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Electron Transfer between Azurin and Cytochrome *c*-551 from *Pseudomonas aeruginosa*

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The electron-transfer reaction between azurin and cytochrome *c*-551 isolated from *Pseudomonas aeruginosa* was investigated by rapid-reaction techniques. Temperature-jump studies clearly reveal two chemical relaxations, the amplitudes of which have identical spectral distributions, but relaxation times show different dependencies on reactant concentrations. Stopped-flow experiments also showed complex kinetics. A model is proposed which is consistent with the kinetic and equilibrium data obtained. The central feature of this model is the proposal that two interconvertible forms of reduced azurin exist in solution, only one of which is able to participate directly in the electron-transfer reaction with cytochrome *c*-551. Support for the hypothesis that two forms of reduced azurin exist is derived from studies on the electron-transfer reaction between azurin and *Pseudomonas* cytochrome oxidase. The possible physiological significance of such a situation is discussed.

The electron-transfer reaction between the blue copper protein azurin and cytochrome *c*-551, isolated from either *Pseudomonas fluorescens* or *Pseudomonas aeruginosa*, has been studied by a number of workers (Antonini *et al.*, 1970; Pecht & Rosen, 1973; Brunori *et al.*, 1974). Although the proteins are relatively small (the molecular weights of azurin and cytochrome *c*-551 are 16000 and 9000 respectively), electron transfer between them has been shown to be complicated. In particular, temperature-jump studies (Pecht & Rosen, 1973; Brunori *et al.*, 1974) have clearly resolved two chemical relaxations, one of which reflects a bimolecular process, and the other reflects some coupled monomolecular reaction. To date, attempts to explain this complicated behaviour have been framed in terms of the formation of a complex or complexes between azurin and cytochrome *c*-551. Brunori *et al.* (1974) have, however, excluded mechanisms in which electron transfer within any such complex gives rise to the slow largely concentration-independent relaxation seen in temperature-jump studies.

The present paper reports further investigations of this system by temperature-jump and stopped-flow techniques. On the basis of these experiments we propose an alternative model for the electron-transfer reaction between these proteins which does not involve postulating the formation of a complex. This alternative model proposes that an equilibrium exists between two forms of reduced azurin and that only one of these two forms can exchange electrons with cytochrome *c*-551. We wish at the outset, however, to stress that this model does not rule out the possibility that complexes between azurin and cytochrome *c*-551 exist in order to facilitate the known high rate of electron transfer between the two but only that such complexes cannot account for the temperature-jump data and may only be present in catalytic amounts.

The model we propose may be written formally as follows:

$$\begin{array}{ccc} & \overset{K_{12}}{\overset{k_{12}}{\rightleftharpoons}} & \\ C_r + A_o & \underset{k_{21}}{} & C_o + A_r \\ & & \Big\downarrow k_{23} \quad \Big\uparrow k_{32} \; K_{23} \\ & & A_r' \end{array} \qquad (1)$$

where C and A stand for cytochrome *c*-551 and azurin respectively and the suffixes o and r denote either oxidized or reduced protein. A_r' represents the form of azurin which is unable to participate directly in electron-transfer reactions with cytochrome *c*-551. k_{12}, k_{21}, k_{23} and k_{32} are the rate constants in the directions indicated and K_{12} and K_{23} are respectively the equilibrium constants for

the electron-transfer reaction and for the interconversion between the two forms of reduced azurin.

The results which follow will be discussed in the light of the behaviour predicted by such a model and, where necessary, contrasted with predictions derived from a model in which transitions between complexes are thought to give rise to the coupled monomolecular relaxations seen in temperature-jump studies.

