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ELECTRON TRANSFER WITHIN THE CYTOCHROME *c*-CYTOCHROME *c* PEROXIDASE COMPLEX: DEPENDENCE OF THE TRANSIENT-STATE AND STEADY-STATE KINETICS ON IONIC STRENGTH

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Abstract Steady-State and transient-state kinetic studies of the cytochrome *c* peroxidase (CcP) catalyzed oxidation of ferrocytochrome *c* by hydrogen peroxide at pH 7.5 as a function on ionic strength have been carried out using both horse heart and yeast iso-1 cytochrome *c*. The ionic strength affects the apparent interaction between cytochrome *c* and the oxidized intermediates of CcP while the intracomplex electron transfer rates are essentially independent of ionic strength. The maximum enzyme turnover rates for the oxidation of yeast iso-1 and horse heart ferrocytochrome *c* are 780 ± 130 s^{-1} and 280 ± 40 s^{-1}, respectively, independent of ionic strength at pH 7.5. The intramolecular electron transfer rates from bound ferrocytochrome *c* to the heme site in CcP compound I is about 1900 s^{-1} and 450 s^{-1} for yeast iso-1 and horse heart cytochrome *c*, respectively.

Keywords: Cytochrome c *peroxidase, electron transfer, ionic strength dependence, cytochrome* c

INTRODUCTION

Cytochrome *c* peroxidase (CcP) catalyzes the oxidation of ferrocytochrome *c* by hydrogen peroxide,[1] Eq. 1.

$$2\,C^{2+} + H_2O_2 + 2\,H^+ \xrightarrow{CcP} 2\,C^{3+} + 2\,H_2O \quad (1)$$

The catalytic mechanism includes at least two oxidized enzyme intermediates[2] as illustrated in Eqs. 2-4.

$$CcP + H_2O_2 \rightarrow CcP{\cdot}I + H_2O \quad (2)$$

$$H^+ + CcP{\cdot}I + C^{2+} \rightarrow CcP{\cdot}II + C^{3+} + H_2O \quad (3)$$

$$H^+ + CcP{\cdot}II + C^{2+} \rightarrow CcP + C^{3+} \quad (4)$$

CcP·I represents compound I, the first intermediate observed in the catalytic cycle. It is oxidized two equivalents above the native Fe(III) state of the enzyme. CcP·I contains an

oxyferryl, Fe(IV), heme group and a stable free radical localized on Trp-191.[3,4] The oxyferryl group of CcP·I is reduced by ferrocytochrome *c* to produce CcP·II which retains the Trp-191 radical,[5] Eq. 3. Reduction of CcP·II is complex and not well characterized.[5]

In an effort to elucidate the kinetic mechanism of CcP catalysis and to measure electron transfer rates between ferrocytochrome *c* and the oxidized intermediates of CcP, we are conducting detailed steady-state and transient-state kinetic studies of the CcP-cytochrome *c* system.

STEADY-STATE KINETIC STUDIES

At saturating hydrogen peroxide concentrations, the steady-state initial velocity reflects the binding of ferrocytochrome *c*, conversions between enzyme-substrate and enzyme-product complexes, and product dissociation. The data for the oxidation of horse ferrocytochrome *c* at 10 and 100 mM ionic strength, pH 7.5, is shown in Fig. 1.

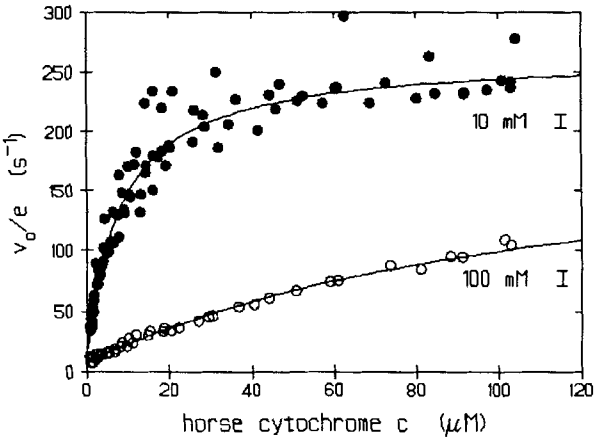

FIGURE 1 The ferrocytochrome *c* dependence of the steady-state velocity is shown at two ionic strengths. Experimental conditions: pH 7.5; 25 °C; $[H_2O_2]$ = 200 μM. Solid circles: 4.2 mM potassium phosphate buffer, 10 mM ionic strength. Open circles: 10 mM potassium phosphate with KNO_3 to adjust ionic strength to 100 mM.

