Milk-ejecting assays were performed using mouse mammary tissue by the method of Hruby and Hadley.[35]

Antidiuretic assays were performed with anesthetized, male, Long-Evans rats (315-345 g) according to the method of Jeffers et al.[36] as modified by Sawyer.[37] Specific antidiuretic potencies were determined using the four-point design or matches against USP posterior pituitary reference standards.

Pressor assays were with urethane-anesthetized, male, Sprague-Dawley rats (250-350 g) as described in the U.S. Pharmacopeia.[38] [D-Gln^4]oxytocin was found to be a partial agonist, showing only about 30% of the maximal response for arginine-vasopressin in this assay system at maximal stimulation. The response also was not parallel to arginine-vasopressin over the linear portion of the dose-response curve and, hence, no accurate determination of potency is possible (Table I).

Acknowledgment. The authors thank Dr. Clark Smith, University of Illinois at the Medical Center, Chicago, IL, for the oxytocic, avian vasodepressor, antidiuretic, and pressor assays, and Dr. Mac Hadley, University of Arizona, Tucson, AZ, for the milk-ejecting assays. We also thank Mr. G. Skala and Mrs. E. Skala for technical assistance and Mr. Jim Ormberg for the amino acid analyses results. This work was supported by Grant AM-17420 from the U.S. Public Health Service and by a grant from the National Science Foundation.

Registry No. Oxytocin, 680-77-9; arginine-vasopressin, 113-79-1; [D-Gln^4]oxytocin, 3196-75-6; [D-Gln^4,Arg^8]vasopressin, 76023-59-1; H-Cys(DMB)-Tyr-Ile-D-Gln-Asn-Cys(DMB)-Pro-Leu-Gly-NH-benzhydrylamine, 80028-66-6; H-Cys(DMB)-Tyr-Phe-D-Gln-Asn-Cys-(DMB)-Pro-Arg(Tos)-Gly-NH_2, 80041-61-8.

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Kinetic Analysis of Cytochrome b_5 Reduction by Fe(EDTA)$^{2-}$

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Abstract: The kinetics of cytochrome b_5 reduction by Fe(EDTA)$^{2-}$ have been studied as a function of temperature, pH, and ionic strength. The second-order rate constant for the reaction is $2.85(6) \times 10^2$ M^{-1} s^{-1} [pH 7.0 (phosphate), μ = 0.5 M, 25 °C] with $\Delta H^{\ddagger}$ = 5.4(2) kcal/mol and $\Delta S^{\ddagger}$ = -29.2(8) eu. The ionic strength dependence between 0.05 and 0.5 M [pH 7.0 (phosphate), 25 °C] has been fitted to the Marcus ionic strength equation and yields a charge of -14.2 for cytochrome b_5 (oxidized). This charge has been used in calculation of the electrostatics-corrected self-exchange rate for cytochrome b_5 in this reaction (k_{11}^{corr}) to obtain a value of 4.3 M^{-1} s^{-1}. Virtually identical (k_{11}^{corr}) values have been reported for cytochrome *c* and cytochrome c_{551}. The pH dependence of the reaction has been analyzed in terms of reduction of a protonated (k_a) and an unprotonated (k_b) form of the protein to yield a pK_a of 5.85 and values of $7.2(10^2)$ M^{-1} s^{-1} for k_a and $2.5(10^2)$ M^{-1} s^{-1} for k_b [25 °C (phosphate), μ = 0.5 M]. The activation parameters for this reaction have been studied as a function of pH and found to reflect an isokinetic relationship. This result suggests that the effect of pH on this reaction arises from the pH dependences of the driving force of the reaction and/or the electrostatic interaction between the protein and reagent.

Introduction

Cytochrome b_5 is presently known to participate in at least three oxidation-reduction processes involving protein-protein electron transfer in vivo: (1) stearyl-CoA desaturation;[1] (2) cytochrome P 450 reduction;[2] and (3) methemoglobin reduction.[3] Despite this functional versatility, the electron-transer properties of cytochrome b_5 remain largely uncharacterized. As a first step toward a better understanding of this facet of cytochrome b_5 function, we have studied the reaction of the proteolytically solubilized form of the protein with Fe(EDTA)$^{2-}$. Fe(EDTA)$^{2-}$ was selected for this purpose because it has been shown to provide useful mechanistic information in studies involving a variety of other metalloproteins.[4]

Experimental Section

Reagent grade chemicals were used throughout except where noted. Glass distilled water was used in initial measurements. In later studies this water was further purified by passage through a Barnstead NANOpure water purification system to produce water that routinely had a resistivity of 17-18 MΩ·cm. Residual oxidizing impurities present in Linde prepurified nitrogen were removed by passing the gas through two vanadous[5] and one photoreduced methylviologen[6] scrubbing towers. Measurements of pH were made with a Radiometer Model PHM 84 pH meter and combination electrode.

