Rate of Intramolecular Reduction of Oxyferryl Iron in Horse Heart Myoglobin

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Like heme peroxidases and other heme enzymes, myoglobin forms oxyferryl (Fe^{IV}=O) on reaction with peroxides.[1] We have recently observed[2] slow intramolecular electron transfer (ET) to the oxyferryl heme of cytochrome *c* peroxidase (CCP) from a_5Ru^{II} (a_5Ru = pentaammineruthenium) bound at His60 and proposed a large reorganizational energy (λ) for oxyferryl heme. An obvious test of this large postulated λ is to directly compare intramolecular ET rates between oxyferryl and a_5Ru centers in myoglobin with the corresponding rates in zinc-substituted sperm whale (SWMb) and recombinant human myoglobins (RHMb).[3,4] Since the oxyferryl heme of horse heart myoglobin (HHMb) is significantly more stable than that of SWMb,[5] the former protein was chosen for this study. A a_5Ru group was attached to the surface His48 of HHMb,[6] and rates of ET over the 12.7-Å distance between the a_5Ru center and the ferric and oxyferryl hemes were measured by pulse radiolysis at Brookhaven National Laboratory.[7]

HHMb (0.5–10 μM) solutions were prepared in N_2O-saturated sodium phosphate buffer at pH 7.0 (40 mM) containing 12 mM HCOONa to generate $CO_2^{\bullet-}$ radicals via reaction with $OH^{\bullet}$. All pulse radiolysis experiments were performed at 25 °C using 2.0- or 6.1-cm path lengths. The dose in each pulse, as calibrated by thiocyanate dosimetry,[8] was chosen to generate sufficient $CO_2^{\bullet-}$ to reduce ≤10% of the protein.

The bimolecular rate constant for the reduction of native HHMb(Fe^{III}–OH_2) by $CO_2^{\bullet-}$ was determined to be 2×10^8 M^{-1} s^{-1} by monitoring the appearance of HHMb(Fe^{II}) at 434 nm.[9] Bimolecular reduction of the a_5Ru^{III}(His48) center in modified HHMb by $CO_2^{\bullet-}$ was found to have a rate constant ~6-fold higher than that for heme reduction;[9] thus, 85% of the reduction occurred at the Ru^{III} center. Since the reduction potentials for the Ru and heme centers are closely matched,[10] the observed rate constant for intramolecular ET from Ru^{II} to the heme follows reversible first-order kinetics:

$$a_5Ru^{III}(His48)—Fe^{III}—OH_2 \xrightarrow[CO_2^{\bullet-}]{fast} a_5Ru^{II}(His48)—Fe^{III}—OH_2 \quad (1)$$

$$a_5Ru^{II}(His48)—Fe^{III}—OH_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} a_5Ru^{III}(His48)—Fe^{II} + H_2O \quad (2)$$

The observed rate constant ($k_{obs} = k_1 + k_{-1}$) is 0.059 ± 0.003 s^{-1}, which is essentially identical to that observed previously for the a_5Ru(His48) derivative of SWMb.[11,12] Also, as with SWMb,[11] addition of CO trapped the Fe^{II} heme and transformed the equilibrium in eq 2 into an irreversible reaction. These results establish that the kinetics and thermodynamics[10] of ET in the a_5Ru(His48) derivatives of SWMb and HHMb are very similar, which is not surprising since the structures of the two proteins are also very similar.[13]

The rate of intramolecular ET to the Fe^{IV}=O heme of HHMb was measured after reaction with excess H_2O_2.[14] Following the 60-ns pulse, rapid reduction of Ru^{III} by $CO_2^{\bullet-}$ occurred, and slow reduction of the oxyferryl to ferric heme was observed at 409 and 421 nm:[9,14]

$$a_5Ru^{II}(His48)—Fe^{IV}=O \xrightarrow{k_{obs}} a_5Ru^{III}(His48)—Fe^{III}—OH_2 \quad (3)$$

