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ANALYSIS OF THE KINETICS OF ELECTRON TRANSFER REACTIONS OF HEMOGLOBIN AND MYOGLOBIN WITH INORGANIC COMPLEXES

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Summary: The kinetics of methemoglobin reduction by $Fe(EDTA)^{2-}$ have been studied and found to follow a second order rate law with $k = 29.0\ \underline{M}^{-1}\ s^{-1}$ [25°C, $\mu = 0.2\ \underline{M}$, pH 7.0 (phosphate)], $\Delta H^{\ddagger} = 5.5 \pm 0.7$ kcal/mol, and $\Delta S^{\ddagger} = -33 \pm 2$ e.u. The electrostatics-corrected self-exchange rate constant (k_{II}^{corr}) for hemoglobin based on the $Fe(EDTA)^{2-}$ cross-reaction is $2.79 \times 10^{-3}\ \underline{M}^{-1}\ s^{-1}$. This rate constant is compared with others reported for a water-soluble iron porphyrin and calculated from published data for the reactions of myoglobin and hemoglobin with $Fe(EDTA)^{2-}$ and $Fe(CDTA)^{2-/-}$. The k_{II}^{corr} values for these systems range over ten orders of magnitude with heme >> myoglobin > hemoglobin.

Our studies of metalloprotein electron transfer reaction kinetics have shown that outer-sphere probe reagents with hydrophilic surfaces (e.g. $Fe(EDTA)^{2-}$)† are unable to penetrate hydrophobic environments. Consequently, exposed or accessible metal sites react more rapidly with $Fe(EDTA)^{2-}$ than do sites that are buried in hydrophobic protein interiors (1). In principle, hydrophilic inorganic redox agents should be useful as probes of the active-site environment of oxygen carriers such as hemoglobin and myoglobin, as all that is required for the analysis is a protein metal center capable of exchanging an electron with the probe reagent. Our analysis of the kinetics of electron transfer between hydrophilic redox reagents and hemoglobin and myoglobin is reported herein.

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†Abbreviations: EDTA, ethylenediaminetetraacetate; CDTA, trans-1,2-diamino-cyclohexane-N,N,N',N'-tetraacetate.

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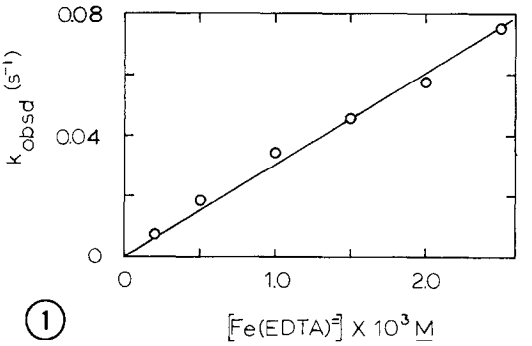

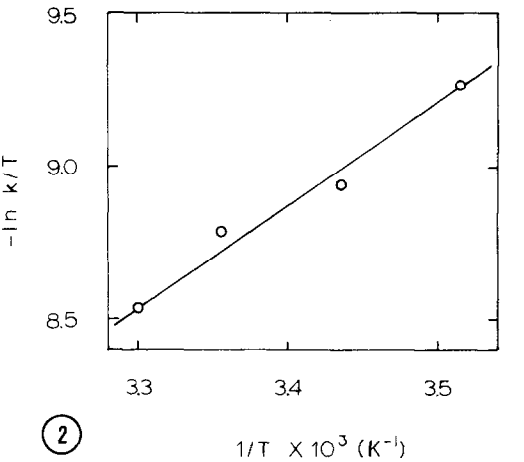

Figure 1. Concentration dependence of k_{obsd} for the reduction of methemoglobin by $Fe(EDTA)^{2-}$ [pH 7.0 (phosphate) μ = 0.2 M, 25° C]. Each point is the mean of at least four measurements.

Figure 2. Eyring plot for the reduction of methemoglobin by $Fe(EDTA)^{2-}$ [pH 7.0 (phosphate) μ = 0.2 M]. [$Fe(EDTA)^{2-}$] = 1.5 x 10^{-3} M. Each point is the mean of at least four measurements.

Methods: Methemoglobin was prepared from fresh hemoglobin by reacting for five minutes with a five-fold molar excess (per heme) of ferricyanide. The solution was then passed over Sephadex G-25 equilibrated with phosphate buffer (pH 7.0, μ = 0.2 M) to remove excess oxidant. Stock $Fe(EDTA)^{2-}$ was prepared in solution under argon as described previously (2) and diluted with degassed buffer using a Hamilton gas tight syringe. Pseudo first order kinetics measurements were made anaerobically on a Durrum model 110 stopped-flow spectrophotometer interfaced to a PDP-10 computer for data acquisition and analysis. Methemoglobin reduction was followed by monitoring the decrease in absorbance at 630 nm. Calculations of electrostatics-corrected rate constants were performed as described previously (3).