The initial velocity is not a hyperbolic function of the cytochrome *c* concentration and the data were fit to the empirical equation shown in Eq. 5.

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$$\frac{v_0}{e} = \frac{n_1[C^{2+}]}{1 + d_1[C^{2+}]} + \frac{n_2[C^{2+}]}{1 + d_2[C^{2+}]} \tag{5}$$

At all ionic strengths between 10 and 100 mM, the first term in Eq. 5 appears to be saturated and only three parameters are needed to fit the data: n_1/d_1, n_2, and d_2.

The steady-state velocities for the oxidation of yeast iso-1 ferrocytochrome *c* at 10 and 100 mM ionic strength are shown in Fig. 2.

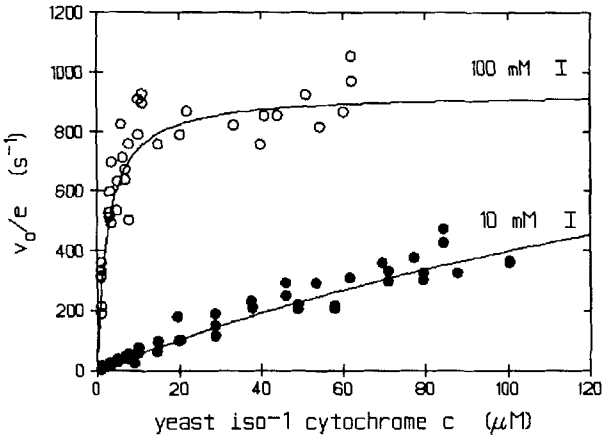

FIGURE 2 The dependence of the steady-state velocity on the concentration of yeast iso-1 ferrocytochrome *c* is shown at two ionic strengths. Experimental conditions and symbols are the same as in Figure 1.

Three parameters are required to fit the yeast cytochrome *c* data between 20 and 70 mM ionic strength, while a two parameter equation is sufficient at 10 and 100 mM ionic strength. The ionic strength dependence of the yeast cytochrome *c* data is very unusual. The data at 10 mM ionic strength do not saturate up to the limits of the cytochrome *c* concentration used in this study, 100 μM, while the data at 100 mM ionic strength reaches a limiting value above 20 μM yeast cytochrome *c*, Fig. 2. This behavior is exactly opposite to that shown by horse cytochrome *c*, Fig. 1. The apparent Michaelis constant for the second term in Eq. 5 is given by $1/d_2$. Values for $1/d_2$ increase with increasing ionic strength as expected for horse cytochrome *c*, with values of 8.3 and 120 μM at 10 and 100 mM ionic strength respectively. For yeast cytochrome *c*, values for $1/d_2$ are 160 and 2.3

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μM at 10 and 100 mM ionic strength, respectively, opposite that expected for the interaction of proteins with opposite charge. The steady-state parameters for the yeast cytochrome *c* data at 10 mM ionic strength have large errors due to the large extrapolation necessary to determine the maximum velocity.

The interpretation of the steady-state parameters depends upon the catalytic mechanism. However, we can determine the maximum enzyme turnover rate in the absence of any particular mechanism. TN_{max} is given by the sum of n_1/d_1 and n_2/d_2. The data for both horse and yeast cytochrome *c* are shown in Fig. 3. Within experimental error, TN_{max}

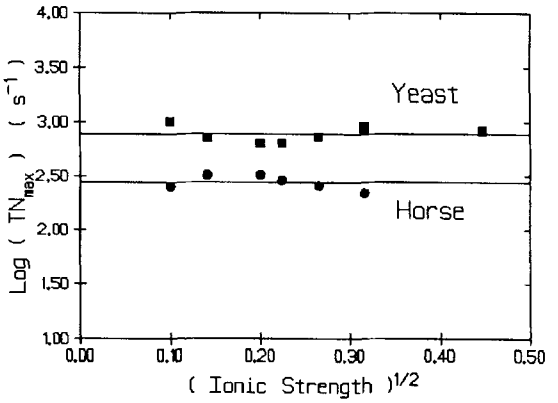

Figure 3 Enzyme turnover rate for the oxidation of yeast iso-1 and horse ferrocytochrome *c* as a function of ionic strength, pH 7.5

is independent of ionic strength. The average value for TN_{max} is 780 $\pm$ 130 s^{-1} and 280 $\pm$ 40 s^{-1} for yeast iso-1 and horse ferrocytochrome *c*, respectively.

