Kinetic Studies of the Reduction of Ferricytochrome *c* by $Fe(EDTA)^{2-}$

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Abstract: The reduction of ferricytochrome *c* by $Fe(EDTA)^{2-}$ follows first-order kinetics for both protein and reductant with a rate constant $k_1 = 2.57 \times 10^4\ M^{-1}\ sec^{-1}$ [25.0°, $\mu = 0.1$ (NaCl), pH 7.0 (phosphate)]. Rate saturation is not observed over a wide range of reducing agent concentrations. Values for $\Delta H^{\ddagger}$ (6.0 kcal/mol) and $\Delta S^{\ddagger}$ (−18 cal/(mol deg)) are comparable to those for outer-sphere cytochrome *c* reductions and redox reactions involving simple iron complexes. The rate data at pH 9 (Tris, 25°) are consistent with $Fe(EDTA)^{2-}$ reduction of two slowly interconverting forms of the protein, native ($k_1 = 2.05 \times 10^4\ M^{-1}\ sec^{-1}$, $\mu = 0.1$ (NaCl)] and high pH ($k_2 = 2.67 \times 10^1\ M^{-1}\ sec^{-1}$, $\mu = 0.2$ (NaCl)] isomers. This is the first observation of a direct reduction pathway for the high pH form of the protein. All of the kinetic results are compatible with outer-sphere attack of $Fe(EDTA)^{2-}$ at the exposed heme edge, but the possibility of adjacent attack through the heme pocket is not ruled out.

Cytochrome *c* is a low molecular weight heme protein which undergoes reversible Fe(II)–Fe(III) oxidation state changes in the mitochondrial electron transport chain of aerobic organisms. The X-ray studies of Dickerson and coworkers[1-3] have established that in addition to four porphyrin nitrogens, the protein ligands to iron are His-18 imidazole and Met-80 sulfur. The ligands attached to iron do not vary with the oxidation state of the enzyme, but some changes in the conformation of the polypeptide chain do occur.[2,3]

A preliminary communication[4] has appeared which describes our early work on the chromous reduction of cytochrome *c* and other metalloproteins. Our recent studies of ferricytochrome *c* reactions have employed the reductant $Fe(EDTA)^{2-}$. Unlike $Cr^{2+}(aq)$, this reductant may be conveniently used under physiological conditions of pH and ionic strength and almost certainly favors some type of outer-sphere electron transfer mechanism. Rate parameters for the reduction of both native and a structurally modified high pH form of cytochrome *c* are reported in this paper.

Experimental Section

Reagent grade chemicals were used throughout, and deionized distilled water was used in the preparation of solutions for kinetics measurements. Nitrogen gas was passed through two chromous scrubbing towers to remove oxidizing impurities. Horse heart cytochrome *c* was purchased from Sigma (Type VI) and was not purified further. Ferricytochrome *c* concentrations were determined by measuring the 550-nm absorbance change accompanying full reduction of the protein with dithionite ($\Delta\epsilon_{550} = 18.5 \times 10^3\ M^{-1}\ cm^{-1}$).[5]

Ferrous chloride solutions were prepared by dissolving high purity iron wire (Allied Chemical) in excess HCl under a stream of nitrogen. The resulting solutions were invariably contaminated with small amounts of unidentified insoluble black material, so care was taken not to include any of this in aliquots taken for analysis or preparation of $Fe(EDTA)^{2-}$. The Fe(II) content of ferrous chloride solutions was routinely assayed by injecting aliquots into excess Ce(IV) and back-titrating to a ferroin end point with As(III).

Buffered solutions in the range pH 5–9 were used for $Fe(EDTA)^{2-}$ kinetics measurements, and their total ionic strength was adjusted to the desired value using sodium chloride. Protein and reducing agent solutions were deoxygenated in serum-capped bottles by purging with N_2. Nitrogen was bubbled slowly through cytochrome *c* solutions to avoid protein denaturation due to frothing.

The $Fe(EDTA)^{2-}$ ion is rapidly attacked by oxygen, so stock solutions of this reductant were prepared anaerobically in a two-necked vessel fitted with an inlet at the bottom for continuous N_2 purging, a Metrohm combination pH electrode, and a rubber serum cap. The vessel initially contained buffer, Na_2H_2EDTA (20% excess over the stoichiometric amount), and enough NaOH to neutralize excess HCl as well as hydrogen ions liberated by the reaction of H_2EDTA^{2-} with Fe^{2+}. Ferrous chloride solution was introduced into the vessel using Hamilton gas-tight syringes. Minor adjustments to the desired pH were then made using deoxygenated solutions of HCl and NaOH while monitoring the pH on a Brinkman pH-101 meter.

Kinetics measurements were performed using a modified Durrum Model D-110 stopped-flow spectrophotometer. Anaerobic solution transfer techniques and other methods pertaining to kinetic determinations have already been described.[4] Absorbance-time data were accumulated in the form of oscilloscope or X–Y recorder traces or as digital output from an analog to digital converter. The A/D converter was used in conjunction with a PDP-10 computer program which produced the analytical plots necessary for the evaluation of observed rate constants (k_{obsd}).