The hydrophilic fragment of cytochrome b_5 was prepared from fresh beef liver by a modified combination of the methods of Strittmatter[7] and Omura and Takesue[8] as follows. All operations were carried out at 4 °C.

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(8) Omura, T.; Takesue, S. *J. Biochem. (Tokyo)* **1970**, *67*, 249.

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Microsomes were prepared from fresh beef liver by precipitation with ammonium sulfate (Sigma, type I) as described by Strittmatter.[7] The resulting pellet was dialyzed against distilled water until the conductivity of the suspension dropped to about 2 mmhos. This microsomal preparation could be stored at -70 °C until required. To facilitate large-scale purification of the cytochrome, the undiluted microsomal suspension (~100 mg of protein/mL[9]) was treated with trypsin (Sigma type I or Worthington 2 times recrystallized/lyophilized). Specifically, a solution containing 5 g of trypsin, 0.15 g of $CaCl_2 \cdot 2H_2O$, and 0.07 g of L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (Sigma) dissolved in 15 mL of 1 mM HCl was added to each liter of microsomes used. This mixture was stirred for 24 h at 4 °C while maintaining the pH at 7.7. Membranous material was precipitated by adding solid ammonium sulfate to 1.8 M[10] while maintaining the pH at 7.2. After stirring for 1 h, the mixture was centrifuged at 16000*g* for 25 min, and 3 molar equiv of a synthetic trypsin inhibitor (*p*-nitrophenyl *p'*-guanidinobenzoate hydrochloride, Sigma) was added to the red supernatant fluid. This solution was dialyzed against distilled water until the conductivity was reduced to 2 mmhos and then loaded onto a column of DEAE-cellulose (Whatman DE-52) as described by Strittmatter.[7] The partially purified cytochrome obtained from this column was rechromatographed on a second DE-52 column employing the same conditions and then concentrated to a small volume by ultrafiltration using an Amicon YM-5 membrane. This concentrated solution (2–3 mL) was then passed over a column of Sephadex G-75 resin (80 × 2.6 cm) to remove a yellow contaminant. The resulting purified protein was homogeneous on $NaDodSO_4$–polyacrylamide gel electrophoresis and had an $A_{412.5}/A_{280}$ ratio of 5.7–6.0. Approximately 50–100 mg of purified cytochrome could be obtained from 1 kg of liver.

$Fe(EDTA)^{2-}$ stock solutions were prepared under nitrogen on the day of use as described by Wherland et al.[4b] and diluted with degassed buffer using Hamilton gas-tight syringes that had been flushed thoroughly with nitrogen. Protein solutions were degassed by bubbling gently with nitrogen for 30 min in bottles fitted with cleaned serum caps; after degassing, the protein was kept under a constant stream of nitrogen at room temperature.

Kinetic measurements were made with a stopped-flow spectrophotometer system based on the Dionex Model D-103 mixing apparatus and employing a data acquisition and analysis system designed and constructed by On-Line-Instrument Systems (Jefferson, Ga). The optical system of this apparatus is comprised of a Jobin-Yvon Model DH-10 double monochromator and a tungsten–halogen source powered by an OLIS XL150 adjustable power supply. A computer-controlled stepping motor attached to the monochromator permits acquisition of the electronic spectrum of the contents of the observation cell (2-cm path length). The signal from the phototube (EMI-Gencom) is fed in turn to an OLIS HVA-SF amplifier, OLIS Model 3620 interface, and finally to a Data General Nova 2/10 minicomputer equipped with a dual floppy disk drive. Data collection software permits signal averaging of input decays and control of data input timing. Graphic data analysis is permitted via a Tektronix digital monitor and a computer-controlled X–Y plotter.

