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Kinetics of Reduction of Horse-Heart Ferricytochrome c by Catechol

Sir:

Ferricytochrome *c* (cyt *c*(III)) has been the subject of numerous studies employing a broad spectrum of reductants which have been selected to probe the mechanism of electron transfer to the ferriprotein. Rate and activation parameters have been determined for the reduction of cyt *c*(III) by relatively simple reductants such as Cr_{aq}^{2+}, $Ru(NH_3)_6^{2+}$, e_{aq}^-, $Fe(EDTA)^{2-}$, ascorbate, superoxide anion, and dithionite.[1] On the other extreme, studies of electron transfer to cyt *c*(III) in enzymatic systems[2] and even from cyt *c*(II)[3] have been undertaken. There is, however, a paucity of information pertaining to the mechanism of reduction of cyt *c*(III) by mild, multielectron reductants such as catechol (1,2-dihydroxybenzene) and catechol derivatives (e.g., 3,4-dihydroxyphenylalanine and epinephrine).[4] Although such studies may not have direct biological significance,[5] they should shed light on the mechanism of electron transfer in cytochrome systems.

This report summarizes the results of a series of experiments designed to examine possible pathways for electron transfer from catechol to ferricytochrome *c*. The rate expression for electron transfer contains terms which are first and second power in $[HC^-]$ and [cyt *c*(III)], respectively, rate = $d[\text{cyt } c(II)]/dt = k[\text{cyt } c(III)]^2[HC^-]$, where HC^- represents catechol anion and $k = (4.51 \pm 0.12) \times 10^7\ M^{-2}\ s^{-1}$.

All experiments were conducted under nitrogen at 25.0 °C, $\mu = 0.10$(NaCl) and pH ranging from 7.53 to 6.02 (phosphate buffer).[6] Concentration of catechol was at least 200-fold mole excess in all runs. Kinetics data were obtained by recording absorbance vs. time for the appearance of the 550-nm band of cyt *c*(II). A few experiments were conducted by attempting to monitor absorbance changes at 556.5, 542, and 526.5 nm, isosbestic points in the cyt *c*(II)–cyt *c*(III) spectra. No change in optical density was observed at any of these wavelengths during the course of reduction, suggesting that any intermediates on the reaction pathway exist in low concentrations.

The results of the kinetics experiments appear in Table I. In spite of the large excess [catechol], linear plots of $-\ln (A_\infty - A_t)$ vs. time[7] were never obtained. Rather, plots of $1/(A_\infty - A_t)$ vs. time were always linear (for at least 85% reaction, Figure 1), implying a rate law with a second-order dependence on [cyt *c*(III)], the reactant in limiting concentration,

$$\text{rate} = d[\text{cyt } c(II)]/dt = k_{obsd}[\text{cyt } c(III)]^2$$

where k_{obsd}[8] is some function of [catechol] and $[H^+]$, and k_{obsd} is a pseudo-second-order rate constant. Plots of k_{obsd} vs. [catechol] at each selected pH were linear, however, demonstrating the first-order dependence of the reaction rate on [reductant]. Indeed, constant values of $k' = k_{obsd}/[\text{catechol}]$ were obtained at each pH and these values also appear in Table I.

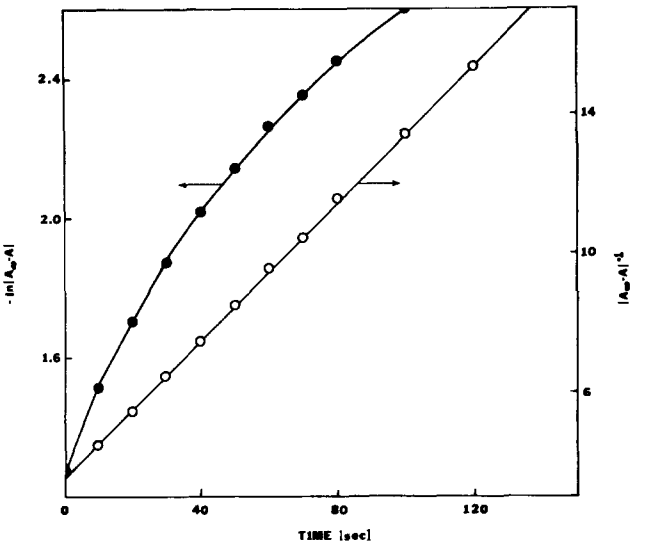

Figure 1. Plots of $-\ln (A_\infty - A)$ vs. time (filled circles) and $(A_\infty - A)^{-1}$ vs. time (open circles) for a representative run. [Catechol] = 1.53×10^{-2} M, [cyt *c*(III)] = 7.57×10^{-6} M, pH 7.01 (phosphate), μ = 0.10 (NaCl), λ 550 nm, and T = 25.0 °C.