Reduction of ferricytochrome *c* was followed at 550 nm and at 695 nm using pseudo-first-order conditions for the metalloprotein. Protein concentrations were typically $2.5–5 \times 10^{-6}$ and $5 \times 10^{-5}\ M$ for measurements at 550 and 695 nm, respectively. Reducing agent concentrations ranged from 100- to 1000-fold excesses in the 550-nm studies, and from ten- to 50-fold excesses in the 695-nm studies. Protein solutions for the kinetic studies at pH 9 were stored for 0.5–3 hr before use, a time well in excess of the 30 sec necessary to achieve equilibrium between the native and alkaline forms of cytochrome *c*.

Results

I. $Fe(EDTA)^{2-}$ Reduction of Native Cytochrome *c*. Fast changes in 550- and 695-nm absorbance were observed when $Fe(EDTA)^{2-}$ and cytochrome *c* were mixed in the stopped-flow apparatus. First-order plots of the 550- and 695-nm data are linear for greater than 95% of the redox reaction. The first-order dependence of observed rate constants on $[Fe(EDTA)^{2-}]$ at pH 7.0 is illustrated in Figure 1 for data measured at 550 nm. Similar results were found at 695 nm. The least-squares

(1) R. E. Dickerson, T. Takano, D. Eisenberg, O. B. Kallai, L. Samson, A. Cooper, and E. Margoliash, *J. Biol. Chem.*, **246**, 1511 (1971).
(2) T. Takano, O. B. Kallai, R. Swanson, and R. E. Dickerson, *J. Biol. Chem.*, **248**, 5234 (1973).
(3) T. Takano, R. Swanson, O. B. Kallai, and R. E. Dickerson, *Cold Spring Harbor Symp. Quant. Biol.*, **36**, 397 (1971).
(4) J. W. Dawson, H. B. Gray, R. A. Holwerda, and E. W. Westhead, *Proc. Nat. Acad. Sci. U. S.*, **69**, 30 (1972).
(5) E. Margoliash and N. Frohwirt, *Biochem. J.*, **71**, 570 (1959).

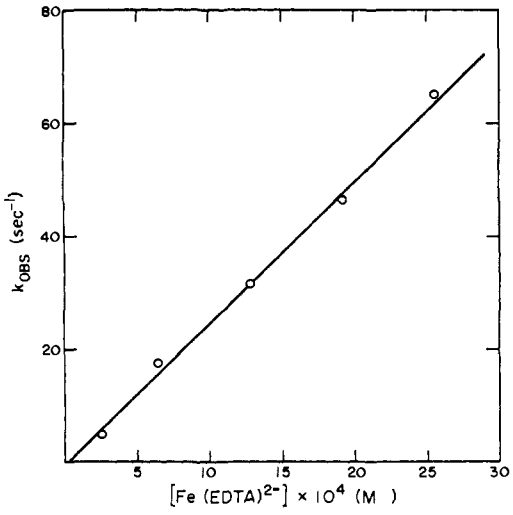

Figure 1. Plot of k_{obsd} *vs.* [Fe(EDTA)$^{2-}$] for the reduction of ferricytochrome *c* (25°, pH 7.0 (phosphate), μ = 0.1 (NaCl)). The reaction was followed at 550 nm. Circles plotted in all figures represent averages of at least four data points.

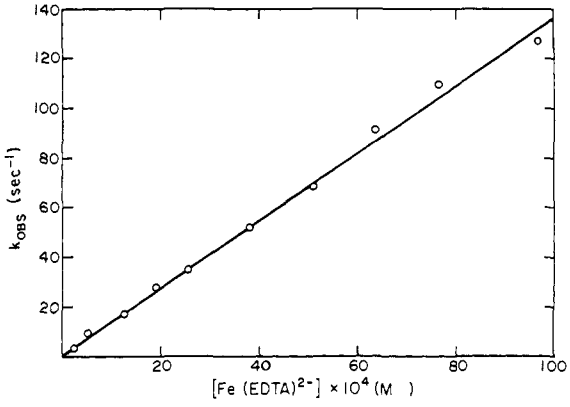

Figure 2. Plot of k_{obsd} *vs.* [Fe(EDTA)$^{2-}$] for the reduction of ferricytochrome *c* (25°, pH 7.0 (phosphate), μ = 0.2 (NaCl)).

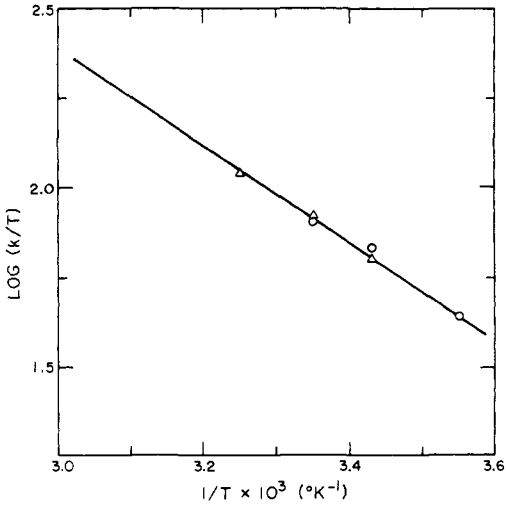

Figure 3. Eyring plot of rate data for the reduction of ferricytochrome *c* by Fe(EDTA)$^{2-}$ (pH 7.0 (phosphate), μ = 0.1 (NaCl)): ○, [Fe(EDTA)$^{2-}$] = 1.92 × 10^{-3} *M*; Δ, [Fe(EDTA)$^{2-}$] = 1.28 × 10^{-3} *M*.

slope of the data in Figure 1 gives the second-order rate constant k_1 for cyt *c* (III) reduction (k_1 = (2.57 ± 0.1) × 10^4 M^{-1} sec^{-1}; 25°, μ = 0.1, pH 7.0 (phosphate)). Values of k_{obsd} are independent of Na_2H_2EDTA concentrations for 20–300% excesses of Na_2H_2EDTA over Fe(II), establishing that the reductant is exclusively the EDTA complex.