Three modifications have been made in the Dionex mixing apparatus to improve anaerobicity. The first involves replacement of the Teflon luer fittings that lead to reservoir syringes with compression fittings machined from nylon. These fittings are used with saran tubing (0.125 in. o.d., 0.031 in. wall, Pyramid Plastics, Hope, Ar), stainless-steel needles, and a second nylon luer/compression fitting adapter to transfer reactant solutions from nitrogen-flushed serum bottles into the drive syringes. The second modification involves replacement of the ceramic-tipped drive syringe plungers with plungers that employ viton o-ring-sealed nylon tips designed to allow adjustment of the pressure exerted by the o-ring against the wall of the drive syringe. This feature not only provides improved anaerobicity but permits compensation for the contraction and expansion of the drive syringe assembly that occurs in variable-temperature studies. Finally, the cone-shaped Teflon adapters that mount between the drive syringes and the Kel-F valve block were replaced with identical adapters machined from nylon.

Temperature was controlled to ±0.1 °C with a Haake Model F3 refrigerated water bath. Solutions were allowed to equilibrate for 25 min at 25 °C and for 45 min at all other temperatures.

(9) Protein concentration was determined by the dye-binding method sold by Bio-Rad. To eliminate interference with this assay by lipid present in the microsomal suspension, it was necessary to saponify the sample to be analyzed prior to analysis as follows: 0.1 mL of a 100-fold dilution of the microsomal preparation was mixed with 0.1 mL of 6 M NaOH and heated at 100 °C for 15 min. The assay mixture was then neutralized with glacial acetic acid and analyzed as described in the instructions accompanying the Bio-Rad reagent. Protein concentration was determined from a standard curve obtained by treating a series of protein solutions of known concentration in an identical manner.

(10) Wood, W. I. *Anal. Biochem.* **1976**, *73*, 250.

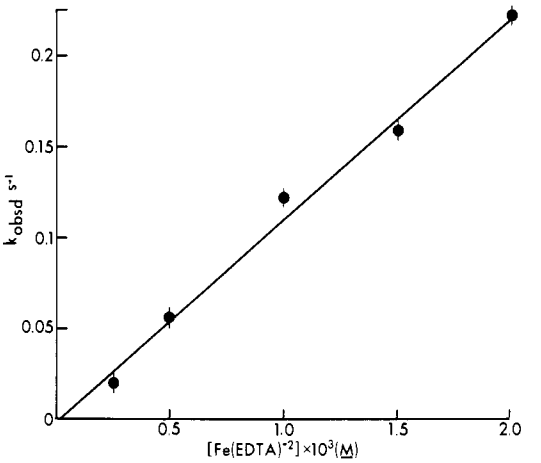

Figure 1. Dependence of observed rate constants for the reduction of cytochrome b_5 by $Fe(EDTA)^{2-}$ on reductant concentration [25 °C, pH 7.0 (phosphate), μ = 0.1 M]. All values of k_{obsd} were calculated from the pseudo-first-order reversible analysis.[11]

Table I. Second-Order Rate Constants for the Reduction of Cytochrome b_5 by $Fe(EDTA)^{2-}$ Determined from Concentration Dependences Measured as a Function of pH and Ionic Strength at 25 °C in Phosphate Buffer

pH	μ, M	k_{12}, M^{-1} s^{-1}
5.5	0.5	5.75(5) × 10²
6.0	0.5	4.63(8) × 10²
6.5	0.5	3.14(2) × 10²
7.0	0.5	2.85(7) × 10²
7.0	0.4	2.54(6) × 10²
7.0	0.3	2.03(8) × 10²
7.0	0.2	1.86(3) × 10²
7.0	0.1	1.12(6) × 10²
7.0	0.05	4.3(1) × 10
8.0	0.5	2.56(2) × 10²

Reactions were run under pseudo-first-order conditions with $Fe(EDTA)^{2-}$ in at least 30-fold excess over cytochrome b_5. Reduction of cytochrome b_5 was monitored at 423 nm (typically, ΔA = +0.15), and at least five tracings were averaged for each first-order rate cited. First-order rate constants were obtained from weighted linear-squares analyses; identical results were obtained from nonlinear regression analyses. Second-order rate constants were obtained from weighted linear least-squares analyses of concentration dependences. Activation parameters were obtained from weighted linear least-squares analyses of Eyring plots.

