# The Reduction of Ferric *o*-Phenanthroline Complexes by Reduced Diphosphopyridine Nucleotide*

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ABSTRACT: The binuclear complex, bisdiferric tetra-*o*-phenanthrolinediol, is a two-electron acceptor. The two adjoining ferric ions are bridged by the two hydroxyl groups. The magnetic properties of this molecule suggest overlapping of d orbitals between the iron atoms (Gaines, A., Jr., Hammett, L. P., and Walden, G. H., Jr. (1936), *J. Am. Chem. Soc. 58*, 1668). The ferric dimer is reduced in slightly acid solution by reduced diphosphopyridine nucleotide. In the reactions 1 mole of reduced diphosphopyridine nucleotide reduces 1 mole of the dimer forming 2 moles of ferrous tri-*o*-phenanthroline. The redox reaction is of first-order kinetics when both reactants are in nearly equimolar concentrations. This indicates that a mixed complex of ferric dimer and reduced diphosphopyridine nucleotide is formed at a rate higher than that of the electron transfer. The over-all reaction velocity decreases with increase of pH, the concentration of ferric dimer needed to saturate the reduced diphosphopyridine nucleotide increases with increasing pH. These two phenomena are well correlated with the relative concentration of hydroxyl and oxo forms of the dimer. This is explained by assuming that the hydroxyl–iron bond is broken and replaced by a reduced diphosphopyridine nucleotide–iron bond, thus forming the mixed complex. The oxo–iron bond is much more resistant to such displacement. By saturating the reduced diphosphopyridine nucleotide molecules with the ferric dimer, both $K_{diss}$ of the mixed complex and the rate constant of the electron transfer step were estimated. We suggest that the redox reaction is carried by two one-electron transfers, both of them from the reduced nicotinamide moiety to the ligated ferric ion. Because of the overlapping d orbitals the first newly gained electron is rapidly transferred to the adjoining ferric ion, thus enabling the ligated iron to accept the second electron.

Reduced diphosphopyridine nucleotide, a two-electron donor, is oxidized in mitochondria in one-electron transfer reactions (Beinert and Palmer, 1965) by a flavoprotein containing nonheme iron (King *et al.*, 1966). A mechanism for this reaction was suggested by Fox and Tollin (1966a,b). According to these authors, the initial step is a two-electron transfer from DPNH[1] to FMN, which is later converted into its semiquinoid form, thus forming the first one-electron donor in the respiratory chain. In this work, we investigate a system in which DPNH is simultaneously oxidized by two one-electron acceptors.

A possible one-electron acceptor in DPNH dehydrogenase is the nonheme iron of the enzyme, which undergoes oxidation reduction cycles during enzymic activity (Beinert *et al.*, 1963; Beinert and Hemmerich, 1965). It was already shown (Gutman *et al.*, 1968) that aqueous ferric ions can react with DPNH to form a colored complex which decomposes into ferrous iron and a one-electron oxidation product of DPNH. By using a binuclear ferric complex, two electrons can be transferred from DPNH, each to a different ferric ion.

Bis(diferric tetra-*o*-phenanthrolinediol) (Gaines *et al.*, 1936) is a stable compound in which the two ferric ions are bridged by two $OH^-$ groups. The complex is readily soluble in water with low absorbance at 510 mμ. The reduction of this complex in the presence of excess of *o*-phenanthroline is $Fe^{2+}$-*o*-$phen_3$, which has a high absorbance at 510 mμ ($\epsilon_m$ $11.10^3$ $M^{-1}$ $cm^{-1}$); thus, the reduction of the complex is readily detected at this wavelength. When $(Fe_2$-*o*-$phen_4(OH)_2)^{4+}$ is reduced by dithionite with excess of *o*-phenanthroline, the red color of the ferrous complex is formed immediately, so that it can be assumed that the addition of the third *o*-phenanthroline to the iron does not interfere with our kinetic measurements.

We found that DPNH rapidly reduces the bisferric *o*-phenanthroline dimer, and the stoichiometry of the reaction is 2 ferrous iron equiv formed/mole of DPNH oxidized. The product of DPNH oxidation is $DPN^+$.

The kinetics of the reaction indicates than an intermediate complex of DPNH and $(Fe_2$-*o*-$phen_4(OH)_2)^{4+}$ is formed and undergoes redox reaction which is the rate-limiting step of the over-all mechanism. The rate of this reaction is pH dependent. The rate constant of the redox reaction and the formation constant of the intermediate complex are reported, and a mechanism for the reaction is suggested.

