The kinetics of electron transfer between *Pseudomonas aeruginosa* cytochrome *c*-551 and its oxidase

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The redox reaction between cytochrome *c*-551 and its oxidase from the respiratory chain of *Pseudomonas aeruginosa* was studied by rapid-mixing techniques at both pH 7 and 9.1. The electron transfer in the direction of cytochrome *c*-551 reduction, starting with the oxidase in the reduced and CO-bound form, is monophasic, and the governing bimolecular rate constants are $1.3(\pm 0.2) \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 9.1 and $4(\pm 1) \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 7.0. In the opposite direction, i.e. mixing the oxidized oxidase with the reduced cytochrome *c*-551 in the absence of O_2 , both a lower absorbance change and a more complex kinetic pattern were observed. With oxidized azurin instead of oxidized cytochrome *c*-551 the oxidation of the *c* haem in the CO-bound oxidase is also monophasic, and the second-order rate constant is $2(\pm 0.7) \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 9.1. The redox potential of the *c* haem in the oxidase, as obtained from kinetic titrations of the completely oxidized enzyme with reduced azurin as the variable substrate, is 288 mV at pH 7.0 and 255 mV at pH 9.1. This is in contrast with the very high affinity observed in similar titrations performed with both oxidized azurin and oxidized cytochrome *c*-551 starting from the CO derivative of the reduced oxidase. It is concluded that: (i) azurin and cytochrome *c*-551 are not equally efficient *in vitro* as reducing substrates of the oxidase in the respiratory chain of *Pseudomonas aeruginosa*; (ii) CO ligation to the d_1 haem in the oxidase induces a large decrease (at least 80 mV) in the redox potential of the *c*-haem moiety.

Azurin and cytochrome *c*-551, two globular metalloproteins that can be extracted from the bacterium *Pseudomonas aeruginosa*, are generally regarded as the physiological electron donors for *Pseudomonas* nitrite reductase (or *Pseudomonas* oxidase) (EC 1.9.3.2), which is known to react with both O_2 (Horio *et al.*, 1958) and NO_2^- (Yamanaka *et al.*, 1961). The steady-state parameters (K_m and $V_{\max}$) obtained with either one of these two proteins as reducing substrates in the reaction of cytochrome oxidase with O_2 are very similar; moreover the accumulation of the oxidized species leads to an inhibition (product inhibition) that is comparable for the two proteins (Barber *et al.*, 1976).

A detailed analysis of the pre-steady-state events in the reaction between azurin and the oxidase has been made previously (Wharton *et al.*, 1973; Parr *et al.*, 1977). However, direct observation on pre-steady-state kinetics of the electron transfer between *Pseudomonas* cytochrome *c*-551 and its oxidase is difficult because of the very great

similarity in the absorption spectra of cytochrome *c*-551 and the haem *c* moiety of the oxidase. This similarity precluded other workers from obtaining results, at least at pH 6.6 (Wharton *et al.*, 1973). On the other hand it is self-evident that direct experimental information would be extremely useful for a complete characterization of the reaction pathway involving these three redox components of *Pseudomonas*.

In the present paper we report a successful attempt to observe directly and characterize the electron transfer between *Pseudomonas* cytochrome *c*-551 and the haem *c* moiety of the oxidase. The relevance of our findings for the overall mechanism of respiratory chain of the bacterium is briefly discussed.

Materials and methods

Cytochrome oxidase, cytochrome *c*-551 and azurin were purified from *Pseudomonas aeruginosa*

(strain N.C.T.C. 6750) by the procedure of Parr *et al.* (1976).

The proteins were more than 95% pure on the basis of spectrophotometric (Parr *et al.*, 1976) and electrophoretic analysis on 7.5% polyacrylamide gels by the method of Davis (1964). We performed the experiments only with cytochrome oxidase preparations characterized by the following absorbance ratios: $A_{410}/A_{280} = 1.15$ and $A_{640}/A_{520} = 1.1$.

Protein concentrations were determined by using the following molar absorption coefficients: oxidized azurin, $\epsilon_{625} = 3500 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Brill *et al.*, 1968); ferrocycytochrome *c*-551, $\epsilon_{551} = 28300 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Horio *et al.*, 1960); cytochrome oxidase, $\epsilon_{411}^{\text{ox}} = 282000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Silvestrini *et al.*, 1979).