Results and Discussion

First-order plots for the reduction of cytochrome b_5 by $Fe(EDTA)^{2-}$ were found to be linear for at least 90% of the reaction under most conditions employed. At $Fe(EDTA)^{2-}$ concentrations less than 2 mM, however, these plots were not linear. In these cases, examination of the spectrum of the reaction mixture after mixing in the stopped-flow apparatus revealed the presence of both oxidized and reduced protein. This suggested that under these conditions the reaction did not proceed to completion. Consequently, these data were analyzed by a nonlinear regression fit to a pseudo-first-order reversible rate equation.[11]

The dependence of observed rates on $Fe(EDTA)^{2-}$ concentration was linear in all cases examined. A representative concentration dependence is shown in Figure 1. At the ionic strength used in the collection of these data, all first-order rates were determined by the pseudo-first-order-reversible analysis. The linearity and the zero intercept of this plot demonstrate that the assumption upon which this analysis is based is quantitatively consistent with the experimental data. The second-order rate constants obtained

(11) (a) Cummins, D., Gray, H. B. *J. Am. Chem. Soc.* **1977**, *99*, 5158. (b) Frost, A. A.; Pearson, R. G. "Kinetics and Mechanism", 2nd ed.; Wiley: New York, 1961; p 185.

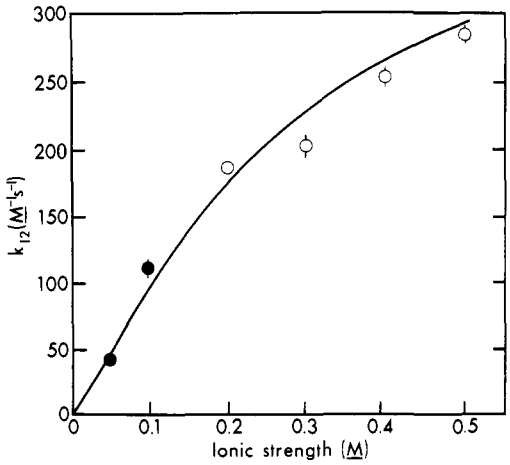

Figure 2. Dependence of second-order rate constants for the reduction of cytochrome b_5 by $Fe(EDTA)^{2-}$ on ionic strength [25 °C, pH 7.0 (phosphate)]. Solid circles represent rates determined from k_{obsd} values obtained by pseudo-first-order reversible analysis. The solid line represents the fit of the data to the Marcus ionic strength equation.[13]

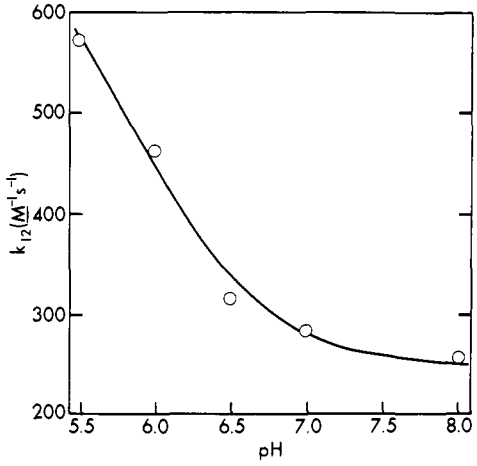

Figure 3. Dependence of second-order rate constants for the reduction of cytochrome b_5 by $Fe(EDTA)^{2-}$ on pH [25 °C (phosphate), μ = 0.5 M]. The solid line is the fit to the data described in the text.

from these data and from other measurements at varying pH and ionic strength are set out in Table I.

The ionic strength dependence of cytochrome b_5 reduction by $Fe(EDTA)^{2-}$ is shown in Figure 2. The observed increase in rate with increasing ionic strength is consistent with the expected behavior for a reaction between two similarly charged reactants. These data have been fitted by a nonlinear least-squares regression to the Marcus equation for ionic strength dependence[12,13] by assuming a charge and radius for $Fe(EDTA)^{2-}$ (-2 and 4 Å, respectively[13a]) and a radius for cytochrome b_5 (15.9 Å[14]) and allowing the charge of the protein to vary. The resulting charge for the oxidized protein was -14.2(6). This result may be compared with the value of -6.5 that we estimate from the amino acid sequence as described by Wherland and Gray.[13b] Interstingly, the value of -14.2 correlates much better with the number of negatively charged groups (aspartyl and glutamyl residues and heme propionate side chains) shown by crystallographic analysis to be clustered around the partially exposed heme edge of this protein.[15] Although it may be tempting to speculate that this