The variation of observed rate constants with [Fe(EDTA)$^{2-}$] was also studied over a wider range of reducing agent concentrations (2.5–100 × 10^{-4} *M*) at μ = 0.2, pH 7.0 (Figure 2). Rate saturation has been found in studies of cytochrome *c* reduction by chromous ion[6,7] and dithionite,[8] but no evidence for such an effect with Fe(EDTA)$^{2-}$ was observed. From Figure 2 it is clear that the cytochrome *c* reduction rate is first order with respect to [Fe(EDTA)$^{2-}$] over the entire concentration interval, and values of k_{obsd} much larger than the saturation rate of 60 sec^{-1} (pH 6.1, 25°) for chromous ion[6] were obtained.

Table I gives room temperature second-order rate constants as a function of pH and activation parameters based on measurements at several temperatures. The observed rate constants upon which the values in Table I are based are available in a supplement to this paper.[9] The value of k_1 is essentially independent of pH and the buffer used for 5.0 ≤ pH ≤ 7.8. The rate law for cyt *c* (III) reduction is

$$\frac{-d[\text{cyt } c \text{ (III)}]}{dt} = k_1[\text{Fe(EDTA)}^{2-}][\text{cyt } c \text{ (III)}]$$

within this pH range. The Eyring plot calculated from the 550-nm pH 7.0 (phosphate) results (Figure 3) is typical of those found throughout the 5.0–7.8 pH range.

Table I. Rate Data[a] for the Fe(EDTA)$^{2-}$ Reduction of Ferricytochrome *c*

Abs, nm	k, M^{-1} sec^{-1} (25°)	$\Delta H^{\ddagger}$, kcal/mol	$\Delta S^{\ddagger}$, eu	pH	Buffer
550	2.81 × 10^4	5.5	−20	5	Acetate
550	2.57 × 10^4	6.0	−18	7	Phosphate
	2.72 × 10^4			6	
	2.62 × 10^4			7.8	
550	2.47 × 10^4	5.1	−21	7	Tris
695	2.65 × 10^4	5.7	−19	7	Tris
550	2.05 × 10^4	4.9	−23	9	Tris
	2.67 × 10^1 (μ = 0.2)				
550	1.10 × 10^4	4.8	−24	9	Carbonate
695	2.29 × 10^4	6.3	−18	9	Tris

[a] μ = 0.1 (NaCl), except where noted. Estimated uncertainties are ±0.05 × 10^4 M^{-1} sec^{-1} in k, ±0.3 kcal/mol in $\Delta H^{\ddagger}$, and ±1.0 eu in $\Delta S^{\ddagger}$.

(6) J. K. Yandell, D. P. Fay, and N. Sutin, *J. Amer. Chem. Soc.*, **95**, 1131 (1973).

(7) In view of the comprehensive report of the reaction of Cr^{2+} (aq) with cyt *c* (III) by Yandell, Fay, and Sutin,[6] we have made no attempt to extend the scope of our experiments in that particular system. However, we are taking this opportunity to correct previously published[4] activation parameters for this reaction ($\Delta H^{\ddagger}$ ≈ 17.4 kcal/mol, $\Delta S^{\ddagger}$ = +18 cal/(mol deg), pH 4.1, μ = 0.1 (NaCl)) which are in error. We have repeated our chromous experiments with several different cytochrome *c* preparations, but now find an apparent activation enthalpy of 10.0 ± 0.2 kcal/mol. The new rate constant (k = 1.9 × 10^4 M^{-1} sec^{-1}, pH 4.1, 25°) is not very different from the value of 1.2 × 10^4 M^{-1} sec^{-1} we reported earlier.[4] Considering the complex pH dependence of the rate of the Cr^{2+} (aq) + cyt *c* (III) reaction, it now seems clear that apparent activation parameters derived from the pH 4.1 data may not be easily interpreted.

(8) (a) C. Creutz and N. Sutin, *Proc. Nat. Acad. Sci. U.S.*, 70, 1701 (1973); (b) D. O. Lambeth and G. Palmer, *J. Biol. Chem.*, **248**, 6095 (1973).

(9) See paragraph at end of paper regarding supplementary material.