The CO derivative of reduced cytochrome oxidase for the stopped-flow experiments was obtained by equilibrating the degassed protein under 0.1 MPa (1 atm) of CO in the presence of a stoichiometric amount of sodium ascorbate. For the static experiments, the same derivative was obtained by auto-reduction under CO (see Barber *et al.*, 1976).

Reduced azurin and cytochrome *c*-551 were obtained by addition of a few grains of $\text{Na}_2\text{S}_2\text{O}_4$, which was afterwards removed by using a Sephadex G-25 column equilibrated with the appropriate buffer.

In view of the known tendency of oxidized azurin to be autoreduced in alkaline solutions (Brill *et al.*, 1968), we checked that under our experimental conditions (maximum pH 9.1), no appreciable auto-reduction occurred during the time necessary to complete the experiments.

Stopped-flow experiments were performed at 20°C in a Gibson–Durrum apparatus equipped with a 2 cm observation chamber (dead time 3–4 ms); static spectrophotometry was conducted with a Cary 219 spectrophotometer.

All chemicals were of analytical grade.

Results

Reaction of *Pseudomonas* cytochrome oxidase with cytochrome *c*-551

To follow the reaction:

a first set of stopped-flow experiments was performed by mixing, under 0.1 MPa of CO, oxidized cytochrome *c*-551 with the reduced CO derivative of *Pseudomonas* cytochrome oxidase; CO was added in order to maintain the d_1 haem in the reduced form and thus eliminate any electron transfer from this site (Wharton *et al.*, 1973; Brunori *et al.*, 1975).

Work by Moore *et al.* (1980) and by Silvestrini *et al.* (1981) has shown that cytochrome *c*-551²⁺ at

$\approx 551 \text{ nm}$ undergoes a pH-induced red-shift of about 3 nm with a pK of 7.2, whereas the Soret band is essentially pH-independent. On the other hand, over the same pH range, no spectral change was observed in the reduced form of *Pseudomonas* cytochrome oxidase at the level of the absorption bands assigned to the *c* haem (M. C. Silvestrini, A. Colosimo & M. Brunori, unpublished work). To take advantage of this different pH behaviour, the initial stopped-flow experiments were performed at pH 9.1 (2% borate), where the difference in absorbance for the two *c* haems should be maximal. Under these conditions a monophasic reaction, in the millisecond time range, was observed (see Fig. 1). The amplitude of the process, reported in Fig. 2 as a kinetic difference spectrum, clearly indicates that the couple $\text{Cyt } c\text{-}551^{3+} + (c^{2+}d_1^{2+}\text{CO})_2$ is spectroscopically different from the couple $\text{Cyt } c\text{-}551^{2+} + (c^{3+}d_1^{2+}\text{CO})_2$ even in the Soret region. The maximum amplitude of the process corresponds to a change in absorbance of approximately 4–5% of the total absorbance at 416 nm. The overall features of the kinetic difference spectrum shown in Fig. 2 were reproduced also by static differential spectrophotometry. To record the static difference spectrum a pair of double-sector absorption cells (1 cm total path length) was used. After the mixing in the sample cuvette of oxidized cytochrome *c*-551 with fully reduced CO-bound

Fig. 1. Reaction of ferricytochrome *c*-551 with cytochrome oxidase

The Figure shows the oscilloscopic trace obtained on mixing oxidized cytochrome *c*-551 with reduced CO-bound cytochrome oxidase (observed at 416 nm). The vertical scale corresponds to ΔA of 0.0054 per grid unit and the horizontal one to a sweep time of 50 ms per grid unit. The concentrations after mixing were: cytochrome oxidase (as dimer), 0.75 μM ; cytochrome *c*-551, 1.5 μM . The buffer was 2% borate, pH 9.1, and the temperature was 22°C.