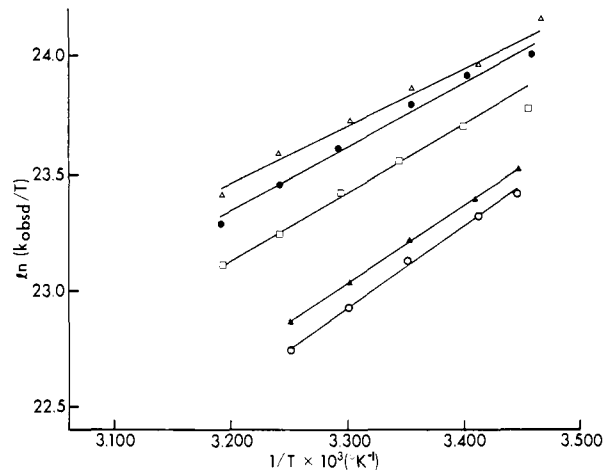

Figure 4. Eyring plots of the rate data for the reduction of cytochrome b_5 by $Fe(EDTA)^{2-}$ (μ = 0.5 M, phosphate): (○) pH 8.0; (▲) pH 7.0; (□) pH 6.5; (●) pH 6.0; (△) pH 5.5. In each case [$Fe(EDTA)^{2-}$] was $1.25(10^{-2})$ M.

Table II. Activation Parameters for the Reduction of Cytochrome b_5 by $Fe(EDTA)^{2-}$ as a Function of pH in Phosphate Buffer, μ = 0.5 M

pH	$\Delta H^{\ddagger}$, kcal/mol	$\Delta S^{\ddagger}$, eu
5.5	7.1(2)	−22.1(8)
6.0	6.5(1)	−24.3(5)
6.5	5.7(2)	−27.8(8)
7.0	5.4 (2)	−29.2(8)
8.0	4.9(2)	−31.1(5)

result defines the active-site charge of cytochrome b_5 that is experienced by $Fe(EDTA)^{2-}$, the relatively high ionic strength used in this study precludes such a conclusion. The ionic strength dependence analysis, however, is useful insofar as it provides an empirical means of estimating the contribution of electrostatic interaction between the reactants to the rate of reaction (vide infra).[13]

The dependence of the second-order rate constant on $Fe(EDTA)^{2-}$ concentration as a function of pH is shown in Figure 3. Since $Fe(EDTA)^{2-}$ does not have an ionizable group with a pK_a that lies within the pH range studied,[16] this rate dependence can be attributed to an ionizable group on cytochrome b_5 that is functionally linked to the redox activity of the protein. With this assumption, the following reactions may be considered:

$$Fe(EDTA)^{2-} + \text{cyt } b_5{\cdot}H^+ \xrightarrow{k_a} \text{products}$$

$$Fe(EDTA)^{2-} + \text{cyt } b_5 \xrightarrow{k_b} \text{products}$$

$$\text{cyt } b_5{\cdot}H^+ \overset{K_a}{\rightleftharpoons} \text{cyt } b_5 + H^+$$

In this case, the following relationship can be derived:[4c]

$$k_{12} = \frac{k_a[H^+] + k_b[K_a]}{[H^+] + K_a}$$

The parameters in this equation were evaluated by a nonlinear regression fit of the data in Figure 3 to this relationship. The

(12) Haim, A.; Sutin, N. *Inorg. Chem.* **1976**, *15*, 476.
(13) (a) Wherland, S.; Gray, H. B. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *79*, 2950. (b) Wherland, S.; Gray, H. B. "Biological Aspects of Inorganic Chemistry"; Addison, A. W.; Cullen, W.; James, B. R.; Dolphin, D., Eds.; Wiley: New York, 1977; p 289.
(14) The radius of cytochrome b_5 is estimated from the following relationship:[4c] $R = 0.717M_T^{1/3}$, where M_T is the molecular weight of the protein (assumed to be 11 000 for cytochrome b_5).
(15) (a) Mathews, F. S.; Czerwinski, E. W., "The Enzymes of Biological Membranes"; Martonosi, A. E.; Plenum Press: New York, 1976; Vol 4, p 143. (b) Mathews, F. S.; Czerwinski, E. W., Argos, P. "The Porphyrins"; Dolphin, D., Ed.; Academic Press: New York, 1979; Vol VII, p 107. (c) Examination of a molecular model of cytochrome b_5 (Vis-Aid Devices, Winnipeg, Man.) reveals the following negatively charged residues within 14–15 Å of the heme edge as estimated along the "surface" of the protein: Glu at positions 37, 38, 43, 44, 48, 56, 59, and 69; Asp at positions 57, 60, and 66.
(16) (a) Schwarzenbach, G.; Heller, J. *Helv. Chim. Acta* **1951**, *34*, 576. (b) Belcher, R.; Gibbons, D.; West, T. S., *Anal. Chim. Acta* **1955**, *12*, 107.

