Reductions by ferrocytochrome *c* peroxidase 4. Kinetics of yeast cytochrome *c* reduction at high buffer phosphate concentration

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Abstract

The kinetics of reduction of yeast ferricytochrome *c* from *Saccharomyces cerevisiae* by ferrocytochrome *c* peroxidase (CCP^{II}) were investigated at high ionic strength. In 200–500 mM phosphate (pH 7.0) first-order kinetics were observed with an average rate of 0.025 ± 0.003 s^{-1} at 24 ± 1 °C. Previously (*Inorg. Chem., 27* (1988) 1078; *Rev. Port. Quim., 27* (1985) 237) it was reported that the reduction of horse cytochrome *c* by CCP^{II} in $\geqslant 100$ mM phosphate also exhibited first-order kinetics with a rate of 0.02 s^{-1}. These observations are consistent with a reduction mechanism in which the rate-limiting step involves a conformation change in free CCP. The fluorescence polarization of yeast porphyrin cytochrome *c* in the presence of CCP^{III} was also examined, and the results indicate that the two proteins do not form a complex in 200 mM phosphate.

Introduction

Electron transfer between CCP** and its biological partner cytochrome *c* (C) is at present being used to probe hemoprotein mediated electron transfer [1–6]. However, the factors which control electron transfer rates in the electrostatically-stabilized, non-covalent complex formed by these two proteins are poorly understood. To identify possible electron transfer pathways, computer graphics modelling of the complex has been carried out [7, 8] using the atomic coordinates from the crystal structures of CCP [9, 10] and tuna C [11]. Optimization of the interactions between positive charges surrounding the exposed heme edge of C and complementary negative charges on the surface of CCP results in a model in which the two heme centers lie in parallel planes separated by a closest approach distance of 16.5 Å and a potential electron transfer pathway of π–π and hydrogen bonding interactions forms a bridge between the hemes.

An electrostatic model of the C/CCP complex was generated since one of the salient features of the interaction of C with its biological partners is the dependence on the ionic strength of the medium [12, 13]. The effect of high phosphate (Pi) concentration ($\geqslant 100$ mM) on the rate of reduction of horse C^{III} by CCP^{II} has been reported [14, 15]. Contrary to expectation, bimolecular kinetics were not observed at high Pi, although direct binding studies between horse C^{III} and CCP^{III} indicated that the complex dissociates at high salt [16–18]. A conformation change in CCP^{II} prior to electron transfer to C^{III} was proposed to explain the observed first-order kinetics at high ionic strength [15].

The present study examines the effects of high Pi concentration on the reaction between CCP^{II} and yeast C^{III}. Substitution of yeast for horse C should not alter the observed kinetics if the rate-limiting step involves only free CCP. The fluorescence polarization of yeast porC (i.e. the iron-free derivative of C) in the presence and absence of CCP was also investigated to directly probe C/CCP complexation at high Pi concentration.

The physiological reactions† between C and CCP involve the reduction of the enzyme intermediates, CpdI and CpdII, by C^{II}. Thus, although the reduction of C^{III} by CCP^{II} is not biologically significant, a comparison of the reactivities of yeast and horse C with the different

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**Abbreviations: CCP, cytochrome *c* peroxidase; ferrocytochrome *c*:H_2O_2 oxidoreductase, EC 1.11.1.5; C, cytochrome *c*; superscripts indicate the ferric(III) and ferrous(II) forms of these proteins; C/CCP, the non-covalent complex formed between the proteins; porC, porphyrin cytochrome *c* (i.e. the iron-free derivative of cytochrome *c*).

†CpdI (compound I) of CCP is formed by the two-electron oxidation of CCP^{III} by H_2O_2, and CpdII (compound II) of CCP is formed by one-electron reduction of CpdI [27, 28].

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oxidation states of CCP is of interest. This report is the fourth in a series which examines the redox reactivity of CCP^{II}; the reduction of horse C^{III} was the subject matter of the first two reports [14, 15], and Cheung *et al.* [3] reported rates of reduction of horse, tuna and yeast C^{III} in 1–10 mM Pi where C/CCP complex formation is extensive.