## Materials and Methods

*Chemicals.* *o*-Phenanthroline hydrochloride, ferric ammonium sulfate, and ferrous ammonium sulfate were commercial preparations of highest degree of purity.

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[1] Abbreviations are listed in *Biochemistry 5*, 1445 (1966).

*(Fe₂-o-phen₄(OH)₂)*$^{4+}$. This compound is the product of reaction between ferric ions and *o*-phenanthroline at acid solutions (Gaines *et al.*, 1936). The complex formed under these conditions has an absorbance spectrum similar to solutions made of crystalline ($Fe_2$-*o*-$phen_4$-$(OH)_2)^{4+}$, therefore, freshly prepared solutions of ($Fe_2$-*o*-$phen_4(OH)_2)^{4+}$ formed by mixing ferric ammonium sulfate and *o*-phenanthroline were employed. In all the experiments the molar ratio of $Fe^{3+}$ to *o*-phenanthroline was 1:3.

*Fe*$^{3+}$*-o-phen₃* was produced by oxidation of $Fe^{2+}$-*o*-$phen_3$ with $PbO_2$ in 0.2 N $HNO_3$ as described by George and Irvine (1954).

*Methods.* The reaction was followed at 510 mμ, where $Fe^{2+}$-*o*-$phen_3$ has an extinction coefficient of $\epsilon_m$ $11.10^3$ $M^{-1}$ $cm^{-1}$, while that of the ferric complex is negligible. Reactions were started by the addition of DPNH to the ferric complex diluted in the desired buffer.

*Spectroscopic* measurements were made with Cary 15 or Beckman DB instruments at room temperature.

*Thin-layer chromatography* was employed to identify $DPN^+$ in the reaction mixture. Silica G plates (0.25 mm) were used, and they were developed with $H_2O$. DPNH was detected by its fluorescence under ultraviolet illumination. $DPN^+$ was detected in the same way after spraying with 1 M NaOH and heating for 15 min.

## Results

*The stoichiometry* and the extent of the reaction were studied by reacting ($Fe_2$-*o*-$phen_4(OH)_2)^{4+}$ and DPNH at various ratios and measuring the amount of $Fe^{2+}$-*o*-$phen_3$ produced. The results are summarized in Table I

TABLE I: The Stoichiometry of the Reduction of ($Fe_2$-*o*-$phen_4(OH)_2)^{4+}$ by DPNH.[a]

| ($Fe_2$-*o*-$phen_4$-$(OH)_2)^{4+}$ (μM) | DPNH (μequiv/l.) | (Fe-*o*-$phen_3)^{2+}$ (μM) |
|---|---|---|
| 1000 | 97 | 98 |
| 500 | 97 | 95 |
| 250 | 97 | 99 |
| 200 | 97 | 97 |
| 100 | 97 | 95 |
| 50 | 97 | 82 |
| 25 | 97 | 46 |

[a] DPNH (48 μM) was oxidized by increasing amounts of ($Fe_2$-*o*-$phen_4(GH)_2)^{4+}$. The reaction was carried at 25°, 0.1 M malate buffer (pH 4.5).

and indicate that all of the DPNH is consumed in the reaction and that ferrous iron is produced in an equivalent amount to the DPNH added to the reaction mixture.

*The Kinetics of the Reaction.* It was found that the velocity of the reaction was dependent upon the concentration of both reactants. In Figure 1a,b, the log of the initial velocity is drawn against the log of concentration of one reactant, while the concentration of the other is kept constant. The straight lines obtained in both cases with a slope of 1 suggest that the over-all reaction would be of second-order kinetics. However, as seen in Figure 2, the experimental results fitted first-order kinetics even when both reactants were at nearly equimolar concentrations. We conclude that the rate-limiting step in the