Fig. 2. Kinetic difference spectra for the reaction between cytochrome *c*-551 and cytochrome oxidase

The difference spectra were calculated by subtracting the absorption values at infinite time from those at zero time as observed in the stopped-flow apparatus (light-path 2 cm). Open symbols refer to the reaction between cytochrome *c*-551³⁺ and the (*c*²⁺*d*₁²⁺CO)₂ oxidase species mixed in a 1:1 ratio in terms of *c* haem concentration, i.e.: □, 0.5 μM; ○, 1.5 μM; ◇, 4.5 μM (after mixing). Black triangles (▲) refer to the reaction between 1 μM-cytochrome *c*-551²⁺ and 0.45 μM-(*c*³⁺*d*₁³⁺)₂ oxidase species (concentrations after mixing). All values were normalized to the lowest concentrations. All other experimental conditions were as for Fig. 1. An approximate estimate of errors may be obtained by observing the scatter of data for experiments performed at the same wavelength, repeated at different concentrations of reactants.

cytochrome oxidase at equimolar concentrations (in terms of *c* haem) the difference spectrum was recorded within 5 min. Any possible complication due to autoreduction processes is excluded by the very slow appearance of these effects as observed in control experiments (*t*₁ ≈ 60 min). These experiments show that the possibility of monitoring reaction (1) is not uniquely related to the pH-dependence of the α-band of cytochrome *c*-551²⁺, but rather depends on differences in the absorption coefficients of the two cytochromes in both oxidation states.

The transients observed were assigned to the direct electron transfer from the (*c*²⁺*d*₁²⁺CO)₂ oxidase species to cytochrome *c*-551³⁺ on the basis of the following results: (a) the time course of the reaction and its dependence on the concentration of the two reactants correspond to a bimolecular process (see Table 1); (b) the total amplitude of the process at constant oxidase concentration is a function of the concentration of cytochrome *c*-551³⁺ (see Fig. 3).

The kinetic titration shown in Fig. 3 is essentially stoichiometric (1 Cyt *c*-551/1 *c* haem in the oxidase), indicating that the equilibrium constant for reaction (1) is largely in favour of the (*c*³⁺*d*₁²⁺CO)₂ oxidase species and therefore not directly accessible. This allows an analysis of the time course of the transients in terms of a second-order quasi-irre-

Table 1. Second-order rate constants for the reaction between cytochrome *c*-551³⁺ and reduced CO-bound cytochrome oxidase calculated on the basis of a second-order irreversible model from data at three different absolute concentrations (after mixing)

The number of independent experiments conducted at each concentration is indicated in parenthesis.

<i>c</i> -551 ³⁺ (μM)	(<i>c</i> ²⁺ <i>d</i> ₁ ²⁺ CO) ₂ (μM)	<i>k</i> _{on} × 10 ⁻⁷ (M ⁻¹ · s ⁻¹)
4.5	2.3	1.2 ± 0.2 (5)
1.5	0.75	1.2 ± 0.1 (4)
0.5	0.25	1.5 ± 0.1 (7)

Fig. 3. Amplitude of the reaction between oxidized cytochrome *c*-551 and reduced CO-bound cytochrome oxidase as a function of cytochrome *c*-551 concentration

The Figure shows the amplitude of the process observed in the stopped-flow apparatus by mixing 2.5 μM reduced and CO-bound cytochrome oxidase with various amounts of oxidized cytochrome *c*-551, under pure CO. The buffer was 2% borate, pH 9.1, and the temperature was 22°C. Appropriate corrections for the dead time of the instrument have been applied (for the highest cytochrome *c*-551 concentration this was found to be about 25% of the observed Δ*A*). Cytochrome *c*-551 concentrations are expressed as those after mixing.

versible process, yielding a rate constant of approx. $1.3 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ (at pH 9.1 and 20°C). Reaction (1) has been observed also at pH 7, although the absorbance changes are even smaller than at pH 9.1. Apart from other considerations, this in itself may account for the failure reported by Wharton *et al.* (1973) in observing a transient on mixing cytochrome *c*-551 with *Pseudomonas* cytochrome oxidase. Since kinetic titration experiments showed also in this case a stoichiometric reduction of cytochrome *c*-551, the time courses were analysed in accordance with a second-order quasi-irreversible model, yielding a kinetic constant of $4(\pm 1) \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$.