Experimental

Yeast (*Saccharomyces cerevisiae*) CCP was isolated by the published procedures [19, 20], and resonance Raman spectroscopy was used to confirm that the heme was in its native five-coordinate, high-spin state [20]. Types VI (horse heart) and VIII (*Saccharomyces cerevisiae*, mainly iso-1) cytochromes *c* were obtained from Sigma. Deamidated forms of C were separated from native C on a 1.5×60-cm CM-Sepharose Cl-6B (Pharmacia) equilibrated with 120 mM Pi (pH 7.0) for horse C and 140 mM Pi (pH 7.0) for yeast C following the procedure of Brautigan *et al.* [21]. The concentrations of the protein stock solutions were determined spectrophotometrically using millimolar absorptivities of 98 at 408 nm [22] for CCP^{III} and 106 at 410 nm [23] for C^{III}.

All reactions were carried out in sodium phosphate buffers at pH 7.0. Because of the extreme O_2 sensitivity of CCP^{II}, the peroxidase was photochemically reduced in the cuvet and C^{III} was transferred by gas-tight syringe as described previously [3]. The reduction of C^{III} was followed at 420 nm ($\Delta\epsilon = \epsilon_{red} - \epsilon_{ox} = 44\ mM^{-1}\ cm^{-1}$ for C [23]; $\Delta\epsilon \sim 0$ for CCP) using a rapid response spectrophotometer (HP model 8451 A; response time 0.1 s). The rate of CCP^{III} oxidation was followed at 440 nm ($\Delta\epsilon \sim 70\ mM^{-1}\ cm^{-1}$ for CCP; $\Delta\epsilon = 7\ mM^{-1}\ cm^{-1}$ for C [23]) to ensure that CCP^{II} oxidation and C^{III} reduction followed the same kinetics.

Horse and yeast porCs were prepared using the reported procedure [17], and stock solutions of known concentration were prepared using a millimolar absorbtivity of 160 at 404 nm [24]. Fluorescence polarization measurements were made on a Perkin-Elmer 44B spectrofluorometer as described previously [15].

Results

The kinetic trace for the reduction of ycast C^{III} by CCP^{II} in 200 mM Pi is shown in Fig. 1. The absorbance growth at 420 nm was fit to a single exponential ($R = 0.9999$) using Asystant (Asyst Software Technologies) and the experimental first-order rate constant (k_{obs}) is $0.034 \pm 0.003\ s^{-1}$. In 500 mM Pi, the kinetics of C^{III} reduction monitored at 420 nm and of CCP^{II} decay monitored at 440 nm were essentially identical to those shown in Fig. 1. Changing the ratio of C^{III} to CCP^{II} concentration did not alter the kinetics, and the average value of k_{obs} measured at high Pi was $0.025 \pm 0.003\ s^{-1}$ (Table 1).

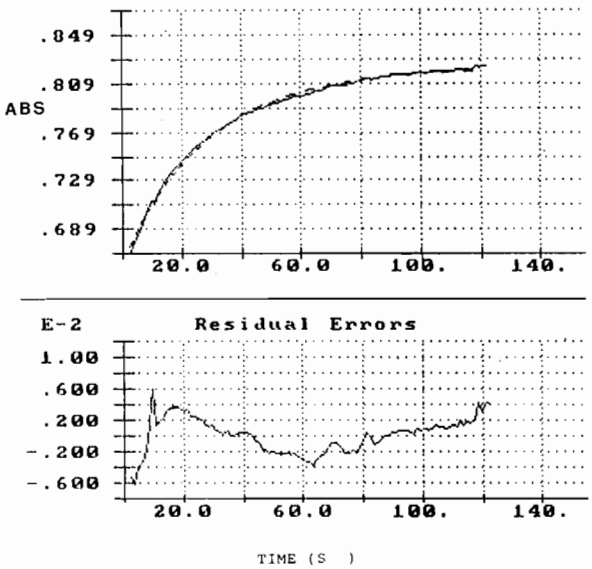

Fig. 1. Reduction of 3 μM yeast C^{III} by 3 μM CCP^{II} in 200 mM Pi buffer at pH 7.0 and 24±1 °C. The solid line is the observed absorbance increase at 420 nm vs. time due to C^{II} accumulation following rapid mixing of the proteins. The dotted line is the fit of the data to a single exponential, and the residual errors ($ABS \times 10^{-2}$) indicate the difference between the calculated and observed absorbance values.