Table I. Kinetics Data for the Reduction of Ferricytochrome *c* by Catechol (25.0 °C, μ = 0.10 (NaCl))

pH	10^2[catechol],[a,b] M	$10^{-3}\ k_{obsd}$,[c] $M^{-1}\ s^{-1}$	$10^{-5}\ k'$,[d] $M^{-2}\ s^{-1}$
7.53	0.589	5.11	8.67
7.53	1.14	10.0	8.77
7.53	2.19	19.4	8.86
7.53	4.02	34.7	8.63
		at pH 7.53 $\bar{k}'$ =	8.73 ± 0.10
7.01	0.411	0.992	2.41
7.01	0.806	1.79	2.22
7.01	0.999	2.21	2.21
7.01	1.91	4.26	2.24
7.01	2.89	7.36	2.55
7.01	3.49	8.73	2.50
		at pH 7.01 $\bar{k}'$ =	2.36 ± 0.15
6.54	1.04	0.747	0.718
6.54	1.49	1.05	0.705
6.54	1.90	1.36	0.716
6.54	3.55	2.56	0.721
		at pH 6.54 $\bar{k}'$ =	0.715 ± 0.007
6.01	2.32	0.670	0.289
6.01	5.47	1.62	0.296
6.01	7.39	2.09	0.283
6.01	10.0	3.08	0.307
		at pH 6.01 $\bar{k}'$ =	0.294 ± 0.010

[a] [catechol] represents total of all catechol species, HC^- and H_2C. [b] [cyt *c*(III)] = 1.80×10^{-5} M for pH 7.53 and 7.01, and 1.63×10^{-5} M for pH 6.54 and 6.02. [c] Each entry is the average of at least three runs. Values of k_{obsd} calculated as $k_{obsd} = sl(\Delta\epsilon)$, where s is the slope of a plot of $1/(A_\infty - A_t)$ vs. time, l is the pathlength (2 cm), and $\Delta\epsilon$ is the difference in molar extinction coefficients for cyt *c*(III) and cyt *c*(II) at 550 nm ($1.85 \times 10^4\ M^{-1}\ cm^{-1}$, E. Margoliash and N. Frohwirt, *Biochem. J.*, **71**, 570 (1959)). [d] k' is defined as $k' = k_{obsd}/[\text{catechol}]$.

The rate of cyt *c*(III) reduction was found to increase with increasing pH, and a linear plot of k' vs. $1/[H^+]$ was obtained. Thus the rate law for electron transfer might be expressed as

$$\text{rate} = k[\text{cyt } c(III)]^2[\text{catechol}][H^+]^{-1}$$

On the other hand, consideration of the nature of the reductant, namely, that catechol is a weak acid, suggests an alternate formulation of the rate expression. If the actual reductant is catechol anion, HC^-, the pH dependence can be explained on the basis of increasing $[HC^-]$ with decreasing acidity. That is, if rate = k[cyt c(III)]^2[HC^-], incorporation of the equilibrium constant for catechol dissociation (pK_a = 9.15)[9] and mass balance in [catechol], $[HC^-]$ = [catechol]$_{total}$ − $[H_2C]$, gives

$$\text{rate} = k[\text{cyt } c(\text{III})]^2[\text{catechol}](1 + [H^+]/K_a)^{-1}$$

for which the pseudo-second-order rate constant is

$$k_{obsd} = k[\text{catechol}](1 + [H^+]/K_a)^{-1}$$

A plot of k_{obsd}/[catechol] vs. $(1 + [H^+]/K_a)^{-1}$ should be linear with zero intercept (in the absence of contributions from catechol species other than HC^-) and slope = k. Such a plot is indeed linear, least-squares analysis giving slope = $k = (4.51 \pm 0.12) \times 10^7\ M^{-2}\ s^{-1}$ and intercept[10] = $(1.5 \pm 1.3) \times 10^4\ M^{-2}\ s^{-1}$ (errors are 1σ).