To obtain a complete description of reaction (1), the rate constant for the back process at both pH values would be necessary. A direct determination of this parameter is, however, not feasible, because it is impossible to obtain a stable mixed-valence CO form

of the oxidase, i.e. $(c^{3+}d_1^{2+}\text{CO})_2$, owing to complete reduction during incubation under CO (see also Parr *et al.*, 1977).

The reaction involving the electron transfer from reduced cytochrome *c*-551 to oxidized *Pseudomonas* cytochrome oxidase

has also been observed in the stopped flow upon mixing equimolar concentrations (in terms of *c* haem) of the two reactants. In this case, however, the time course of the process (which was followed, in the absence of both O₂ and CO, at pH 7 and 9) was found to be more complex. As expected (Wharton *et al.*, 1973; Parr *et al.*, 1977), a slow intramolecular electron transfer from the *c* haem to the *d*₁ haem in the oxidase follows the initial reduction of the *c* haem and accounts for most of the observed absorbance change (in no case less than 70% of the total). The wavelength-dependence of the faster process (▲ in Fig. 2) is consistent with the spectral results obtained for the reaction followed starting with the CO derivative of reduced oxidase (see eqn. 1 and above). This is in complete agreement with the assignment of the fastest event to a direct electron-transfer process between the two cytochromes. It is clear from Fig. 2 that the amplitude of this process (fast phase of reaction 2) is smaller than in the case of reaction (1) (Δ*A*_{fast} at 416 nm ≈ 1.8% of the total absorbance at pH 9.0 and 2–3% at pH 7.0). Given the value of the equilibrium constant, which is about 1 at the two pH values (see the Discussion section), the conditions used in these experiments, i.e. equal concentrations of the two reactants (which proved to be the most suitable to look at the reaction), do not allow a complete kinetic analysis to get second-order rate constants.

The *t*₁ of the fastest process observed at different reactant concentrations are summarized in Table 2.

Table 2. Half-times (*t*₁) for the faster process observed in the reaction between cytochrome *c*-551²⁺ and the *c*³⁺*d*₁³⁺ oxidase species, at different reactant concentrations (after mixing)

Experimental conditions were as follows: 0.1 M-phosphate buffer, pH 7.0, or 2% borate buffer, pH 9.1; temperature 22°C.

<i>c</i> -551 ²⁺ (μM)	(<i>c</i> ³⁺ <i>d</i> ₁ ³⁺) ₂ (μM)	<i>t</i> ₁ (ms)	pH
3.4	1.7	70	7.0
1.7	0.85	120	7.0
0.8	0.4	150	7.0
0.4	0.2	230	7.0
1.5	0.75	130	9.1

Reaction of *Pseudomonas* cytochrome oxidase with azurin

The reaction between *Pseudomonas* cytochrome oxidase and azurin has been previously investigated in both directions, at pH 7 (Parr *et al.*, 1977). To obtain information directly comparable with the process in eqn. (1) analysed above, the kinetics of the reaction of the CO derivative of reduced *Pseudomonas* cytochrome oxidase with oxidized azurin was followed at pH 9.1 by rapid-mixing experiments under conditions identical with those used for Fig. 3. The electron transfer is easily followed under these conditions, and can be analysed in terms of a single bimolecular process, showing the expected dependence on the azurin concentration. An essentially stoichiometric kinetic titration, similar to that obtained with cytochrome *c*-551 and shown in Fig. 3, indicates that the reaction between oxidized azurin and the $(c^{2+}d_1^{2+}CO)_2$ oxidase species proceeds essentially to completion. This is perfectly reasonable considering that the redox potential of azurin is, at every pH value, higher than that of cytochrome *c*-551. From these experiments a second-order rate constant of $2(\pm 0.7) \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ was calculated. The stoichiometric character of the titration with oxidized azurin impaired the determination of an equilibrium constant and thus of the redox potential of the *c* haem in the oxidase, under conditions in which the reduced d_1 haem is CO-bound.

A series of static titrations in which oxidized azurin was added to reduced CO-bound *Pseudomonas* cytochrome oxidase ($0.1\text{--}0.5 \mu\text{M}$) was at-

tempted. Although no reducing agents were added to the system, slow processes leading to reduction of both proteins occurred during the titration, thus preventing a reliable estimate of the equilibrium constant.