The change in heme absorbance at 409 nm and the fit to first-order kinetics are shown in Figure 1A. The dependence of the k_{obs} on the initial concentration of a_5Ru^{III}(His48)—Fe^{IV}=O is shown in Figure 1B. The rates are essentially independent of protein concentration, establishing that bimolecular ET processes are insignificant, and the average value of k_{obs} is 0.19 ± 0.02 s^{-1} for reaction 3. This rate constant is 5–6 orders of magnitude smaller than those measured for Ru/Zn SWMb and RHMb,

$$a_5Ru^{III}(His48)—{}^3ZnP^* \xrightarrow{k_f} a_5Ru^{II}(His48)—ZnP^+ \xrightarrow{k_b} a_5Ru^{III}(His48)—ZnP \quad (4)$$

where k_f and k_b are 7×10^4 and 1×10^5 s^{-1} at $-\Delta G°$ values of 0.82 and 0.96 eV, respectively.[3,4] Thus, at the same driving force (0.96 eV)[15] and over the same pathway, ET to the oxyferryl heme

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(1) (a) George, P.; Irvine, D. H. *Biochem. J.* **1952**, *52*, 511. (b) George, P.; Irvine, D. H. *J. Colloid Sci.* **1956**, *11*, 327. (c) Fox, J. R., Jr.; Nicolas, R. A.; Ackerman, S. A.; Swift, C. E. *Biochemistry* **1974**, *13*, 5178. (d) King, N. K.; Winfield, M. E. *J. Biol. Chem.* **1963**, *238*, 1520. (e) King, N. K.; Looney, F. D.; Winfield, M. E. *Biochim. Biophys. Acta* **1967**, *133*, 65.

(2) Fox, T.; Hazzard, J. T.; Edwards, S. L.; English, A. M.; Poulos, T. L.; Tollin, G. *J. Am. Chem. Soc.* **1990**, *112*, 7426.

(3) Axup, A. W.; Albin, M.; Mayo, S. L.; Crutchley, R. J.; Gray, H. B. *J. Am. Chem. Soc.* **1988**, *110*, 435.

(4) Casimiro, D. R.; Wong, L.-L.; Colón, J. L.; Zewert, T. E.; Richards, J. H.; Chang, I.-J.; Winkler, J. R.; Gray, H. B. *J. Am. Chem. Soc.* **1993**, *115*, 1485.

(5) Uyeda, M.; Peisach, J. *Biochemistry* **1981**, *20*, 2028.

(6) Ruthenated HHMb was prepared as previously described,[3] except that $a_5Ru^{II}(H_2O)$ and Mb(Fe^{III}) were left to react for 1 h rather than 30 min.

(7) Pulse radiolysis was performed using the 60-ns electron pulses from a 2-MeV van de Graaff accelerator. Transient absorption data were obtained using a conventional halogen lamp–filter–sample–monochromator–PMT detection system and fit to first-order kinetics.

(8) Dosimetry was performed by measuring the initial absorbance of the di(thiocyanate) radical anion generated by radiolysis of N_2O-saturated 0.01 M KSCN solution (G = 6.13 radicals/100 eV absorbed, ϵ_{472} = 7950 M^{-1} cm^{-1}).

(9) HHMb(Fe^{III}–OH_2) and HHMb(Fe^{II}) exhibit Soret maxima at 409 (ϵ = 188 mM^{-1} cm^{-1}) and 434 nm (ϵ = 121 mM^{-1} cm^{-1}), respectively. The ratio of $CO_2^{\bullet-}$ reacting with the Ru^{III} and Fe^{III} centers was determined by monitoring at 434 nm the amounts of Mb(Fe^{II}) formed on millisecond and second time scales due to direct reduction by $CO_2^{\bullet-}$ and to intramolecular ET from Ru^{II}, respectively.

(10) HHMb(Fe^{III}–H_2O/Fe^{II}) $E°'$ (pH 6.5) = 57 mV vs NHE (Taniguchi, I.; Watanabe, K.; Tominaga, M. *J. Electroanal. Chem.* **1992**, *333*, 331); $E°'$ for $a_5Ru^{III/II}$(His48)Mb was found to be 76 ± 1 mV in 50 mM sodium phosphate buffer at pH 7.0 by differential pulse voltammetry. Peak potentials were measured at pulse amplitudes of 10–50 mV, and $E°'$ was obtained from the intercept of the potential vs amplitude plot (Bard, A.; Faulkner, L. R. *Electrochemical Methods*; John Wiley & Sons: New York, 1980; p 194). The $Fe^{III/II}$ and $a_5Ru^{III/II}$(His48) potentials for SWMb are 58.8 and 85.5 mV,[11] respectively.