TABLE 1. Observed first-order rate constants for the reduction of C^{III} by CCP^{II} in phosphate buffers[a]

C^{III}	[Pi] (mM)	k_{obs} (s^{-1})[b]
Yeast[c]	1[e]	3.4
	200	0.03(0.3)
	500	0.02(0.3)
Horse[d]	1[e]	0.3
	200	0.02(0.2)
	500	0.02(0.2)

[a]Experimental conditions: phosphate buffers, pH 7.0, 24±1 °C. [b]The numbers in brackets are the errors in the rate constants in units of the last digit. [c]Yeast C from *Saccharomyces cerevisiae* (iso-1). [d]Data for reduction of horse C^{III} from refs. 14 and 15. [e]Data for reduction of C^{III} at 1 mM Pi from ref. 3.

Table 2 gives the results of the fluorescence polarization measurements on the porCs in the presence and absence of CCP^{III}. The polarization of free horse porC is $\sim 0.138 \pm 0.007$ between 5 and 200 mM Pi, similar to the value published previously [15]. In the absence of CCP^{III}, the Soret absorption band of yeast porC broadens considerably in 5 mM Pi and its polarization varies with time, indicating that free yeast porC is unstable at low ionic strength. However, in the

TABLE 2. Observed polarization of yeast and horse porCs in the presence and absence of CCP^{III}[a]

PorC	[Pi] (mM)	Polarization[b, c]	
		$+CCP^{III}$	$-CCP^{III}$
Horse	5	0.210	0.132
	200	0.139	0.146
Yeast	5	0.204	[d]
	200	0.137	0.147

[a]Measurements were carried out in sodium phosphate buffers, pH 7.0, at 24 ± 1 °C. The porC concentrations were ~3 μM and ~3 μM CCP^{III} was present in the $+CCP^{III}$ solutions. Horse and yeast porCs were prepared from horse heart and *Saccharomyces cerevisiae* Cs. [b]The excitation and emission wavelengths were 515 and 620 nm, respectively, and the G factor, which corrects for the polarization response of the detector system, was 0.48. A 570-nm cut-off filter was placed at the emission slit to block scattered light. [c]The deviations in the observed polarizations are ≤5%. [d]Yeast porC is unstable at low ionic strength in the absence of added CCP^{III}.

presence of CCP^{III} or in 200 mM Pi, the polarization value obtained for yeast porC is the same within experimental error as that for horse porC, as expected for two proteins of similar shape and molecular weight. Also in the presence of CCP^{III}, the observed polarization values for yeast and horse porCs are significantly higher in 5 mM Pi, but drop to the free porC values in 200 mM Pi. Thus, the presence of CCP^{III} does not alter the rotational correlation time of the porCs in high phosphate, indicating that the proteins are not complexed in 200 mM Pi.

Discussion

The reaction investigated in the present study is

$$C^{III} + CCP^{II} \longrightarrow C^{II} + CCP^{III} \qquad \Delta E° = 0.45\ V \qquad (1)$$

where C is from *Saccharomyces cerevisiae* and $\Delta E°$ was calculated from $E°$ for C^{III}/C^{II} [25] and $E°$ for CCP^{III}/CCP^{II} [26]. First-order kinetics are observed at 200 and 500 mM Pi indicating that bimolecular association of the proteins is not involved in the rate-limiting step. Furthermore, the observed rate constants (k_{obs}) are similar to those observed for horse C^{III} reduction, and the values for both cytochromes *c* are listed in Table 1. The lack of dependence of k_{obs} on the sequence of C corroborates the high salt mechanism proposed previously for the reduction of horse C^{III} by CCP^{II} [15]

$$CCP^{II} \underset{k_2\ (\text{slow})}{\overset{k_1}{\rightleftharpoons}} {}^*CCP^{II} + C^{III} \underset{k_4\ (\text{fast})}{\overset{k_3}{\rightleftharpoons}} C^{III}/{}^*CCP^{II} \xrightarrow[(\text{fast})]{k_5} C^{II}/CCP^{III} \qquad (2)$$

The rate-limiting step in eqn. (2) was assigned to the slow conversion of CCP^{II} to $^*CCP^{II}$, which rapidly reduces C^{III} on the formation of a transient $C^{III}/^*CCP^{II}$ complex. Thus, this scheme predicts kinetics that are independent of the sequence and concentration of the cytochrome as observed here, and also that $k_{obs} = k_1$.