Kinetic experiments aimed at measuring the reaction between reduced azurin and oxidized cytochrome oxidase were performed under anaerobic conditions at pH 9.1 and 7.0, always with a large excess of azurin. Under all conditions the time course (followed at 420 nm) was biphasic, with the slower process related, in agreement with previous observations (Wharton *et al.*, 1973; Parr *et al.*, 1977), to the internal electron transfer between the haem *c* and the haem d_1 components of the oxidase. The faster phase analysed as a single pseudo-first-order process; we have therefore been unable to reproduce the observation by Parr *et al.* (1977) that the faster process is itself biphasic. However, since the variability in the amplitude of the intermediate phase described by Parr *et al.* (1977) is, according to those authors themselves, dependent on the preparations and/or on the solvent conditions, we have not pursued this matter any further.

Fig. 4(a) shows the azurin-concentration-dependence of the pseudo-first-order rate constant for the fast phase associated with the electron transfer between the two proteins (Wharton *et al.*, 1973; Parr *et al.*, 1977), at pH 9.0, from which a second-order rate constant of $1.5 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ can be calculated.

The slow process, associated with the intramolecular electron transfer between haems *c* and d_1 in the oxidase (Wharton *et al.*, 1973; Parr *et al.*,

Fig. 4. Reaction of reduced azurin with oxidized cytochrome oxidase

The reaction between reduced azurin and oxidized cytochrome oxidase was followed anaerobically in the stopped-flow apparatus at 420 nm in 2% borate buffer, pH 9.1, at 22°C. Cytochrome oxidase was $1 \mu\text{M}$ before mixing. (a) shows the dependence on azurin concentration of the pseudo-first-order rate constant of the fast phase. (b) shows the amplitudes (in 2 cm path length) of the fast phase as a function of azurin concentration.

Table 3. Redox potentials of three components of the respiratory chain of *Pseudomonas aeruginosa*

Key to references: ^aLappin *et al.* (1979), results obtained in phosphate or borate buffer, 10.1, at 25°C, ^bMorton *et al.* (1970), results obtained in phosphate buffer, 10.02, at 20°C; ^cMoore *et al.* (1980), results obtained in phosphate or glycine/NaOH buffer, 10.006, at 25°C, ^dKamen & Horio (1970), results obtained at 20°C; ^epresent work.

pH	Azurin	Redox potential (mV)	
		Cytochrome c-551	Haem c in cytochrome oxidase
7	320 ^a	255 ^b	288 ^d
		260 ^c	288 ^e
9	300 ^a	235 ^c	255 ^e

1977), is independent of both azurin concentration and pH. The value of the first-order constant, $1(\pm 0.3)\text{s}^{-1}$, compares reasonably well with that given by Wharton *et al.* (1973), and is not wholly inconsistent with the value (0.25s^{-1}) reported by Parr *et al.* (1977). From the amplitude of the fast phase as a function of azurin concentration (results obtained at pH 9.1 shown in Fig. 4b) the equilibrium constant for the reaction of reduced azurin with the oxidized c haem of oxidase was estimated to be $K = 0.28$ at pH 7 and $K = 0.16$ at pH 9.1. From these equilibrium constants the redox potential of the c haem in the oxidase was calculated at both pH values (see Table 3).

Discussion

The kinetics of electron transfer between *Pseudomonas* cytochrome c-551 and the c haem of its oxidase were followed directly, both at pH 9.1 and 7.0, by rapid-mixing experiments. The choice of the high pH for the initial attempt to observe the reaction time course was suggested by the fact that the spectrum of reduced cytochrome c-551 is different from that of the reduced c haem of *Pseudomonas* cytochrome oxidase in the α -band region (Moore *et al.*, 1980; Silvestrini *et al.*, 1981). However, the presence of a net absorbance difference also in the Soret region, that is largely pH-independent, implies that the observed effect stems from intrinsic differences between the absorption coefficients of the two c haems, obviously reflecting different environments. The observed absorbance change associated with the initial electron transfer varies with pH, and it is considerably smaller for the reaction of the reduced cytochrome c-551 with the oxidized oxidase, in view of the unfavourable equilibrium conditions.