(11) Crutchley, R. J.; Ellis, W. R., Jr.; Gray, H. B. *J. Am. Chem. Soc.* **1985**, *107*, 5002.

(12) Lieber, C. M.; Karas, J. L.; Gray, H. B. *J. Am. Chem. Soc.* **1987**, *109*, 3778.

(13) Evans, S. V.; Brayer, G. D. *J. Mol. Biol.* **1990**, *213*, 885.

(14) Oxyferryl myoglobin was prepared by reacting HHMbFe^{III} with 10-fold excess H_2O_2. When the sample was fully converted to the Fe^{IV}=O state (λ_{max} = 421 nm, ϵ = 111 mM^{-1} cm^{-1}), excess peroxide was removed with a catalytic amount of bovine catalase (Sigma).

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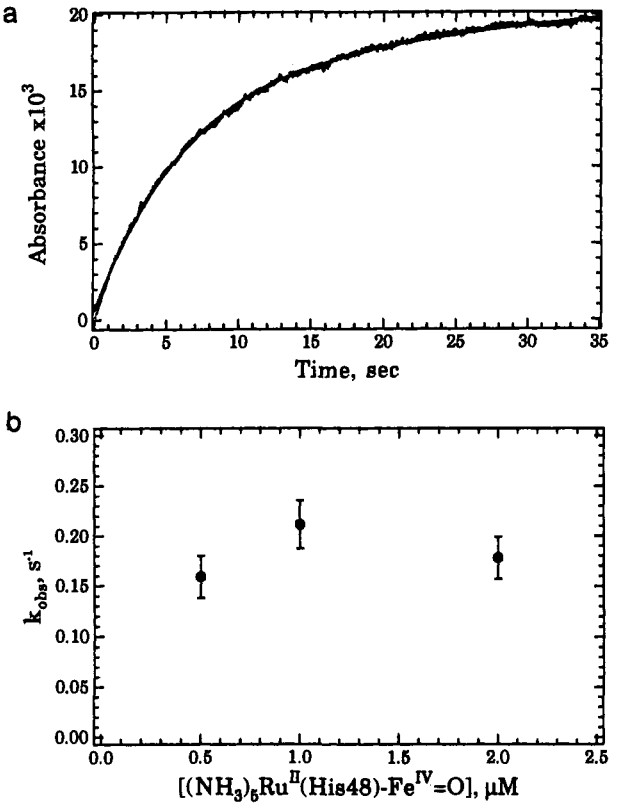

Figure 1. (A) Observed absorbance change at 409 nm vs time following pulse radiolysis of 2 μM a_5Ru^{III}(His48)—Fe^{IV}=O horse heart myoglobin in N_2O-saturated, 40 mM sodium phosphate, 12 mM sodium formate, pH 7.0, I = 0.1 M, 25.2 °C, path length 2.0 cm. The concentration of $CO_2^{\bullet-}$ generated in the pulse was 0.37 μM, and the observed $\Delta\epsilon_{409} \sim 23$ mM^{-1} cm^{-1} is only 25% of that expected for HHMb(Fe^{IV}=O) reduction due to competition from $CO_2^{\bullet-}$ self quenching and scavengers. The solid line shows the fit of the experimental points to first-order kinetics. (B) Dependence of the observed first-order rate constant k_{obs} for intramolecular ET [a_5Ru^{II}(His48) → Fe^{IV}=O] on protein concentration.

of HHMb is ~10^6-fold slower than that to the Zn^+ porphyrin center. To ensure that reaction of HHMb(Fe^{III}) with H_2O_2, which also generates a short-lived, unidentified radical,[16] did not alter the polypeptide between the Ru and heme centers, the ET rate was remeasured following reduction of the Fe^{IV}=O heme.[17] After reduction, k_{obs} (eq 2) was 0.063 ± 0.016 s^{-1}, indicating that radical formation and decay do not retard ET. Consistent with slow intramolecular reduction of the oxyferryl heme, the bimolecular rate constant for the reduction of *unmodified* HHMb(Fe^{IV}=O) by $CO_2^{\bullet-}$ was observed to be $<10^5$ M^{-1} s^{-1}, which is >3 orders of magnitude smaller than that observed for HHMb(Fe^{III}—OH_2) under the same conditions.