The fluorescence polarization of porC increases on binding to CCP [15] because the observed polarization depends on the rotational time of the fluorophore. Since the polarization decreases to the free porC values in 200 mM Pi (Table 2), the fluorescence results indicate that neither cytochrome forms a complex with CCP at high salt. This is consistent with a C^{III} reduction mechanism at high Pi that involves a rate-limiting conformation change in free CCP^{III}.

In the CCP enzymic cycle, a ferryl heme ($Fe^{IV}=O$) and a protein cation radical are formed (CpdI) when H_2O_2 binds to the heme [27, 28]. The enzyme is converted to its resting form by the oxidation of two C^{II} molecules. Using flash photolysis, Hazzard *et al.* [1, 29, 30] followed the kinetics of electron transfer from horse and yeast C^{II} to CpdI in Pi buffers and proposed the following scheme

$$CpdI + C^{II} \overset{K}{\rightleftharpoons} C^{II}/CpdI \xrightarrow{k_t} C^{III}/CpdII \qquad (3)$$
$$\Delta E° \sim 1\ V$$

At high salt concentrations the reduction of CpdI by C^{II} clearly exhibited bimolecular kinetics [1, 29]. Hence, both yeast and horse C^{III} reduction by CCP^{II} follow first-order kinetics under conditions where the oxidation of C^{II} by CpdI is bimolecular. Therefore, the slow conformation change in the peroxidase proposed here (eqn. (2)) is not required for CpdI reduction by C^{II}. This indicates that the mechanism of electron transfer between C and CCP at high salt is highly dependent on the oxidation level of the peroxidase.

It is also of interest to examine the effects of ionic strength on the rates of electron transfer between the different redox forms of C and CCP. From the data in Table 3 it appears that formation of electrostatically-stabilized complexes impedes electron transfer from C^{II} to CpdI at low ionic strength. However, the non-physiological electron transfer between horse and yeast

TABLE 3. Literature values for the first-order rate constants for intracomplex electron transfer from C^{II} to CpdI of CCP[a]

C^{II}	Ionic strength (mM)	k_t (s^{-1})[b]
Horse	8	730
Horse	30	3300
Yeast	8	150
Yeast	260	1460[c]

[a]From refs. 1, 29 and 30; Pi buffers, pH 7. [b]See eqn. (3) in the text. [c]This value was determined using excess CpdI where the onset of rate saturation was observed [29].

C^{III} and CCP^{II} is slowed down by factors of 10 and 100, respectively, in high Pi (Table 1). Also, yeast and horse C show opposite electron-transfer reactivities with CCP^{II} and CpdI at low salt since yeast C^{III} is more reactive with CCP^{II} (Table 1), whereas horse C^{II} is more reactive with CpdI (Table 3) under these conditions. Obviously, there are structural differences between the C^{III}/CCP^{II} and C^{II}/CpdI complexes formed at low ionic strength that control intracomplex electron transfer reactivity. These results also support the cytochrome-specific conformational responses of CCP^{III} to complexation with horse and yeast C^{III} observed by resonance Raman spectroscopy [31].

Finally, it is of note that no kinetic evidence has been found for complex formation at high salt between proteins which form electrostatically-stabilized complexes. The kinetics of reduction of horse C^{III} by ferrocyt c_1 are first-order and independent of ionic strength over a wide range of salt concentrations [32]. However, in the presence of the hinge protein, which is a negatively-charged subunit of cyt c_1 and speculated to be the normal C binding site, the kinetics are bimolecular at high salt. In the putidaredoxin-cyt P_{450} complex [33, 34], electron transfer also occurs via a unimolecular, intracomplex step at both high and low salt. Its X-ray structure [35] shows that, unlike CCP and other partners of C, cyt P_{450} does not possess an obvious ionic recognition site for its partner; thus, the stabilization and orientation forces in the P_{450} complex must be non-electrostatic in origin.

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