Electrostatic Control of Electron Transfer between Myoglobin and Cytochrome b_5: Effect of Methylating the Heme Propionates of Zn-Myoglobin

Zhao-Xun Liang,[†] Judith M. Nocek,[†] Igor V. Kurnikov,[‡] David N. Beratan,[‡] and Brian M. Hoffman*,[†]

Department of Chemistry
Northwestern University, 2145 Sheridan Road
Evanston, Illinois 60208
Department of Chemistry, University of Pittsburgh
Pittsburgh, Pennsylvania 15260

Received November 9, 1999

Electron transfer (ET) between metmyoglobin (metMb)/methemoglobin (metHb) and cytochrome b_5 (cyt b_5) is critical for maintaining the biological functions of Mb/Hb in living systems.[1−3] Detailed knowledge of where and how cyt b_5 binds to Mb and Hb is still lacking,[4,5] although Brownian dynamics calculations have suggested a broad, positively charged docking patch on Mb which encompasses almost the entire hemisphere surrounding the exposed heme edge.[6] Within this region, the two heme propionates of Mb present a pair of negative charges that would tend to repel cyt b_5. Hence, simple electrostatics considerations suggest that neutralizing the two propionates by esterification should enhance ET by increasing the overall binding affinity. Herein, we report a dramatic observation of electrostatic control of protein recognition and the discovery that neutralizing the heme propionates of Mb sharply enhances photoinduced ET between zinc-substituted Mb and cyt b_5 *without* altering the net binding constant, and we offer an interpretation of this phenomenon.

ZnDMb (zinc-deuteroporphyrin Mb) and the variant with heme-propionates esterified, ZnD(dme)Mb (zinc deuteroporphyrin dimethylester Mb), were prepared from horse heart Mb.[7] Their incorporated Zn-porphyrins exhibit identical absorption maxima at 414 nm, emission maxima at 586 nm, singlet lifetimes of about 4.2 ns, and intrinsic triplet state decay rate constant of $k_d = 56 \pm 4\ s^{-1}$. Although it has been suggested that the two propionates help to orient the Mb heme in the protein pocket through salt bridges with residues Ser92, His97, and Lys45,[8−10] such results indicate that the heme largely retains its orientation after losing these salt bridges.

Photoinduced ET from Zn-substituted Mb to (Fe^{3+})cyt b_5 was studied by monitoring quenching by cyt b_5 (recombinant[11]) of the photoexcited ^{3*}ZnDMb and ^{3*}ZnD(dme)Mb with transient absorption spectroscopy (450 nm).[5] In both cases the triplet decay remains exponential in the presence of b_5, but the same concentration of cyt b_5 causes far greater quenching of ZnD(dme)Mb than of ZnDMb.[12] For example, at pH 6.0, a 2-fold excess of cyt b_5 gives a quenching rate constant for ZnDMb of $k_q = 4.1 \times 10^2\ s^{-1}$ ($k_q = k_{obs} - k_d$; k_{obs} is the measured decay constant), while that for ZnD(dme)Mb is about 30-fold greater, $k_q = 1.2 \times 10^4\ s^{-1}$; at pH 7.0 the increase is almost 100-fold (Figure 1, left)! In the case of ZnDMb, the quenching was shown to arise primarily from ET by the observation of the charge-transfer intermediate.[13] This intermediate is readily detected because the back-ET is slowed by dissociation of the protein partners, and the intermediate thus builds up over the course of the triplet decay. For ZnD(dme)Mb, the ET intermediate also can be observed, but its signal is small compared with that of ZnDMb. We interpret this as indicating that back-ET now is faster than dissociation, although a contribution to k_q from energy transfer cannot be ruled out.[14]

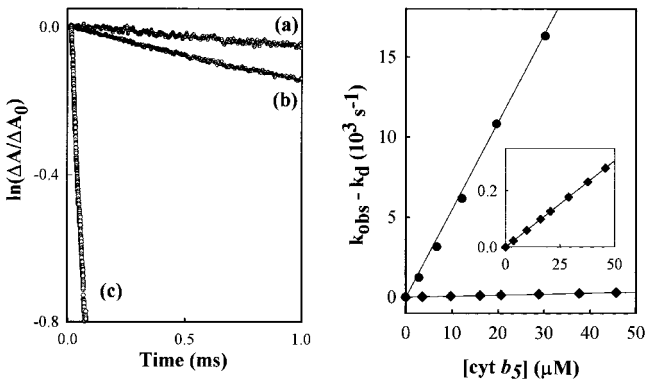

Figure 1. (Left) Triplet decay traces for (a) ZnDMb (4 μM), (b) ZnDMb-(4 μM) + cyt b_5 (8 μM), and (c) ZnD(dme)Mb(4 μM) + cyt b_5 (8 μM). (Right) Quenching titration curves for ZnDMb (●) and ZnD(dme)Mb (◆) with cyt b_5 (Inset: quenching titration curve for ZnDMb). Conditions: 10 mM potassium phosphate buffer at pH 7.0, 20 C.