Assuming the same electronic coupling terms in a_5Ru(His48)-Mb and the corresponding Zn-substituted Mbs,[18] rate-limiting ET would require a reorganizational energy (λ) of 3.1 eV for reaction 3, compared to $\lambda \sim 1.3$ eV for the Zn-Mbs.[4,19] For a_5Ru^{II}(His)CCP(Fe^{IV}=O), a k_{obs} of 10^6 s^{-1} is predicted for ET over 12.7 Å,[20,21] suggesting a small λ as in the Zn-Mbs.[4] However, the surprisingly slow intra- and bimolecular reduction of HHMb oxyferryl heme suggests that ET may not be rate-limiting in this case. A possible explanation is that protonation of the oxygen atom *precedes* ET to oxyferryl hemes. Thus, a lack of proton donors in the hydrophobic Mb heme pocket, unlike in the CCP pocket, would give rise to rate-limiting protonation in the former and rate-limiting ET in the latter (as in the Zn-Mbs). Experiments (driving force and temperature dependence, H/D isotope effects, etc.) to determine the nature of the rate-limiting step for reaction 3 are in progress.

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(15) HHMb(Fe^{IV}=O/Fe^{III}—OH_2) $E^{\circ\prime}$(pH 6.82) = 1.04 V (George, P.; Irvine, D. H. *Biochemistry* **1954**, *58*, 188).

(16) (a) Davies, M. J. *Biochim. Biophys. Acta* **1991**, *1077*, 86. (b) Newman, E. S. R.; Rice-Evans, C. A.; Davies, M. J. *Biochem. Biophys. Res. Commun.* **1991**, *174*, 3, 1414. (c) Catalano, C. E.; Choe, Y. S.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1989**, *264*, 10534.

(17) Oxyferryl myoglobin was prepared as previously described[14] and then titrated back within 1 h to Mb(Fe^{III}—OH_2) with 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS), and the oxidation products were removed by ultrafiltration.

(18) Assuming that $k_{et} = A \exp[-(\Delta G^\circ + \lambda)^2/4\lambda RT]$ and that A remains constant for reactions 3 and 4, λ is estimated to be 3.1 eV for reaction 3 from the ratios of k_3 (=10^5 s^{-1})[3,4] and k_{obs} (=0.19 s^{-1}).

(19) Winkler, J. R.; Gray, H. B. *Chem. Rev.* **1992**, *92*, 369.

(20) At 21.8 Å, k_{obs} = 3.2 s^{-1} for a_5Ru^{II}(His60)CCP(Fe^{IV}=O);[2] hence, k_{obs} = 10^6 s^{-1} at 12.7 Å assuming exponential ($\exp[-\beta(d-3)]$) decay with distance d and a distance decay factor β of 1.4 $Å^{-1}$ as in Mb.[4,22] The value calculated for k_{obs} depends strongly on the value used for β; for example, if β = 1.0 $Å^{-1}$ in CCP, k_{obs} at 12.7 Å would still be large (~10^4 s^{-1}).

(21) Although ET across 21.8 Å in a_5Ru^{II}(His60)CCP(Fe^{IV}=O) is slower[2] than in Zn-Mb at 19–22 Å,[3] reported k_f values for reaction 4 at these large distances are considered upper limits because of unresolved bimolecular contributions.[4] Hence, our previous comparison[2] of ET rates in a_5Ru^{II}(His60)-CCP(Fe^{IV}=O) with these values may not be meaningful.

(22) Beratan, D. N.; Betts, J. N.; Onuchic, J. N. *Science* **1991**, *252*, 1285.