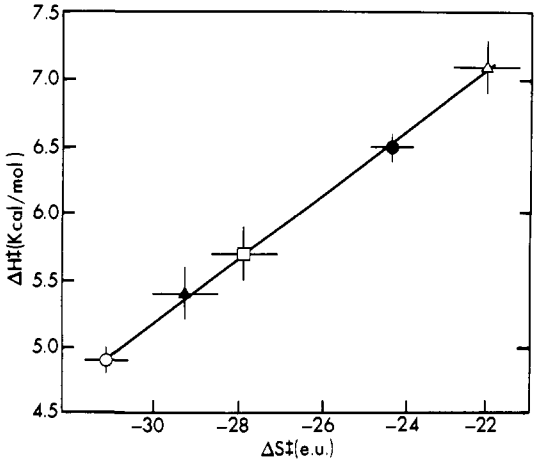

Figure 5. Compensation plot for the reduction of cytochrome b_5 by $Fe(EDTA)^{2-}$. Conditions and symbols as in Figure 4.

resulting values are $pK_a = 5.85$, $k_a = 717\ M^{-1}\ s^{-1}$, and $k_b = 248\ M^{-1}\ s^{-1}$. The identity of the functional group on the protein that is responsible for this pH dependence cannot, of course, be surmised from these data alone. Although pK_a values in this range or higher are frequently taken as being suggestive of the involvement of an imidazole group, carboxyl groups have been reported to have pK_a values in this range or higher attributable to specific environmental effects within proteins.[17] With the plethora of carboxyl groups adjacent to the putative active site of cytochrome b_5, this possibility cannot be dismissed. One particularly intriguing possibility along this line is the heme propionate group located on the D ring of the porphyrin.[15,18] Crystallographic data have indicated that this group is involved in neutralizing the charge on the heme iron in the oxidized form of the protein. If this interpretation is correct, protonation of this propionate would destabilize the oxidized form and effectively increase the reduction potential of the protein, thereby increasing the rate of reaction with $Fe(EDTA)^{2-}$. Significantly, NMR studies[19] indicate that this heme propionate becomes protonated in the pH range studied here.

The temperature dependence of cytochrome b_5 reduction by $Fe(EDTA)^{2-}$ has been studied as a function of pH as shown in the family of Eyring plots illustrated in Figure 4. The activation parameters derived from these plots are set out in Table II. Compared with similar data for the $Fe(EDTA)^{2-}$/cytochrome c reaction, the activation entropy is relatively large and negative. One major possibility that may contribute to this difference is the proposed cation binding near one of the heme propionates that is thought to occur as cytochrome b_5 is reduced.[15] This notion is consistent with the approach of $\Delta S^{\ddagger}$ toward zero as the pH is lowered. Similar anion or cation building is not thought to occur in cytochrome c.[20]

The pH dependence of the activation parameters also suggests that the mechanism of cytochrome b_5 reduction by $Fe(EDTA)^{2-}$ is invariant with pH as shown by examination of these data in terms of a compensation plot[21] (Figure 5). This plot suggests that an isokinetic relationship exists with a compensation temperature (slope) of 246(4) K.[22] If this is the case, then the origin of the rate dependence on pH lies either in the variation of reactant reduction potentials with pH or in the variation of protein–reagent electrostatic interaction with pH, although both factors may, of course, contribute.

The data presented here have been analyzed further by the use of relative Marcus theory to estimate the apparent self-exchange rate demonstrated by cytochrome b_5 in its reaction with $Fe(EDTA)^{2-}$. By using the effective electrostatic charge on the protein defined by the analysis shown in Figure 2, the published reduction potentials for the reactants,[14,23] and the self-exchange rate of $Fe(EDTA)^{2-/1-}$,[13] this rate may be estimated for an ionic strength of 0.1 M (k_{11}^{corr}) by the method of Wherland and Gray.[13] As second-order rates are available for a variety of ionic strengths (pH 7, 25 °C), we have used all of them to calculate an average value of 4.3 $M^{-1}\ s^{-1}$ for k_{11}^{corr}. The range of values calculated from data obtained at various ionic strengths is small (3.3–5.8 $M^{-1}\ s^{-1}$) as anticipated from the reasonably good fit of the data seen in Figure 2. This value of k_{11}^{corr} is remarkably similar to those reported previously for the same reactions of cytochrome c ($k_{11}^{corr} = 6.2\ M^{-1}\ s^{-1}$)[14a] and cytochrome c_{551} ($k_{11}^{corr} = 2.0\ M^{-1}\ s^{-1}$),[4d] implying that the mechanisms of reaction in all three cases are very similar. Apparently the structural differences between c- and b-type cytochromes do not produce kinetically detectable alterations in the mechanism of electron transfer employed by these proteins with this inorganic complex.[24]