The quenching rate constant, k_q, both for ZnDMb and ZnD(dme)Mb increases linearly with increasing [cyt b_5]. (Figure 1, right), which means that the slope of the plots of k_q vs [cyt b_5] corresponds to a bimolecular quenching rate constant, k_2. At pH 6.0, k_2(ZnD(dme)Mb) $= 1.2 \times 10^9\ M^{-1}\ s^{-1}$, which is about 30-fold greater than k_2 (ZnDMb) $= 4.4 \times 10^7\ M^{-1}\ s^{-1}$. At higher pH values, the difference between the quenching rate constants is even more striking, as indicated above, with the difference approaching almost 100-fold at pH 7.0: k_2 (ZnD(dme)Mb) $= 5.5 \times 10^8\ M^{-1}\ s^{-1}$; k_2 (ZnDMb) $= 6.1 \times 10^6\ M^{-1}\ s^{-1}$. Surprisingly, however, calculations that model the titration with a simple 1:1 binding isotherm, defined in terms of a 1:1 binding constant, K_a, and intracomplex rate constant, k_c, indicate that the difference cannot be attributed to a change in K_a. This is supported by a steady-state fluorescence, energy-transfer quenching[15] titration, which showed no significant change in second-order quenching constant, and it is confirmed by isothermal titration calorimetry measurements, which show that the binding constant

[†] Northwestern University.
[‡] University of Pittsburgh.

(1) Hultquist, D. E.; Passon, P. G. *Nature New Biol.* **1971**, *229*, 252−254.
(2) Hagler, L.; Coppes, R. I., Jr.; Herman, R. H. *J. Biol. Chem* **1979**, *254*, 6505−6514.
(3) Goto-Tamura, R.; Takesue, Y.; Takesue, S. *Biochim. Biophys. Acta* **1976**, *423*, 293−302.
(4) Qiao, T.; Witkowski, R.; Henderson, R.; McLendon, G. *J. Bio. Inorg. Chem.* **1996**, *1*, 432−438.
(5) Naito, N.; Huang, H.; Sturgess, A. W.; Nocek, J. M.; Hoffman, B. M. *J. Am. Chem. Soc.* **1998**, *120*, 11256−11262.
(6) Nocek, J. M.; Sishta, B. P.; Cameron, J. C.; Mauk, A. G.; Hoffman, B. M. *J. Am. Chem. Soc.* **1997**, *119*, 2146−2155.
(7) The reconstitution procedures for the preparation of ZnDMb and ZnD(dme)Mb are slightly different. ZnDMb was prepared by adding 10% NaOH solution containing ZnD porphyrin into the apoMb solution. ZnD(dme)Mb was prepared by adding DMSO/CH_3OH(v/v 3:1) containing ZnD(dme) porphyrin into the apoMb solution; the final concentration of the organic solvent was kept below 8% to prevent denaturation.
(8) Hunter, C. L.; Lloyd, E.; Eltis, L. D.; Rafferty, S. P.; Lee, H.; Smith, M.; Mauk, A. G. *Biochemistry* **1997**, *36*, 1010−1017.
(9) La Mar, G. N.; Emerson, S. D.; Lecomte, J. T. J.; Pande, U.; Smith, K. M.; Craig, G. W.; Kehres, L. A. *J. Am. Chem. Soc.* **1986**, *108*, 5568−5573.
(10) Neya, S.; Funasaki, N.; Igarashi, N.; Ikezaki, A.; Sato, T.; Imai, K.; Tanaka, N. *Biochemistry* **1998**, *37*, 5487−5493.
(11) Funk, W. D.; Lo, T. P.; Mauk, M. R.; Brayer, G. D.; MacGillivray, R. T. A.; Mauk, A. G. *Biochemistry* **1990**, *29*, 5500−5508.
(12) The decay of ^{3*}ZnD(dme)Mb actually exhibits a major component (95−98%) which responds to the presence of cyt b_5 and a minor component (2−5%) that does not and is hence ignored.
(13) The transient absorption spectrum at times longer than the triplet decay has a feature around 670 nm assigned to the radical cation of ZnDMb/ZnD(dme)Mb, along with one at 562 nm attributed to reduced cyt b_5.
(14) ^{3}Zn-porphyrin → Fe^{3+}P Forster energy transfer depends on the emission spectrum of the energy donor, which is the same for ZnDMb and ZnD(dme)Mb (Turro, N. J. *Modern Molecular Photochemistry*; Benjamin/Cummings Publishing Co.: Menlo Park, CA, 1978). Consequently any increase in energy transfer with esterification must come from a change in the distribution of binding geometries, as discussed below.
(15) McLendon, G.; Zhang, Q.; Wallin, S. A.; Miller, R. M.; Billstone, V.; Spears, K. G.; Hoffman, B. M. *J. Am. Chem. Soc.* **1993**, *115*, 3665−3669.

