b) Mattevi, A.; Gatti, G.; Coda, A.; Rizzi, M.; Ascenzi, P.; Brunori, M.; Bolognesi, M. *J. Mol. Recognit.* **1991**, *4*, 1. (c) Conti, E.; Moser, C.; Rizzi, M.; Mattevi, A.; Lionetti, C.; Coda, A.; Ascenzi, P.; Brunori, M.; Bolognesi, M. *J. Mol. Biol.* **1991**, *233*, 498.

(23) Tavaglini Allocatelli, C.; Cutruzzolá, F.; Brancaccio, A.; Brunori, M.; Qin, J.; La Mar, G. N. *Biochemistry* **1993**, *32*, 6041.

(24) La Mar, G. N.; Budd, D. L.; Sick, H.; Gersonde, K. *Biochim. Biophys. Acta* **1978**, *537*, 270−283. (b) Krishnamoorthi, R.; La Mar, G. N. *Eur. J. Biochem.* **1984**, *138*, 135−140.

(25) Hunter, C. L.; Lloyd, E.; Eltis, L.; Rafferty, S.; Lee, H.; Smith, M.; Mauk, A. G. *Biochemistry* **1997**, *36*, 1010.

(26) Le Brun, N. E.; Keech, A. M.; Mauk, M. R.; Mauk, A. G.; Andrews, S. C.; Thomson, A. J.; Moore, G. R. *FEBS Lett.* **1996**, *397*, 159.

(27) Fersht, A. *Enzyme Structure and Mechanism 2nd ed.*; W. H. Freeman; 1985; p 3.

ticularly when various ligand-bound derivatives of myoglobin are considered as in the present study. In this case, the reduction potential can, however, be understood as a consequence of the driving force for maintenance of electroneutrality of the heme core.

Acknowledgment. This work was supported by funds from the Protein Engineering Network of Centres of Excellence (to A.G.M.) and by NSF Grant CHE-9508640 (to F.M.H.).

IC9710824