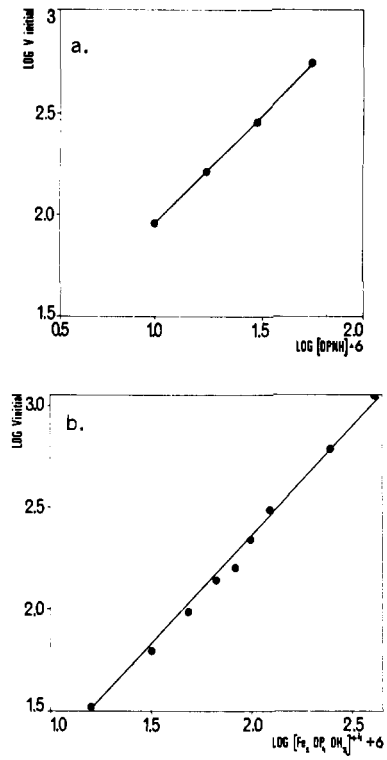



FIGURE 1: Initial velocity data. (a) The variation of log of initial velocity with respect to log [DPNH]. [$Fe_2$-*o*-$phen_4$-$(OH)_2]^{4+}$, 0.50 mM; 0.1 M acetate buffer (pH 4.25). (b) The variation of log of initial velocity with respect to log [$Fe_2$-*o*-$phen_4(OH)_2]^{4+}$. DPNH, 69 μM; 0.1 M acetate buffer (pH 4.5).

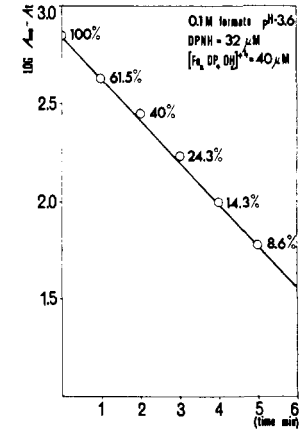



FIGURE 2: Logarithmic plot of $A_\infty - A_t$ at 510 mμ *vs.* time. [$Fe_2$-*o*-$phen_4(OH)_2]^{4+}$, 40 μM; [DPNH], 32 μM; 0.1 M formate buffer (pH 3.6). At each point the percentage of unreacted DPNH is indicated.

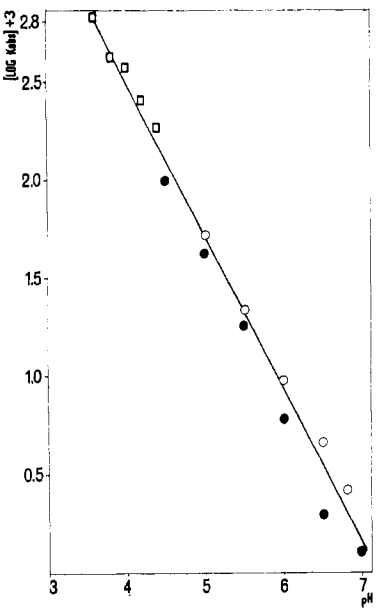



FIGURE 3: The pH dependence of log $k_{obsd}$ in 0.1 M formate, acetate, and malate buffers. (○) $[Fe_2\text{-}o\text{-}phen_4(OH)_2]^{4+}$, 50 μM; [DPNH], 212 μM. (●) $[Fe_2\text{-}o\text{-}phen_4(OH)_2]^{4+}$, 185 μM; [DPNH], 56 μM. (□) $[Fe_2\text{-}o\text{-}phen_4(OH)_2]^{4+}$, 225 μM; [DPNH], 45 μM.

over-all reaction is not the formation of the complex between the reacting molecules, but the electron transfer that occurs within the complex itself.

The effect of pH on over-all reaction velocity is shown in Figure 3. The logarithm of the observed rate constant is linearly dependent upon pH, with a slope of −0.6. The observed first-order rate constant of the over-all reaction was found to increase with the concentration of $(Fe_2\text{-}o\text{-}phen_4(OH)_2)^{4+}$. The shapes of the concentration dependence plots are similar to those of saturation curves (Figure 4).

*The Product of DPNH Oxidation.* A direct determination of $DPN^+$ in the reaction by enzymic reduction was unreliable because of the inhibitory effect of *o*-phenanthroline on alcohol dehydrogenase.

Thin-layer chromatography revealed in the reaction products a spot with fluorescence and $R_F$ value similar to that of marker $DPN^+$. After extraction of the spot, enzymic reduction, and correction for the recovery of the marker $DPN^+$, the amount of $DPN^+$ detected in the products was about 70% from that of DPNH added.

*The Reduction of the $Fe^{3+}$-o-phen$_3$ by DPNH.* It was found that the addition of DPNH to a solution of $Fe^{3+}$-*o*-phen$_3$ resulted in the instantaneous reduction of the iron, evidenced by the change in color observed. The amount of ferrous ions formed upon addition of a known amount of DPNH, was estimated colorimetrically, and it was found to correspond to a stoichiometry of 2 iron equivs reduced/mole of added DPNH.