To complete our analysis of these data, we have used the method of Mauk, Scott, and Gray[25] to estimate the distance from the point of electron transfer within the protein to the protein surface from the rates measured at pH 7.0 and 25 °C. The average value of the self-exchange rate for cytochrome b_5 at infinite ionic strength (k_{11}^{∞}) calculated in this manner is $1.4(10^2)\ M^{-1}\ s^{-1}$, which is consistent with an R_p value of 4.5 Å and a distance of 2.7 Å from the heme edge to the protein surface. Significantly, this value is consistent with similar values calculated for c-type cytochromes and within previously discussed error limits[23] of any value that might be deduced from crystallographic data.[15]

The present study represents the first systematic analysis of an oxidation–reduction reaction between cytochrome b_5 and an inorganic complex. The results are fully consistent with a mechanism for the reaction in which $Fe(EDTA)^{2-}$ approaches the partially exposed heme edge of the protein and transfers an electron at the shortest distance permitted by local steric considerations. This picture is qualitatively consistent with the observation of simple second-order kinetics, the large negative effective charge estimated by the ionic strength dependence, and the distance estimated from k_{11}^{∞} and is essentially identical with the proposed mechanism for the reaction of $Fe(EDTA)^{2-}$ with c-type cytochromes. Remarkably, the k_{11}^{corr} calculated for this reaction is the same as that calculated for the same reactions of cytochrome c and cytochrome c_{551}[4d,13a] despite widely divergent isoelectric points for the proteins and despite a 240-mV difference in protein reduction potentials.[13b,23]

With the information available from this study it is now feasible to take advantage of experimental options that are available uniquely through use of cytochrome b_5 for the functional char-

(17) Fehrst, A. "Enzyme Structure and Mechanism"; W. H. Freeman: San Francisco, 1977; p 154.

(18) (a) Keller, R. M.; Wüthrich, K. *Biochim. Biophys. Acta* **1980**, *621*, 204. (b) Mathews, F. S. *Biochim. Biophys. Acta* **1980**, *622*, 375.

(19) Keller, R. M.; Groudinsky, O.; Wüthrich, K. *Biochim. Biophys. Acta* **1976**, *427*, 497.

(20) Takano, T.,; Dickerson, R. E. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 6371.

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(22) The limited temperature range accessible in studies such as these restricts the number of data points that can be collected and thereby compromises the statistical validity of frequently employed error analysis as used here. For this reason, estimates such as ±3 eu for the activation entropy and ±1 kcal/mol for the activtion enthalpy may be more realistic. We note also that compensation plots are not infallible and should be regarded with some caution (Davis, W. H.; Pryor, W. A. *J. Chem. Educ.* **1976**, *53*, 285; Krug, R. R.; Hunter, W. G.; Grieger-Block, R. A. "Chemometrics: Theory and Applications"; Kowalski, B. R., Ed.; American Chemical Society, Washington, DC, 1977; p 192).

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(24) In addition to differences in the protein environment of the heme binding sites, the principal differences between c and b cytochromes are threefold: (1) c hemes are covalently bound to the protein through two thioether linkages, while b hemes are bound noncovalently; (2) the axial ligands to the heme iron are methionine sulfur and imidazole nitrogen in c hemes, while b heme iron ligands are both imidazole nitrogens; and (3) the orientation of the prosthetic groups in the heme pocket are nonidentical.

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acterization of cytochrome oxidation-reduction behavior. In particular, it will be of interest to examine the influence of heme substituents through use of heme-substitution techniques. These studies are now in progress.

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Supplementary Material Available: Listing of observed first-order rate constants (3 pages). Ordering information is given on any current masthead page.