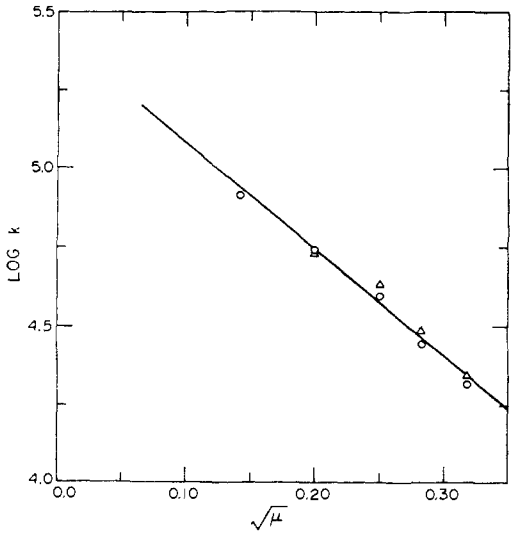

Figure 4. Plot of log k *vs.* $\sqrt{\mu}$ for the reduction of ferricytochrome *c* by Fe(EDTA)$^{2-}$ at pH 7 (phosphate, 25°): ○, [Fe(EDTA)$^{2-}$] = 2.63×10^{-4}; △, [Fe(EDTA)$^{2-}$] = 6.56×10^{-4}.

It is evident that a rate-determining step characterized by $\Delta H^{\pm} \cong 6$ kcal/mol and $\Delta S^{\pm} \cong -20$ cal/(mol deg) governs the absorbance changes at both 550 and 695 nm.

The reduction rate of cyt *c* (III) by Fe(EDTA)$^{2-}$ decreases with increasing ionic strength at pH 7.0. The logarithms of observed rate constants at low ionic strength are expected to decrease linearly with $\sqrt{\mu}$ for a reaction between oppositely charged species,[10] and the slope of the line is theoretically related to the charges of the reactants. This relationship should hold only for ionic strengths smaller than 0.01. Nevertheless, a plot of log k_1 *vs.* $\sqrt{\mu}$ for cytochrome *c* reduction at pH 7 (Figure 4) covering the interval $0.02 \leq \mu \leq 0.1$ is linear. As the charge on the reductant is -2, an apparent cyt *c* (III) "active site charge" of $+1.7$ may be calculated from the observed slope of -3.4. In view of the differences between our conditions and those demanded by the theoretical treatment, the exact value cannot be taken seriously. It seems fair to conclude, however, that for encounters with Fe(EDTA)$^{2-}$ the effective active site charge is far smaller than the overall charge[11] of $+7$–8 for cytochrome *c* in neutral solution. A similar difference between effective and overall charge has been observed for the ionization of metmyoglobin.[12,13]

II. Fe(EDTA)$^{2-}$ Reduction of Structurally Modified Cytochrome *c* at High pH. A biphasic absorbance change occurs at 550 nm when Fe(EDTA)$^{2-}$ reduces cyt *c* (III) at pH 9 in Tris buffer. Although the initial phase (*ca.* 50% of the total 550-nm absorbance change) is 1000 times as fast as the second one, a well-defined base line could not be determined for this component. A first-order rate process was assumed for the fast reaction, and observed rate constants were calculated by the Guggenheim method.[14] These rate parameters are similar to those found for the native protein at pH 7.0 (Table I).

(10) A. A. Frost and R. G. Pearson, "Kinetics and Mechanisms," 2nd ed, Wiley, New York, N. Y., 1961, p 150.
(11) E. Margoliash and A. Schejter, *Advan. Protein Chem.*, 21, 113 (1966).
(12) P. George and G. Hanania, *Biochem. J.*, 52, 517 (1952).
(13) P. George and G. Hanania, *Biochem. J.*, 65, 756 (1957).
(14) Reference 10, p 49.

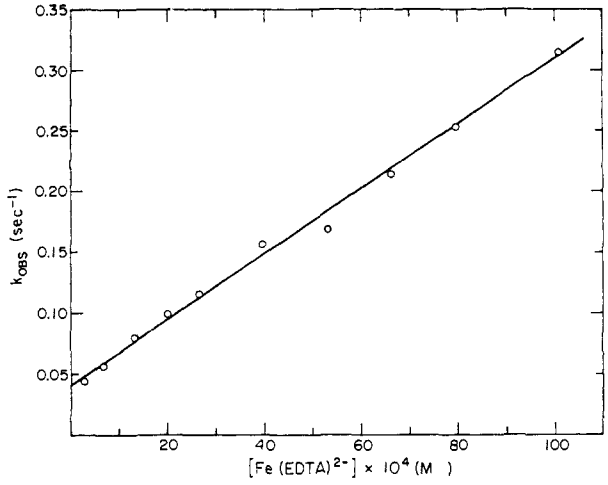

Figure 5. Plot of k_{obsd} *vs.* [Fe(EDTA)$^{2-}$] for the reduction of alkaline ferricytochrome *c* (25°, pH 9.0 (Tris), μ = 0.15 (NaCl)).

The slow phase of the 550-nm absorbance change at pH 9.0 is attributed to the reduction of a high pH form of cyt *c* (III) which interconverts relatively slowly with the native enzyme. Interpretation of the kinetic data is complicated by the fact that slow increases in absorbance continue much longer than expected for a first-order reaction. Observed rate constants were again calculated using the Guggenheim method. Guggenheim plots are linear up to 50–70% of the total slow absorbance change; observed rate constants as a function of [Fe(EDTA)$^{2-}$] are shown in Figure 5. The k_{obsd} values for the slow reaction are consistent with the rate law

$$\frac{-\mathrm{d[cyt\ c\ (III)]}}{\mathrm{d}t} = k_0 + k_1[\mathrm{Fe(EDTA)^{2-}}][\mathrm{cyt\ c\ (III)}]$$

with $k_0 = (4.1 \pm 0.1) \times 10^{-2}$ sec^{-1}, $k_1 = (2.67 \pm 0.06) \times 10^1$ M^{-1} sec^{-1} (25°, μ = 0.2, pH 9 (Tris)).