The direct electron transfer from reduced CO-bound oxidase to oxidized cytochrome c-551 (see eqn. 1) is monophasic under all conditions; the corresponding bimolecular rate constant has a

value of $1.3(\pm 0.2) \times 10^7 \text{M}^{-1} \cdot \text{s}^{-1}$ at pH 9.1 and a value of $4(\pm 1) \times 10^6 \text{M}^{-1} \cdot \text{s}^{-1}$ at pH 7.0.

Table 3 summarizes the redox potentials for the relevant components of the respiratory system of *Pseudomonas aeruginosa* at two pH values. The values for the c haem of the oxidase were obtained from the known mid-point potential of azurin and the equilibrium constant(s) reported above for the reaction involving the oxidase in the absence of extrinsic ligands, such as CO. Therefore it is possible to show that, given the potential(s) of cytochrome c-551, the equilibrium constant(s) for the redox process between oxidized cytochrome c-551 and reduced oxidase in the absence of CO will be $K(\text{pH } 7) = 0.34$ and $K(\text{pH } 9.1) = 0.45$. These estimates are consistent with the finding that the amplitude of the faster kinetic process corresponding to eqn. (2) is greater at pH 7 than at pH 9.1.

However, these equilibrium constants, both smaller than unity, are definitely inconsistent with the titration shown in Fig. 3 for the reaction of oxidized cytochrome c-551 with the reduced oxidase in the presence of CO (eqn. 1). The straightforward conclusion is that the redox potential of the c haem of the oxidase is strongly dependent on the oxidation (and ligation) state of the d_1 haem on the same molecule. In particular, the combination of reduced d_1 haem with CO induces a large decrease in the redox potential of the c haem (ΔE at least -80mV), a form of site-site interaction between the different haems in *Pseudomonas* cytochrome oxidase. This conclusion is consistent with previous results reported for the same enzyme by Parr *et al.* (1977) and for mammalian cytochrome c oxidase by Wilson & Leigh (1972), although the underlying molecular mechanism is still matter of debate (for a review see Brunori *et al.*, 1981).

Azurin, cytochrome c-551 and cytochrome oxidase from *Pseudomonas aeruginosa* are all soluble redox proteins and thus, in principle, may all transfer electrons within the cell. A possible scheme for electron flow in the system may be indicated as shown in Scheme 1.

It has been well established (Wilson *et al.*, 1975; Silvestrini *et al.*, 1981) that electron transfer between azurin and cytochrome c-551 (reaction I) is rapid and involves a complex mechanism characterized by pH-dependent effects to which a regulatory significance has been assigned. Although steady-state kinetic experiments have shown that azurin and cytochrome c-551 are both substrates for *Pseudomonas* cytochrome oxidase (Barber *et al.*, 1976), the relative efficiency of the bimolecular electron transfer along pathways (II) and (III) was unknown. Reaction (II) has been extensively investigated by rapid mixing and relaxation methods (Parr *et al.*, 1977; Brunori *et al.*, 1975), and, in spite of some complexities, the kinetics of electron flow from

Scheme 1. Possible electron pathways in *Pseudomonas aeruginosa*

reduced azurin to the *c* haem component of the oxidase has been fully characterized. The experiments reported in the present paper, and particularly those starting from the fully oxidized oxidase, are the first direct measurements to provide information on reaction (III). The basis for the attribution of the observed kinetic process to the electron transfer between cytochrome *c*-551 and the oxidase has been outlined above. The relevant half-times for the same reaction are in the range of 0.1–0.2 s at 3.5–0.5 μM concentration, as compared with values of 0.4–0.6 s for the electron donation from reduced azurin under comparable conditions (reaction II). Therefore it appears that azurin and cytochrome *c*-551 are not equally efficient electron donors for the oxidase, although the relatively small difference observed between the two does not imply a sequential pathway of electrons in the respiratory chain of *Pseudomonas aeruginosa*.

The validity of this conclusion for considerations of the situation *in vivo* demands, however, a reliable estimate of the concentration of the three redox proteins within the cell, as well as a quantitative analysis of the steady-state kinetics in the presence of variable concentrations of all three components. These problems require further investigation.

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