Communications to the Editor

Stereochemistry and Mechanism of an Acid-Catalyzed 1,2-Phospho Group Migration: Evidence for Pseudorotation in the Reaction of a Phosphoric Monoester

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In the past few years, there has been much work on the determination of the stereochemical consequence at phosphorus of enzyme-catalyzed reactions that involve esters of phosphoric acid.[1] There have been almost no reports, in contrast, on the stereochemical course of *non-enzymic* reactions of phosphoric monoesters. Such stereochemical results are of particular interest since phosphoric monoesters may in principle undergo nucleophilic substitution at phosphorus by three mechanisms,[2] each of which has a different predicted stereochemical outcome. First, the reaction may follow the unimolecular "dissociative" pathway via a monomeric metaphosphate intermediate. If this species is free and symmetrically solvated, we expect the product will have suffered *racemization* at phosphorus. Secondly, if the "in-line associative" mechanism is followed, the reaction will go through a pentacoordinate transition state, and by analogy with the S_N2 reaction at carbon, *inversion* of the configuration at phosphorus will result. Thirdly, in the "adjacent associative" process, adjacent attack of the entering nucleophile forms a pentacoordinate intermediate that must undergo pseudorotation to allow expulsion of the leaving group from an apical position.[3] The stereochemical course of such a reaction is predicted to be *retention*. We report here experimental results on a reaction where the entering nucleophile is constrained to attack phosphorus "adjacent" to the leaving group and show that the stereochemical results are qualitatively and quantitatively in accord with the above prediction for a pathway that involves pseudorotation of an intermediate that is pentacoordinate at phosphorus.

The reaction we have chosen is the 1,2-phospho group migration that occurs when 2-[(*R*)-^{16}O,^{17}O,^{18}O]phosphopropanediol (**2**) is heated in acid. Many years ago, Bailly[4] described the analogous rearrangement of 2-phosphoglycerol to 1-phosphoglycerol and observed that hydrolysis of the phosphoric ester was slow compared to the isomerization reaction. Subsequently, Fordham and Wang[5] demonstrated that two pathways exist for this isomerization, and in a series of elegant kinetic experiments these workers evaluated the rate constants for the minimal kinetic scheme shown in Scheme I. Most important, while the isomerization route via the cyclic diester resulted in the incorporation of solvent ^{18}O into the product, the direct isomerization caused no such incorporation. This is consistent with the latter path involving a pseudorotating pentacoordinate intermediate and is a necessary (though insufficient) condition for this mechanism.

Scheme I. Pathways for the Isomerization of 2-Phosphopropane-1,2-diol and 1-Phosphopropane-1,2-diol

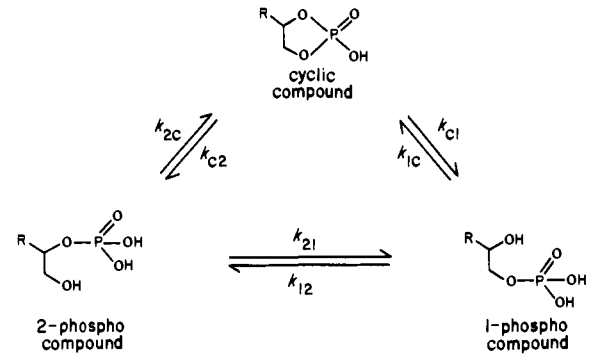

Table I. Kinetic Parameters for the Isomerization of 2 and 1[a,d]

Rate Constant[b]
k_{21}, 1.02×10^{-4} s^{-1}
k_{12}, 5.64×10^{-5} s^{-1}
k_{2c}, 4.84×10^{-4} s^{-1}
k_{1c}, 1.57×10^{-4} s^{-1}
k_{c2}/k_{c1}, 1.70
K_{eq},[c] 1.81

[a] 85 °C, 0.5 N $HClO_4$. [b] See Scheme I. [c] [1]/[2]. [d] The estimated precision of the individual rate constants is ±7%; of the rate ratio, ±5%; and of the equilibrium constant, ±4%.

We first determined conditions under which the rearrangement of **2** to **1** would proceed at a reasonable rate with minimal label incorporation from $H_2{}^{18}O$. At 85 °C in 0.5 M $HClO_4$, the contribution of the upper pathway of Scheme I is minimized, and the rate of loss of phosphoric ester by hydrolysis is negligible. Since, however, the overall equilibrium constant for the **2** to **1** isomerization is not far from unity (K_{eq} = [**2**]/[**1**] = 0.55), the

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