10.1021/ja993951p CCC: $19.00
Published on Web 03/24/2000

changes negligibly: ZnDMb and ZnD(dme)Mb bind cyt b_5 at pH 6.0 with $K_a = 3.5(\pm 0.7) \times 10^2\ M^{-1}$ and $5.4(\pm 0.7) \times 10^2\ M^{-1}$ respectively.[16]

To interpret this result microscopically, we recall that in the rapid-exchange limit evidenced here by the observation of exponential triplet decay traces, the binding constant, K_a, and the second-order and intracomplex quenching constants, k_2 and k_c respectively, can be written as averages over the accessible encounter complex configurations (eq 1)[17]

$$K_a = \sum_i K_a^i \qquad k_2 = \sum_i K_a^i k_{et}^i \qquad k_c = \sum_i \frac{K_a^i}{K_a} k_{et}^i = \frac{k_2}{K_a} \quad (1)$$

where K_a^i is the binding constant of a specific encounter configuration, determined by the binding (free) energy of the configuration through the Boltzmann factor, $\exp[-E_i/kT]$, and k_{et}^i is the rate constant for the configuration.

The affinity (K_a) will be dominated by the set of conformations with the most favorable binding energy, whereas the rate constant, k_2, can by dominated by configurations with large ET rates, or those with favorable binding constants, or both. Thus the observed change in k_2 might arise from changes in binding *or* in reactivity. However, it does not appear that the effects of charge neutralization arise from changes in the ET rate constants, k_{et}^i, of the individual conformation. These can be factored into nuclear and electronic coupling terms. The nuclear term depends on the energetics of the ET reaction, and neutralization of the propionates might cause a very small shift in $-\Delta G°$, less than 50 mV.[18] However, the photoinitiated ET reaction between ZnDMb and cyt b_5 is almost barrierless: we estimated $-\Delta G° = \sim 0.8-0.9$ eV,[19] and reorganization energy in the range of $\lambda = 0.8-1.0$ eV.[20] In such a case, even a far larger shift in driving force would yield a negligible change in the nuclear term. Neither should propionate methylation have a substantial influence on the electronic coupling term of a given conformation. Indeed, if we assume that the encounter complex places the two hemes in van der Waals contact, adding a methyl group would actually *decrease* the coupling and ET rate, not increase it as seen.

Charge neutralization of the propionates must therefore be influencing the affinities of individual configurations, the K_a^i values, despite the puzzling experimental result that it does not substantially change the overall thermodynamic binding constant, K_a. To explore the changes in K_a^i for this charge-neutralizing 'mutation', we just performed Poisson−Boltzmann calculations of the Mb surface electrostatic potential: Figure 2 shows the electrostatic contour plots for the native Mb (Figure 2b) and dimethylated Mb (Figure 2c). Neutralization of the propionates creates a region of positive electrostatic potential around the heme edge, which is expected to increase the K_a^i for complex configurations with b_5 bound near the heme edge, and most particularly with the propionate groups in contact. To estimate this effect we computed the difference in the electrostatic stability energy of

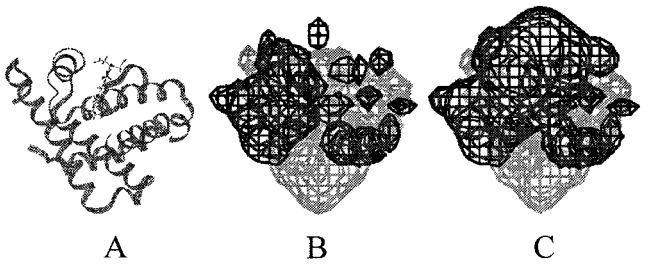

Figure 2. Poisson−Boltzmann electrostatic calculations. (A) Structure of Fe^{2+} Mb to indicate the orientation. (B) Electrostatic contour plot for Fe^{2+}Mb. (C) Electrostatic contour plot for Fe^{2+}(dme)Mb (black = +2 kcal/(mol e); gray = −2 kcal/(mol e)). The results were computed with a protein dielectric constant of 4 and a solution dielectric constant of 80; the solution ionic strength was set to zero.

b_5/Mb complexes caused by neutralizing the Mb propionates, using several conformations found to be favorable in the Brownian dynamics simulation,[6] as well as a Mb/cyt b_5 model complex constructed with the heme edges in close proximity, a geometry chosen to exhibit maximal electronic coupling. Intriguingly, in each case the native complex has an *un*favorable binding energy, while the neutralized complex has a binding energy near zero.