TRANSIENT-STATE KINETICS

We have previously published the transient-state oxidation of horse heart ferrocytochrome *c* by CcP·I at pH 7.5, 10 mM ionic strength.[5] The concentration dependence of the pseudo-first order rate constant for the reduction of CcP·I by excess horse ferrocytochrome *c* is consistent with rapid complex formation between the two proteins followed by intracomplex electron transfer from ferrocytochrome *c* to the heme site in CcP·I. The electron transfer rate is 450 $\pm$ 20 s^{-1} and the apparent K_D determined from the kinetic studies is 3.3 $\pm$ 0.3 μM.

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Fig. 4 shows the observed pseudo-first order rate constant for the oxidation of yeast iso-1 ferrocytochrome *c* by CcP·I at 10 and 100 mM ionic strength. The observed rate

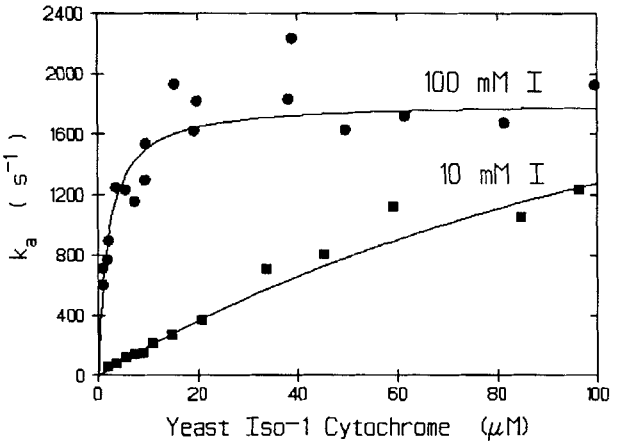

Figure 4 The observed pseudo-first order rate constants for the oxidation of yeast iso-1 ferrocytochrome *c* by CcP·I. Experimental conditions are the same as in Fig. 1.

constant is a hyperbolic function of the yeast cytochrome *c* concentration. The apparent K_D values are 150 ± 50 and 2.0 ± 0.3 μM at 10 and 100 mM ionic strength, respectively. The electron transfer rates are 3200 ± 1000 and 1800 ± 300 s^{-1} at 10 and 100 mM ionic strength, respectively.

The transient-state studies of the oxidation of yeast iso-1 ferrocytochrome *c* by CcP·I, Fig. 4, corroborates the unusual ionic strength dependence for the steady-state oxidation, Fig. 2, in which the observed rate at 100 mM ionic strength reaches a limiting value at much lower yeast cytochrome *c* concentrations than the data at 10 mM ionic strength. This suggests that a mechanism involving rapid complex formation, followed by intracomplex electron transfer, may not be appropriate for the CcP·I oxidation of yeast iso-1 ferrocytochrome *c*.

The limiting oxidation rate, at infinite yeast cytochrome *c* concentration , is independent of ionic strength within experimental error. The weighted average for the intracomplex electron transfer rate from yeast iso-1 ferrocytochrome *c* to the heme site of CcP·I is 1630 ± 140 s^{-1} for six different values between 10 and 200 mM ionic strength. This must be a minimum value since the mixing time of the stopped-flow apparatus causes

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the observed rate constants to be less than the true values for the very large rate constants observed with yeast iso-1 ferrocytochrome *c*.[6]

The rate of CcP·II reduction by horse and yeast cytochrome *c* is identical. The reduction rate is independent of cytochrome *c* concentrations between 1 and 100 μM. At low enzyme concentrations, the rate limiting step for the reduction of CcP·II is an internal electron transfer from the Fe(III) heme in CcP·II to the Trp-191 radical site with a rate of 5 s^{-1}. This is too slow to support the catalytic turnover of CcP.

CONCLUSIONS

The maximum turnover rate for the CcP-catalyzed oxidation of both horse and yeast iso-1 cytochrome *c* and the maximum rate for oxidation of yeast ferrocytochrome *c* by CcP·I are independent of ionic strength. Oxidation of horse ferrocytochrome *c* by CcP·I is consistent with rapid complex formation between the two proteins, followed by intracomplex electron transfer, suggesting that the intracomplex electron transfer rate is independent of ionic strength for the horse cytochrome *c*-CcP·I complex. Complex formation followed by intracomplex electron transfer is not consistent with the ionic strength dependence for yeast iso-1 ferrocytochrome *c* oxidation. Finally, CcP·II, formed during the transient reduction of CcP·I, cannot be an obligatory intermediate in the CcP-catalyzed steady-state oxidation of ferrocytochrome *c* by hydrogen peroxide.

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