Results: First order kinetic plots were linear for at least 90% of the reaction. The concentration dependence of the kinetics of methemoglobin reduction by $Fe(EDTA)^{2-}$ is shown in Figure 1. No evidence of rate saturation is apparent over the concentration range examined. The second order rate constant obtained from a weighted least squares fit to these data is 29.0 M^{-1} s^{-1}. The temperature dependence of this reaction is shown as an Eyring plot in Figure 2. A weighted least squares fit to these data yields $\Delta H^{\ddagger}$ = 5.5 ± 0.7 kcal/mol and $\Delta S^{\ddagger}$ = -33 ± 2 e.u.

Table 1. Kinetic Parameters for Hemoglobin (Hb) and Myoglobin (Mb) Redox Reactions

	Protein reduction				Protein oxidation	
	Hb(III)	Mb(III)	Hb(III)	Mb(III)	Hb(II)	Mb(II)
Parameter	$Fe(EDTA)^{2-}$	$Fe(EDTA)^{2-}$	$Fe(CDTA)^{2-}$	$Fe(CDTA)^{2-}$	$Fe(CDTA)^{-}$	$Fe(CDTA)^{-}$
$E°_{protein}$ (V)[a]	0.150	0.050	0.150	0.050	0.150	0.050
$E°_{reagent}$ (V)[b]	0.120	0.120	0.090	0.090	0.090	0.090
Z_1[c]	9	4.5	9	4.5	5	3.5
Z_1'	5	3.5	5	3.5	9	4.5
Z_2	-2	-2	-2	-2	-1	-1
Z_2'	-1	-1	-1	-1	-2	-2
R_1(Å)[d]	28.2	18.4	28.2	18.4	28.2	18.4
R_2(Å)[e]	4	4	4.6	4.6	4.6	4.6
w_{12}(kcal/mol)	-0.1394	-0.1783	-0.1256	-0.1609	-0.0349	-0.0570
w_{21}(kcal/mol)	-0.0387	-0.0693	-0.0349	-0.0626	-0.1256	-0.1466
w_{11}(kcal/mol)	0.0457	0.0935	0.0457	0.0917	0.0457	0.0917
w_{22}(kcal/mol)	0.4931	0.4931	0.3858	0.3858	0.3858	0.3858
$\Delta E°$ (V)	+0.030	-0.070	+0.060	-0.040	-0.060	+0.040
$k_{12}(M^{-1}s^{-1})$	2.90×10^{1}	3.1×10^{1}[f]	1.25×10^{1}[g]	2.8×10^{1}[f]	2.7[g]	1.48×10^{2}[f]
$k_{22}(M^{-1}s^{-1})$[h]	3.0×10^{4}	3.0×10^{4}	3.0×10^{4}	3.0×10^{4}	3.0×10^{4}	3.0×10^{4}
$\Delta H^{\ddagger}$(kcal/mol)	5.5 ± 0.7	12 ± 1[f]	10.5[g]	13 ± 1[f]	---	---
$\Delta S^{\ddagger}$(e.u.)	-33 ± 2	-13 ± 5[f]	-18.5[g]	-11 ± 5[f]	---	---
$k_{11}^{corr}(M^{-1}s^{-1})$	2.79×10^{-3}	1.26×10^{-1}	1.91×10^{-4}	3.88×10^{-2}	9.52×10^{-4}	4.97×10^{-2}

a. References 6 and 7.

b. References 4 and 8.

c. Protein charges estimated from sequence data as described in reference 3.

d. Protein radii calculated from the relationship $R = 0.717\ M^{1/3}$.

e. $Fe(EDTA)^{2-}$ radius obtained from crystallographic data (9). $Fe(CDTA)^{2-}$ radius estimated from space-filling molecular model.

f. Reference 4.

g. Reference 5.

h. Estimated from the cross-reaction of $Fe(EDTA)^{2-}/Fe(CDTA)^{-}$ (10).

These results and those of Cassatt and co-workers (4,5) have been analyzed according to the Marcus formalism (3) to obtain the electrostatics-corrected self-exchange rate constant, k_{11}^{corr}, for the proteins based on their reactions with $Fe(EDTA)^{2-}$ and $Fe(CDTA)^{2-/-}$. The relevant protein and reagent properties and the work terms calculated for each case are set out in Table 1 with the corresponding kinetic parameters.