These considerations suggest the following interpretation of the experiments. The repulsive interaction between the negatively charged propionates of b_5 and native Mb inhibits the proteins from forming encounter complexes in precisely those conformations with the largest electronic couplings, in particular conformations having the propionates of the two hemes in contact (i.e., small K_a^i for these geometries). Neutralization of the Mb propionates, which we argue above does not significantly alter the k_{et}^i themselves, does increase the K_a^i for the strongly coupled conformation, but still without making these conformations highly favored; it has a minimal influence on the majority set of *non*reactive conformations, which continue to dominate the overall binding.[21] In short, the complex exhibits a form of gating:[22] the binding constant for the b_5/Mb complex is determined by conformations with very small associated ET constants (small k_{et}^i), while quenching is associated with a small minority of highly reactive conformations (large k_{et}^i), and the dramatic influence of the electrostatic perturbation on the ET process reflects a large *percentage* increase in the occurrence of the reactive conformations without substantial changes in the *net* binding constant. It is intriguing to speculate that some physiological protein−protein electron-transfer processes might be tuned or even switched on/off by reversible protein surface modifications, which are well-known to play an important role in regulating protein−protein recognition events in living systems.[23,24]

Acknowledgment. This work has been supported by the National Institutes of Health (HL62303 [B.M.H.] and GM-48043 [D.N.B.]. We are grateful to Professor M. Wasielewski for use of his ns-laser-flash setup and Mr. R. Hayes for assistance. We acknowledge the use of the calorimeter and fluorescence spectrometer in the Keck Biophysics Facility at Northwestern University. We also thank Dr. Yijeng Liu for her help in cyt b_5 expression.

JA993951P

(16) Binding constants were measured on a MicroCal isothermal titration calorimeter; experimental conditions were the same as in the photolysis experiments except the protein concentration is in the mM range.

(17) Nocek, J. M.; Zhou, J. S.; De Forest, S.; Priyadarshy, S.; Beratan, D. N.; Onuchic, J. N.; Hoffman, B. M. *Chem. Rev.* **1996**, *96*, 2459−2489.

(18) Reid, L. S.; Mauk, M. R.; Mauk, A. G. *J. Am. Chem. Soc.* **1984**, *106*, 2182−2185.

(19) Based on the redox potential and excited-state energy for ZnMb and cyt b_5, $-\Delta G° \sim 0.8-0.9$ eV for the forward ET between ZnDMb and cyt b_5 (Cowan, J. A.; Gray, H. B. *Inorg. Chem.* **1989**, 28, 2074−2078. Reid, L. S.; Mauk, M. R.; Mauk, A. G. *J. Am. Chem. Soc.* **1984**, *106*, 2182−2185).

(20) The reorganization energy, λ, is estimated from the λ obtained from the self-exchange reaction of cyt b_5 and intraprotein ET reaction of ruthenium-modified ZnMb (Winkler, J. R.; Gray, H. B. *Chem. Rev.* **1992**, *92*, 369−379. McLendon, G.; Miller, J. R. *J. Am. Chem. Soc.* **1985**, *107*, 7811−7816. Dixon, D. W.; Hong, X.; Woehler, S. E.; Mauk, A. G.; Sishta, B. P. *J. Am. Chem. Soc.* **1990**, *112*, 1082−1088).

(21) This is consistent with the observation that neutralization has no effect on the steady-state fluorescence, energy-transfer quenching titration, which should be dominated by the majority conformations. Time-resolved fluorescence quenching studies will be performed to give further insight into this issue.

(22) Hoffman, B. M.; Ratner, M. R. *J. Am. Chem. Soc.* **1987**, *109*, 6237−6243.

(23) Pawson, T.; Scott, J. D. *Science* **1997**, *278*, 2075−2080.

(24) Lu, P. J.; Zhou, X. Z.; Shen, M. H.; Lu, K. P. *Science* **1999**, *283*, 1325−1328.