Additional data were obtained at 695 nm. At this wavelength the native form of the protein exhibits an absorption maximum, whereas the high pH form, originally characterized as type IV ferricytochrome *c* by Theorell and Åkesson, does not.[15] Our 695-nm results confirm that the slowly reacting component at 550 nm corresponds to the high pH isomer. Only a fast reaction is observed at pH 9 and the absorbance change is 50% of that expected if all of the protein present were in the native form. Second-order rate constants obtained from the 695-nm data are in excellent agreement with those for the fast phase at 550 nm (Table I).

To confirm further that the rate of conversion of native cytochrome *c* to the high pH form is slow compared with its rate of reduction by Fe(EDTA)$^{2-}$, a pH jump experiment was performed. Weakly buffered ferricytochrome *c* at pH 7.0 was mixed with strongly buffered Fe(EDTA)$^{2-}$ at pH 9, and the reaction was followed at 550 nm. A single, fast reaction was observed, and the absorbance change associated with it accounted for the entire increment expected for full reduction of the protein. The second-order rate constant of 1.3×10^4 M^{-1} sec^{-1} (μ = 0.15) obtained from this experiment is in reasonable agreement with the

(15) H. Theorell and Å. Åkesson, *J. Amer. Chem. Soc.*, 63, 1812 (1941).

value found earlier for the initial phase of the pH 9 biphasic sequence.

Discussion

Electron transfer to the iron atom of ferricytochrome *c* reasonably could take place either by remote attack, probably at the heme edge, or by adjacent attack at some point in the limited space available[1] in the heme pocket. Sutin and coworkers have suggested that both remote and adjacent attack may occur with the reductant Cr^{2+} (aq).[6] In a study of the chromous reduction of cytochrome *c* in chloride media, the observed rate saturation with increasing [Cr(II)] was attributed to the influence of a crevice-opening preequilibium, and adjacent attack leading to an inner-sphere activated complex of the form Fe(III)–Cl–Cr(II) was proposed. This interpretation is supported by the agreement between the saturation rate derived from chromous ion kinetic results (60 ± 20 sec^{-1}, pH 6.1, 25°) and the first-order rate constant for crevice-opening obtained from anation studies.[16] Azide and thiocyanate catalyze the Cr(II) reduction, possibly indicating a pathway involving anion bridging at the remote attack site.

Crevice opening apparently is not a requirement for the $Fe(EDTA)^{2-}$ reduction of cytochrome *c*. Pseudo-first-order rate constants greater than 60 sec^{-1} were observed, and no evidence was found for the reducing agent saturation predicted in the rate laws for adjacent inner-sphere attack such as proposed[6,8a] for chromous and dithionite ions. The kinetic results for the $Fe(EDTA)^{2-}$ reduction of cytochrome *c* strongly suggest that an outer-sphere electron transfer mechanism of some type is involved. The rate constant, for example, is identical with the value of 2.6×10^4 M^{-1} sec^{-1} reported for ferrocyanide as reductant ($7.0 \leq$ pH ≤ 9.4 at 22°, $\mu = 0.2$).[17] In addition, the $Fe(EDTA)^{2-}$ rate parameters are very similar to those characterizing the acid-independent pathway for the reaction of the outer-sphere reductant $Ru(NH_3)_6^{2+}$ with cyt *c* (III) ($k = 3.78 \times 10^4$ M^{-1} sec^{-1}, 25°, pH 7.0, $\mu = 0.1$ (Tris); $\Delta H^{\pm} = 2.86$ kcal/mol, $\Delta S^{\pm} = -28$ cal/(mol deg)).[18] It is also important to note that k_{obsd} values as high as 293 sec^{-1} have been observed[18] for the $Ru(NH_3)_6^{2+}$ reaction.

Rate constants and activation parameters for the $Fe(EDTA)^{2-}$ reduction of cytochrome *c* are also comparable to those for outer-sphere reactions between simple inorganic iron complexes. For example, electron transfer between $Fe(EDTA)^{2-}$ and $Fe(CyDTA)^{-}$ takes place with $k(25°) = 3.0 \times 10^4$ M^{-1} sec^{-1}, $\Delta H^{\pm} = 4.0$ kcal/mol, and $\Delta S^{\pm} = -25$ cal/(mol deg).[19] The activation parameters for the ferro–ferricyanide exchange reaction, after correction to zero cation concentration, are $\Delta H^{\pm} = 8.4$ kcal/mol, $\Delta S^{\pm} = -24$ cal/(mol deg).[20] From these comparisons it is apparent that no special "protein effect" such as a conformational change need be invoked to explain the activation parameters for the $Fe(EDTA)^{2-}$ + cyt *c* (III) reaction. The observed $\Delta H^{\pm}$ value is consistent with an activation process requiring only minor nuclear positional rearrangements in the coordination environments of iron atoms in the oxidant and reductant molecules. Our $\Delta S^{\pm}$ value is not much different from that expected (*ca.* −13 cal/(mol deg))[21] considering only losses of translational and rotational entropy accompanying formation of the collision complex from the reactants.