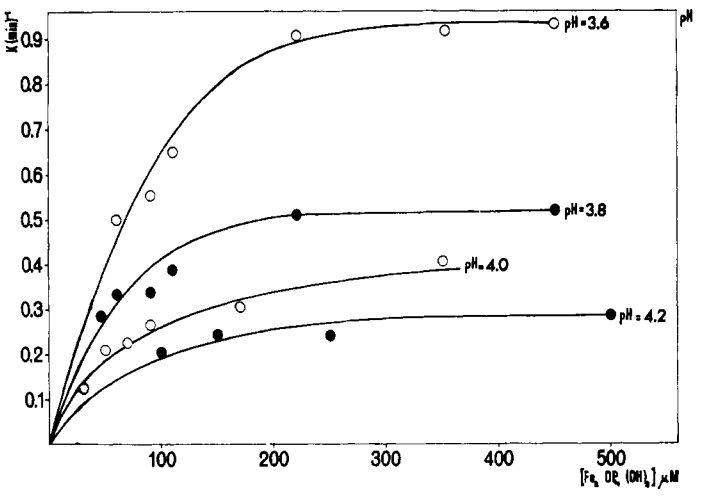



FIGURE 4: The variation of $k_{obsd}$ with respect to the initial concentration of $[Fe_2\text{-}o\text{-}phen_4(OH)_2^{4+}$, measured at different pH's; 0.1 M formate and acetate buffers; [DPNH], 50 ± 5 μM.

## Discussion

It was shown in the preceding paper (Gutman *et al.*, 1968) that the oxidation of DPNH by ferric ions is preceded by the very rapid formation of a DPNH–$Fe^{3+}$ complex. In this investigation, the $Fe^{3+}$-*o*-phenanthroline complex was used as a molecule that can accept simultaneously two electrons, each of them at a different site. The structure of bisdiferric tetra-*o*-phenanthrolinediol is adequate for this purpose, since it consists of two iron atoms joined by a double–OH bridge (Gaines *et al.*, 1936). This provides an acceptor site small enough to accommodate a single reducing molecule of DPNH in an environment able to accept two electrons.

By analogy with the reaction between DPNH and ferric ions in solution (Gutman *et al.*, 1968), the suggestion can be made that the complex, $Fe^{3+}$-*o*-phenanthroline–DPNH, is an intermediate species in the reaction under consideration. The kinetics of the reaction are in keeping with this assumption, as it will be shown below. We have been unable to detect the presence of such a complex by direct measurement of a physical property, and, therefore, other possible kinetic pathways should not be disregarded.

There is a large difference in the rate of reduction of the ferric dimer and that of the monomer, $Fe^{3+}$-(*o*-phen)$_3$, the latter being reduced instantaneously. The electronic configuration of the iron in the two complexes differs by the fact that the monomer is low spin, while the dimer is high spin (Mulay and Hofmann, 1966). The product of the reaction, in both cases, is the low-spin complex, $Fe^{2+}$(*o*-phen)$_3$. Thus, in the reduction of the dimer, two factors act decreasing the rate of the reaction: first, the rearrangement of the electronic configuration, and second, the breakage of the dimer bonds.

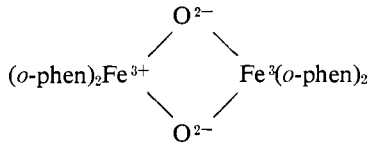
The stoichiometric measurements described above indicate that two electrons are transferred from DPNH to ferric ions under all conditions, even when the reactants are present in equimolar concentrations. This is to be contrasted with the abnormal stoichiometries observed in the reaction of DPNH with free ferric ions under similar conditions (Gutman *et al.*, 1968). Thus, while free ferric ions are one-electron acceptors, the ferric dimer complex is a two-electron acceptor.

The transfer of the reducing electrons to the ferric

ions orbitals can take place through different paths: through the $\pi$-electron system of the *o*-phenanthroline ligand, through the hydroxyl bridges, or directly after displacement of either one of them.