At least two mechanisms are consistent with the observed rate expression. The first involves a rapid equilibrium association of two cyt c(III) species, followed by two electron reduction of the associate by HC^-,

$$\text{cyt } c(\text{III}) + HC^- \rightleftharpoons \text{complex (rapid)}$$

$$\text{complex} + \text{cyt } c(\text{III}) \rightarrow 2\text{cyt } c(\text{II}) + \text{P (rate determining)}$$

where P represents oxidized catechol (presumably *O*-diquinone).[11]

The second mechanism involves initial complexation of cyt c(III) by catechol anion, followed by attack of a second cyt c(III) species.

$$\text{cyt } c(\text{III}) + HC^- \rightleftharpoons \text{complex (rapid)}$$

$$\text{complex} + \text{cyt } c(\text{III}) \rightarrow 2\text{cyt } c(\text{II}) + \text{P (rate determining)}$$

In either case, (1) the precursor equilibrium constants must be very small or (2) association or complexation must have no effect on the optical density of cyt c(III) to be consistent with the observed isosbestic behavior. Furthermore, the precursor equilibrium must be obtained fairly rapidly compared to the rate determining step else saturation of the pseudo-second-order rate constant would have been observed at high [catechol]. The present study does not provide sufficient information to discriminate between these possible mechanisms. The demonstrated ability of such ligands as azide, imidazole, and pyridine to bind cyt c(III) and the fairly rapid electron exchange rate in the cyt c(II)–cyt c(III) system[3] enhance the plausibility of the second mechanism.

Consideration of the nature of the reactants suggests that the kinetic behavior observed is not needlessly complex. Catechol is a two-electron reductant, whereas cyt c(III) is a one-electron oxidant. If cyt c(III) cannot oxidize catechol in a one-electron process (presumably to a semiquinone) then participation of an additional cyt c(III) as second electron acceptor site becomes necessary. The reduction of V(V), a one-electron oxidant, by the two-electron reductants ascorbic acid and catechol, appears to occur via analogous mechanisms. Thus the rate law for V(V) oxidation of ascorbic acid exhibits a first-order dependence on V(V),[12] and a radical intermediate is produced, whereas the rate expression for V(V) oxidation of catechol (a weaker reductant by ca. 0.3 V) exhibits a second-order dependence on V(V).[11]

The most striking feature of the present study is the evidence for a pair of cyt c(III) species in the encounter complex leading to products. Assuming first that catechol binds an iron center prior to cyt c(III) reduction, *direct* transfer of the second electron to the second iron center must be sterically impossible. On the other hand, catechol may transfer electrons to the metalloprotein via the exposed porphyrin edge. A pair of cationic cytochrome molecules can approach closely enough in solution for fairly rapid electron transfer.[3] An anionic HC^- species would be expected to stabilize such an interaction through formation of a 2:1 complex leading to subsequent transfer of one catechol electron to each cytochrome. In either case both electrons cannot be transferred to iron via direct transfer to the respective metal center. Further research in this area is required for the development of a clear and consistent picture of the mechanism of electron transfer to cytochrome c(III).

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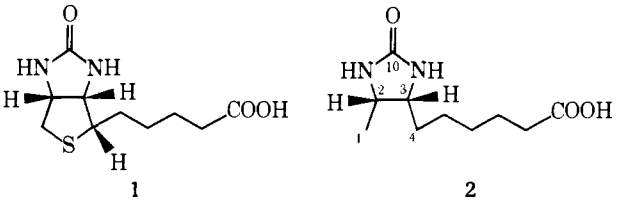
Biotin Biosynthesis. 1. The Incorporation of Specifically Tritiated Dethiobiotin into Biotin

Sir:

The vitamin (+)-biotin (**1**) is widely distributed in plant and animal tissues where it functions as the cofactor for a variety of enzymic carboxylation reactions.[1] A number of fungi and bacteria synthesize biotin from pimelic acid via a metabolic



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