It must be emphasized that crevice opening need not necessarily be a requirement for adjacent attack by outer-sphere reductants. Considering the steric limitations for adjacent attack, however, we favor the exposed heme edge as the most reasonable site for $Fe(EDTA)^{2-}$ attack on cytochrome *c*. Some support for our proposal of edge attack may be drawn from the similarity between the $Fe(EDTA)^{2-}$ rate parameters and results for electron transfer reactions between oxidized and reduced cytochrome *c* molecules. Kowalsky[22] has reported an electron exchange rate of $5 \pm 3 \times 10^4$ M^{-1} sec^{-1} (pD 7.0, 0.1 *M* KCl, 0.1 *M* potassium phosphate) for horse cytochrome *c* based on measurements of the Met 80 methyl proton relaxation time. Using pulsed nmr techniques, Gupta, *et al.*,[23-25] have determined a significantly smaller value (*ca.* 1×10^3 M^{-1} sec^{-1}) at pH 7.0, $\mu = 0.1$. The activation energy varies significantly with ionic strength[25] ($E_a = 13 \pm 1$ kcal/mol at $\mu = 0.1$, $E_a = 7 \pm 1$ kcal/mol at $\mu = 1.0$, pH 7.0), and it has been suggested[25] that electrostatic repulsion between charged groups on ferro- and ferricytochrome *c* limits the distance of closest approach between the two molecules. Near the isoelectric point of cytochrome *c* (pH 10) where electrostatic factors are of lesser importance, the second-order rate constant for cyt *c* (II, III) self-exchange (2×10^4 M^{-1} sec^{-1})[24] is nearly identical with our value for $Fe(EDTA)^{2-}$ reduction of the protein. It is probable, therefore, that the point of outer-sphere attack on cyt *c* (III) is the same for both reactions. As steric constraints on reductant penetration into the heme pocket are expected to be even more prohibitive in the self-exchange reaction than for $Fe(EDTA)^{2-}$ reduction, an edge attack site is indicated. An edge-to-edge mechanism also is likely for the reaction between horse cyt *c* (III) and cyt c_{551} (II) from *Pseudomonas*, as the rate ($k = 4.9 \times 10^4$ M^{-1} sec^{-1}, 20°, pH 7, $\mu = 0.2$) for these two oppositely charged cytochromes does not depend significantly on the ionic strength of the medium.[26] In this case the results imply minimal interaction of the charged regions of the two proteins in the transition state.[21]

A number of workers have found kinetic evidence for the presence of two functionally distinct isomers of cytochrome *c* at high pH. Biphasic reduction curves have been reported in studies of the reaction of ascorbate[27,28] and ferrocyanide[17] with cytochrome *c* near pH 9. It was noted in these studies that the high pH form of cytochrome *c* was not reduced directly but was

(16) N. Sutin and J. K. Yandell, *J. Biol. Chem.*, **247**, 6932 (1972).
(17) K. G. Brandt, P. C. Parks, G. H. Czerlinski, and G. P. Hess, *J. Biol. Chem.*, **241**, 4180 (1966).
(18) R. X. Ewall and L. E. Bennett, *J. Amer. Chem. Soc.*, **96**, 940 (1974).
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(20) R. J. Campion, C. F. Deck, P. King, Jr., and A. C. Wahl, *Inorg. Chem.*, **6**, 672 (1967).
(21) L. E. Bennett, *Progr. Inorg. Chem.*, **18**, 1 (1973).
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(24) R. K. Gupta, S. H. Koenig, and A. G. Redfield, *J. Magn. Resonance*, **7**, 66 (1972).
(25) R. K. Gupta, *Biochim. Biophys. Acta*, **292**, 291 (1973).
(26) R. A. Morton, J. Overnell, and H. A. Harbury, *J. Biol. Chem.*, **245**, 4653 (1970).
(27) C. Greenwood and G. Palmer, *J. Biol. Chem.*, **240**, 3660 (1965).
(28) M. T. Wilson and C. Greenwood, *Eur. J. Biochem.*, **22**, 11 (1971).

attacked only after slowly reverting to the native enzyme. An nmr study based on observations of the Met-80 methyl resonance has indicated that the high pH form of the protein is differently ligated, possibly having Lys-79 N in place of Met-80 S.[29] This conclusion is consistent with the assignment of the 695-nm absorption band in native cytochrome *c* to a transition associated with the Fe(III)–S unit.[30-34] A quantitative correlation between the extent of the slow phase of the ascorbate reaction and loss of protein 695-nm absorbance (relative to pH 7) has been made,[28] and nmr[29] and pH[35] titration data have indicated that loss of the Met-80 ligand is linked to a protein ionization with $pK \sim 9$. Only a limited conformational change is thought to accompany formation of the alkaline cytochrome *c* derivative.[28]