Transfer through the *o*-phenanthroline ligand appears implausible in this case, because the reaction is much slower than that observed when DPNH is added to the highly reactive $Fe^{3+}$-*o*-$phen_3$ complex, where such a path is obligatory. Transfer through the $OH^-$ ligands is very unlikely, since this ligand cannot act as an electron acceptor. Displacement of some of the ligands appears therefore a most probable alternative.

The affinity of *o*-phenanthroline for $Fe^{3+}$ is so large, that it appears unlikely that this is the displaced ligand. Furthermore, the effect of pH on the observed rate constants indicate that ionization of the reactants play a role in the determination of the over-all rate. Now, within the pH region of our experiments, neither the ligand, *o*-phenanthroline, nor the reduced nicotinamide moiety undergo ionization but instead, the two $OH^-$ groups loose their protons with p*K*'s 4.3 and 6.4 (Gaines *et al.*, 1936) becoming oxo bridges




If we assume that only the molecules containing hydroxyl bridges react with DPNH, the hydrogen ion dependence of the observed rate constants should parallel the concentration of hydroxyl groups in the ferric dimers; thus, log $k_{obsd}$ *vs.* pH should result in linear plots. This was actually observed (Figure 3).

The total concentration of hydroxyl groups in the ferric dimer species at various pH values can be estimated from the known p*K* values, using the expressions (see Taqui-Khan and Martell, 1967)

$$[Fe_2(o\text{-phen})_4(OH)_2] = \frac{[Fe]\ (total)}{1 + \frac{K_1}{[H^+]} + \frac{K_2K_1}{[H^+]^2}}$$

$$[Fe_2\text{-}o\text{-}phen_4(O)OH] = \frac{Fe\ (total)}{1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]^2}}$$

where $K_1$ and $K_2$ are the first and second ionization constants given by Gaines *et al.* (1936). In the case under consideration, the sum: $2(Fe_2^{3+}(o\text{-phen})_4(OH^-)_2) + (Fe_2^{3+}(o\text{-phen})_4(OH^-)(O^{2-})$ varies linearly with pH, between pH 3 and 7, with a slope of $-0.41$. This is indeed close to the slope measured in Figure 3, $-0.6$. The difference between the two slopes indicates that other pH-dependent factors operate on $k_{obsd}$, this point will be discussed below.

The effect of the ionizations of the bridging hydroxyls is revealed also in another property of the system: the concentration of the ferric dimer for which the initial velocity is half-maximal. For each pH, the rate of the reaction is proportional to the degree of saturation of DPNH by the hydroxyl-containing ferric complexes.

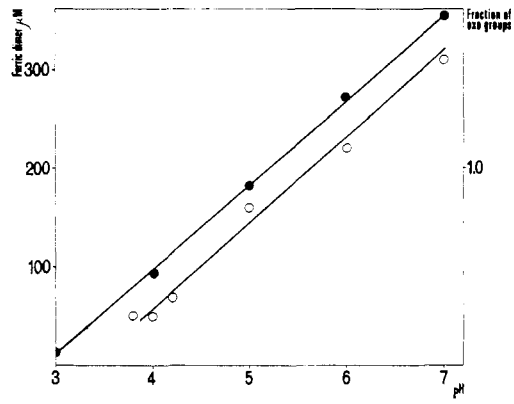



FIGURE 5: The concentrations of ferric dimer giving half-maximal initial velocity at different pH (O—O). [DPNH] (50 ± 5 μM) in 0.1 M formate or acetate buffers. Relative concentration of oxo groups (●—●).

As the relative concentration of oxo groups increases, the concentration of ferric dimer complexes needed to obtain half-maximal velocity should also increase. This behavior is exemplified by the parallel lines shown in Figure 5. One of them represents the concentrations giving half-maximal velocities of various pH values, while the other represents the relative concentration of oxo groups in the complexes.

The way in which the observed rate constants depend upon the concentration of the various reactants also indicates that the rate-limiting step of the over-all mechanism is the intramolecular oxidation–reduction of an intermediate complex.

For a mechanism of the type

$$A + B \underset{k_2}{\overset{k_1}{\rightleftharpoons}} C \xrightarrow{k_3} \text{products}$$

in which the measured parameter is the appearance of products, and the conversion of C into products, is the rate-limiting step, it can be written (Czerlinski, 1966) as

$$k_{obsd} = \frac{k_3}{1 + \frac{k_2}{k_1}\frac{1}{C_0^1}} \qquad (1)$$

where $C_0^1$ is the initial concentration of the reactant in excess, namely, of A or B.