Discussion: The strength of the Marcus analysis is demonstrated by comparing the k_{12} and k_{11}^{corr} values for the six reactions in Table 1. Consideration of the unadjusted second order rate constants alone gives the impression that the four reduction reactions are mechanistically similar and that the two oxidation reactions are markedly different. That such a conclusion is unreasonable is apparent from the large range in thermodynamic driving force. By correcting the second order rate constants for this difference and for electrostatic effects, more reasonable reactivity patterns emerge. For example, both $Fe(EDTA)^{2-}$ and $Fe(CDTA)^{2-}$ show greater inherent reactivity with myoblobin than with hemoglobin (i.e., k_{11}^{corr} (Mb) > k_{11}^{corr} (Hb)). Also, the k_{11}^{corr} values based on $Fe(CDTA)^{2-}$ are smaller than those based on $Fe(EDTA)^{2-}$ for both proteins. Although the origin of this latter effect is uncertain, it could arise from steric factors. Importantly, the two k_{11}^{corr} values obtained for reactions with the couple $Fe(CDTA)^{2-/-}$ are virtually the same for a given protein, indicating that confidence may be placed in our method of analyzing electron transfer between charged species.

The k_{11}^{corr} values set out in Table 1 for the proteins are substantially smaller than the value of 1.2×10^6 $\underline{M}^{-1}s^{-1}$ estimated by Pasternack and Spiro for the self-exchange rate of a water-soluble high-spin iron porphyrin complex (11). Thus it is apparent that incorporation of the heme group into the apo-protein profoundly reduces the rate with which it can exchange electrons with external reagents. Stellwagen has recently reported extensive calculations on the heme environment in several heme proteins based on x-ray crystallographic data (12). From these calculations he has concluded that heme protein reduction potentials are inversely related to the exposure of the heme to solvent and unrelated to the hydrophobicity of the heme pocket. As Stellwagen's correlation did not take into account the known effect of heme spin state changes on reduction potential (13), we view it, at best, as incomplete. Furthermore, our attempts to correlate the present kinetic results with any of the values reported by Stellwagen were unsuccessful. For example, the values for % heme exposed in the protein of 18%

(Mb) and 17% (average of 14% Hb_α and 20% Hb_β) or the values for % heme crevice that is apolar of 79% (Mb) and 81% (average of 79% Hb_α and 82% Hb_β) do not reflect the two orders of magnitude difference in redox reactivity found in the present analysis. Thus it appears that our kinetic approach is sensitive to factors not apparent from Stellwagen's analysis of the crystallographic data.

We have shown that a detailed analysis of the reactions of myoglobin and hemoglobin with inorganic outer-sphere electron transfer reagents is useful in probing the relative reactivities of the heme centers in these proteins. Extension of these studies to other redox-active metalloproteins whose function is not electron transfer should provide valuable new information about their active site structures.

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REFERENCES

1. Cummins, D. and Gray, H. B. (1977) J. Am. Chem. Soc., 99, 5158-5167.

2. Wherland, S., Holwerda, R. A., Rosenberg, R. C., and Gray, H. B. (1975) J. Am. Chem. Soc., 97, 5260-5262.

3. Wherland, S. and Gray, H. B. (1977) in Biological Aspects of Inorganic Chemistry (Addison, A. W., Cullen, W. R., Dolphin, D., and James, B. R., eds.) Wiley, New York, p. 289-368.

4. Cassatt, J. C., Marini, C. P., and Bender, J. W. (1975) Biochemistry, 14, 5470-5475.

5. Yamada, T., Marini, C. P., and Cassatt, J. C. (1978) Biochemistry, 17, 231-236.

6. Antonini, E., Wyman, J., Brunori, M., Taylor, J. F., Rossi-Fanelli, A., and Caputo, A. (1964) J. Biol. Chem., 239, 907-912.

7. Brunori, M., Saggese, U., Rotilio, G. C., Antonini, E., and Wyman, J. (1971) Biochemistry, 10, 1604-1609.

8. Schwarzenbach, G. and Heller, J. (1951) Helv. Chim. Acta, 34, 576-691.

9. Lind, D. and Hoard, J. L. (1964) Inorg. Chem., 3, 34-43.

10. Wilkins, R. G. and Yelin, R. E. (1968) Inorg. Chem., 7, 2667-2669.

11. Pasternack, R. F. and Spiro, E. G. (1978) J. Am. Chem. Soc., 100, 968-972.

12. E. Stellwagen (1978) Nature, 275, 73-74.

13. G. R. Moore and R. J. P. Williams (1977) FEBS Lett., 79, 229-232.