From Greenwood and Wilson's tabulation[28] it is estimated that approximately 45% of cytochrome *c* at pH 9 is present in the ascorbate-irreducible form. This percentage accords well with our estimate of the extent of the slow $Fe(EDTA)^{2-}$ reduction path at this pH. Unlike ascorbate and ferrocyanide, $Fe(EDTA)^{2-}$ is able to attack the high pH form of cytochrome *c* directly. The following mechanism is proposed to account for the kinetic results at pH 9

$$\begin{array}{ccc} \text{cyt } c\text{ (III)}_S & \underset{k_{NS}}{\overset{k_{SN}}{\rightleftharpoons}} & \text{cyt } c\text{ (III)}_N \\ + & & + \\ Fe(EDTA)^{2-} & & Fe(EDTA)^{2-} \\ \downarrow k_1 & & \downarrow k_2 \\ \text{cyt } c\text{ (II)}_S + Fe(EDTA)^{-} & \xleftarrow{k_3} & \text{cyt } c\text{ (II)}_N + Fe(EDTA)^{-} \end{array}$$

where cyt *c* $(III)_S$ and cyt *c* $(III)_N$ refer respectively to native and high pH forms of the protein. This mechanism differs from that proposed for the ascorbate reduction of cyt *c* (III)[27,28] only in that cyt *c* $(III)_N$ now is susceptible to attack by the reducing agent. For $k_1[Fe(EDTA)^{2-}] \gg k_{NS}$ and k_{SN}, two parallel paths of reduction are predicted. The first, of course, corresponds to reduction of the native protein with rate = $k_1[Fe(EDTA)^{2-}][\text{cyt } c\text{ (III)}_S]$. The second path, which is observed after cyt *c* $(III)_S$ is fully reduced, should follow the rate law

$$\frac{-d[\text{cyt } c\text{ (III)}_N]}{dt} = (k_{NS} + k_2[Fe(EDTA)^{2-}])[\text{cyt } c\text{ (III)}_N]$$

where the k_{NS} term refers to reduction of cyt *c* $(III)_N$ accomplished *via* isomerization of the high pH derivative to the native form. Our rate constant for the $Fe(EDTA)^{2-}$ independent path (0.041 sec^{-1}) is in excellent agreement with the value of 0.045 sec^{-1} (20°, μ = 0.2, pH 9) determined by Wilson and Greenwood[28] for the slow phase in the ascorbate reduction of cytochrome *c*.

The assumption made in deriving the above rate law is supported by our pH jump experiment which shows that native cyt *c* (III) is reduced before measurable isomerization to cyt *c* $(III)_N$ takes place. That $k_1[Fe(EDTA)^{2-}] \gg k_{NS}$ and k_{SN} is also clear considering published values[28] of the forward and reverse isomerization rate constants.

The k_3 step in the proposed mechanism is included as a possible explanation of the apparent base line instability found for the slow 550-nm reduction phase at pH 9. It is suggested that ferrocytochrome *c* produced with a new ligand to iron in place of Met-80 slowly reverts to the native form. There is good justification for believing this should happen, as nmr studies[29] have established that the Fe–S bond of ferrocytochrome *c* does not dissociate over the range $4 \leq pH \leq 12$. Reduction of cyanoferricytochrome *c* gives the Fe^{II}–CN^- derivative which is unstable relative to cyt *c* $(II)_S$ at pH 7; dissociation of CN^- occurs with $k = 4 \times 10^{-3}$ sec^{-1} at 25°, and an increase in 550-nm absorbance is observed.[36] An analogous ligand interconversion process is the most likely source of the observed deviations from first-order kinetics as reduction of cyt *c* $(III)_N$ by $Fe(EDTA)^{2-}$ nears completion.

Some intriguing mechanistic questions are posed by the observation that of the three reductants, $Fe(EDTA)^{2-}$, ferrocyanide, and ascorbate, only the first is able to reduce cyt *c* $(III)_N$ directly. The reduction potential of cyt *c* $(III)_N$ is much lower than that of the native protein ($E^0 \leq 90$ mV for cyt *c* $(III)_N$ compared with $E^0 = +261$ mV at pH 7, μ = 0.01, 25° for cyt *c* $(III)_S$).[37] Ferrocyanide ($E^0 = +0.36$ V)[38] is too weak a reductant to attack the high pH derivative, but the reduction potentials of both ascorbate ($E^0 = -0.012$ V, pH 8.7)[39] and $Fe(EDTA)^{2-}$ ($E^0 \cong 0.03$ V, pH 9)[40] are such that full reduction of cyt *c* $(III)_N$ by large excesses of reducing agent is predicted. The inability of ascorbate to reduce cyt *c* $(III)_N$ before it interconverts to cyt *c* $(III)_S$ may be the result of its preference for an inner-sphere mechanism. Ligand substitution may cause the crevice-opening step required for inner-sphere attack to be slow relative to isomerization of the Fe–N derivative to the native form. Unlike ascorbate, $Fe(EDTA)^{2-}$ favors outer-sphere electron transfer mechanisms; its ability to react with cytochrome *c* is not expected to depend quite as critically on the nature of the iron(III) coordination environment as long as sufficient driving force for reduction is present.