In these studies, the reactant in excess was in all cases the ferric dimer. From eq 1, a plot of $1/k_{obsd}$ *vs.* 1/ferric dimer should be linear, with intercept $1/k_3$ and slope $k_2(k_1k_3)^{-1}$. Linear plots of this type were obtained from the experimental data of Figure 4, at different pH values and they are shown in Figure 6.

The values of $k_3$ and $k_2/k_1$ are estimated from Figure 6 and listed in Table II.

The variation of $k_3$ with pH may explain the difference between the pH dependences of $k_{obsd}$ and of the total amount of hydroxyl-containing fractions indicated

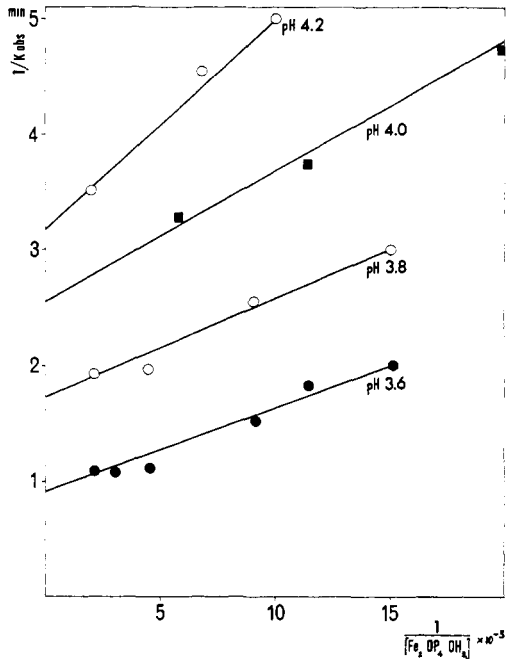
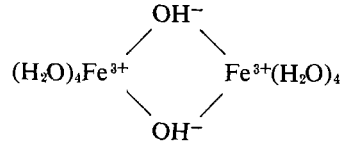



FIGURE 6: The variation of $1/k_{obsd}$ *vs.* $1/[Fe_2$-*o*-$phen_4(OH)_2]^{4+}$ at different pH. Data obtained from Figure 4.

above, since the data given in Figure 3 are not based on $k_3$ but on $k_{obsd}$.

The binding of DPNH to the ferric dimer most probably involves the nicotinamide ring nitrogen, since it is followed immediately by electron transfer. For steric reasons, it is impossible that a second bond should be formed, involving the adenine nitrogens. Thus, if a complex is formed, only one hydroxyl bridge is broken, the other remaining intact. In this situation, the coplanarity of the ferric atoms and the bridge, should cause overlapping of identical d orbitals of the metal, facilitating rapid electron transfer between them. A configuration of this type was suggested already for the ferric *o*-phenanthroline dimer (Gaines *et al.*, 1936), on the basis of the anomalous magnetic properties of the complex, and by Hedström (1953) for a hydroxyl-bridged, aquated ferric dimer.




Therefore, one interesting possibility is that the reduction of the ferric dimer occurs by two consecutive one-electron transfers. In the first, the DPNH-bound iron is reduced, but it donates the newly gained electron to the vicinal ferric ion, and becomes available for the second electron that is transferred by the $DPNH^+$ free radical. A mechanism including metal-to-metal electron transfer was suggested by Beinert and Hemmerich (1965) for the participation of nonheme iron in metalloflavoenzyme catalysis. On the other hand, inspection of a space-filling model of $(Fe^{3+})_2$(*o*-phen)$_4$(DPNH) shows that there is enough proximity between the nicotinamide ring and the two metal atoms to enable simultaneous transfer of two electrons. In both cases, this model provides a mechanism for splitting the electron pathways at the nonheme iron site of a metalloflavoprotein in the respiratory chain.

TABLE II: The Values of $k_3$ and $K_{diss}$ Estimated from Figure 6 at Various pH.

| pH | $k_3$ $(min)^{-1}$ | $K_{diss}$ $(M^{-1})$ |
|---|---|---|
| 3.6 | 1.1 | $77 \times 10^{-6}$ |
| 3.8 | 0.6 | $53 \times 10^{-6}$ |
| 4.0 | 0.4 | $46 \times 10^{-6}$ |
| 4.2 | 0.3 | $60 \times 10^{-6}$ |

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