The rate of reaction of $Fe(EDTA)^{2-}$ with cyt *c* (III), nevertheless, is dramatically affected when ligand substitution at high pH occurs. Expressions based on the relative Marcus theory suggest that the rate of an outer-sphere redox reaction should decrease by about one order of magnitude for a drop of 0.12 V in the

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(30) As the 695-nm band is abolished by iron-binding ligands and denaturing agents, Schejter and George[31] concluded that this absorption is linked to some conformational feature of the protein. Several subsequent studies have cast considerable doubt on the conformational sensitivity of the 695-nm band and have linked it directly to the presence of an Fe(III)–S bond involving Met-80 as ligand. Addition of *N*-acetyl-DL-methionine to ferriheme undecapeptide restores the 695-nm band,[32] and carboxymethylation of Met-80 yields a product lacking this peak.[33] Furthermore, the 695-nm band disappears upon reaction of CN^- with cyt *c* (III) under conditions where the observed protein conformational change is minimal.[34]

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driving force (expressed as ΔE^0).[21] On this basis a decrease in k_1 from 2×10^4 to 4×10^3 M^{-1} sec^{-1} may be accounted for in the reaction of Fe(EDTA)$^{2-}$ with cyt c (III)$_N$ at pH 9, but this leaves a factor of about 150 in the observed decrease in k_1 to 2.67×10^1 M^{-1} sec^{-1} unexplained. Further rate inhibition may arise from steric factors originating in the limited conformational change known to accompany formation of the high pH derivative.[28] The heme pocket presumably is the region in the protein most directly affected by displacement of the Met-80 ligand, but structural perturbations in the vicinity of the heme edge are possible as well. More structural information as well as a better estimate of the E^0 for the high pH cyt c (III) derivative will be required before a choice can be made among the several possible explanations of the observed rate decrease.

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Supplementary Material Available. A listing of k_{obsd} values will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N.W., Washington, D. C. 20036. Remit check or money order for $3.00 for photocopy or $2.00 for microfiche, referring to code number JACS-74-3132..

Reaction of Chromium(VI) with Hydrazinium Ion

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Abstract: The study of the stoichiometry and kinetics of oxidation of hydrazinium ion ($N_2H_5^+$) by chromium(VI) is extended to higher acidities than heretofore. The reaction proceeds through an N-bonded chromate ester *via* two equivalent redox steps. The reaction is catalyzed by acids H^+, H_3PO_4, $H_2PO_4^-$, and $HCrO_4^-$. Cr(IV) is found to disproportionate, but it is not trapped by Cr(VI) as previously supposed. Revised estimates of Cr(VI)/Cr(V) and Cr(VI)/Cr(IV) potentials are given. The Mn^{2+} effect on Cr(VI) oxidations is critically examined.

Chromium(VI) oxidations are characterized by preequilibria involving chromate(VI) esters[2] or one-electron reductions to chromium(V)[3] and by a variety of steps involving active intermediates Cr(V) and Cr(IV).[2,3] Hydrazine is of interest as a reducing agent because of the absence of information on N–Cr bonded esters and because of its ability to discriminate between two-equivalent substrates[4]

$$N_2H_4 \longrightarrow N_2 + 4H^+ + 4e^- \quad (1)$$

and one-equivalent substrates[4]

$$N_2H_4 \longrightarrow {}^1/_2N_2 + NH_4^+ + e^- \quad (2)$$

Durham and Beck[5] have reported on the kinetics of the reaction over the pH range 1–3. We have reported

$$13H^+ + 4HCrO_4^- + 3N_2H_5^+ \longrightarrow 4Cr^{3+} + 3N_2 + 16H_2O \quad (3)$$

on the fate of Cr(IV) intermediates as deduced from stoichiometric studies and Mn^{2+} trapping experiments.[6] We report here the kinetics of the reaction at high acidity, $2.2\ M \geq [H^+] \geq 0.2\ M$, and in phosphoric acid buffers of pH 1.6–3.0 and reexamine inferences drawn from the Mn^{2+} trapping experiments.

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Experimental Section

Analytical grade reagents were used without further purification. Kinetics were measured by following the disappearance of Cr(VI) at the absorption maximum of 350 nm. Both $HCrO_4^-$ and H_2CrO_4 are present over much of the $[H_3O^+]$ range studied, so absolute determination of Cr(VI) was made after making solutions alkaline at 372 nm at the absorbance maximum of CrO_4^{2-}.

Fast reactions were studied in a Sutin stopped-flow apparatus previously described.[7] Detection of a chromium(V) intermediate was made by employing a continuous flow of reacting mixtures through the cavity of an esr spectrometer as previously described.[6]

Ionic strength was controlled using $NaClO_4$ (or $LiClO_4$) and $HClO_4$.

Results

The Rate Law. Figure 1 shows that all runs with excess hydrazine exhibit first-order kinetics in Cr(VI) over several half-lives, giving the pseudo-first-order rate law

$$\frac{-d[Cr(VI)]}{dt} = k_{obsd}[Cr(VI)] \quad (4)$$

Order in $NH_3NH_2^+$. The slopes of the lines in Figure 1 are roughly proportional to $[NH_3NH_2^+]$. However, the values of k_{obsd} given in Table I show a small but distinct departure from first-order behavior at high $[NH_3NH_2^+]$ consistent with the equation

$$k_{obsd} = k_b[NH_3NH_2^+]/(1 + 3.2[NH_3NH_2^+]) \quad (5)$$

This result is consistent with the formation of a

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