Materials and Methods

The isolation procedures developed by Finazzi-Agro *et al.* (1970) for azurin and for cytochrome *c*-551 by Ambler (1963) were slightly modified as described by Parr *et al.* (1974). The concentrations were determined by using the following molar extinction coefficients; azurin $\varepsilon_{625} = 3500$ litre·mol^{-1}·cm^{-1} (Brill *et al.*, 1968); cytochrome *c*-551 (reduced) $\varepsilon_{551} = 28300$ litre·mol^{-1}·cm^{-1} (Horio *et al.*, 1960). Ferrocytochrome *c*-551 and reduced azurin were obtained by careful reduction of the oxidized proteins with a minute excess of sodium dithionite, which was afterwards removed by passage through a Sephadex G-25 column (15cm × 1cm) equilibrated with the appropriate buffer. Ferrocytochrome *c* was found to be stable against oxidation for many days at 4°C, whereas a slow oxidation of the reduced azurin was observed over the course of 2–3 days, even at 4°C.

Most of the experiments were performed in 0.1M-potassium phosphate buffer, pH7.0 although in some instances experiments were run in 0.1M-Tris–HCl, pH7.4.

Stopped-flow experiments were performed by using either an apparatus identical with that described by Gibson & Milnes (1964), fitted with 2mm and 2cm cells having dead times of 1ms and 3ms respectively, or its commercial equivalent (Durrum–Gibson, Techmation Ltd., Edgware, Middx. MA8 8JP, U.K.) equipped with a 2cm path-length cell, dead time 4ms.

Temperature-jump experiments were carried out with an instrument built by Messanlagen Gesellschaft (Gottingen, Germany). Two types of temperature-jump cell were used, either a 1cm path-length cell of 7ml capacity or a 0.5cm path-length cell holding 3ml.

Spectrophotometry was carried out with a Cary 14 instrument, modified to take the temperature-jump cell, thus permitting direct observation of the equilibrium state of the mixture.

Results

Equilibrium data

It has previously been reported (Brunori *et al.*, 1974) that static redox titrations of reduced cytochrome *c*-551 with oxidized azurin conform, over the temperature range 20–30°C, to the simple Mass–Action Law for a two-component redox system. This simple behaviour is entirely compatible with the model in eqn. (1), which predicts that the general form of the titration curve should be 'simple', that is uninfluenced by the presence of the equilibrium between A_r and A_r'. This equilibrium does, however, influence the overall measured equilibrium constant (K'), which characterizes the titration curve (see eqn. 3).

Titration experiments (Brunori *et al.*, 1974) have shown that $K' = 3.7$ at 25°C, in good agreement with previous results (Antonini *et al.*, 1970) and with expectations based on redox measurements, which show that the E'_0 value for azurin is 40mV more positive than the value for cytochrome *c*-551.

Temperature-jump experiments

Equilibrium mixtures were made by adding oxidized azurin to reduced cytochrome *c*-551 solutions. Two types of experiment were performed, (i) those in which temperature-jump experiments were carried out during the titration of reduced cytochrome *c*-551 with azurin (i.e. at fixed cytochrome *c* concentrations), and (ii) others in which the ratio of azurin to cytochrome *c* was kept constant, the mixture being diluted before each of a series of temperature-jump experiments.

As reported previously, rapid temperature perturbation of mixtures of azurin and cytochrome *c*-551 in redox equilibrium results in two chemical relaxation processes being observed (Pecht & Rosen, 1973; Brunori *et al.*, 1974). The dependence of the reciprocal relaxation times of these processes on the total azurin concentration is shown in Figs. 1(*a*) and 1(*c*). These results are in general agreement with previous findings (Brunori *et al.*, 1974; Pecht & Rosen, 1973). The rate of the fast process is linear with total azurin concentration and shows no tendency to reach a limit up to very high rates (2600s^{-1}) (see Fig. 1*b*), whereas the slow phase rapidly becomes independent of azurin concentration.

It has been stated above that the titration data conforms to a simple Mass–Action Law formulation for a two-component electron-transfer system. In temperature-jump experiments the redox system should therefore be 'jumping' between two such titration curves characterized by different K' values. Fig. 2 compares the amplitudes of the relaxations calculated on this assumption with those found in temperature-jump experiments. It is clear that the dependencies of the amplitudes on the total azurin concentration are compatible with the expectations derived from the model in eqn. (1). In addition, the relative amplitude of the two relaxation processes is invariant along the titration curve (see Table 1).

Fig. 3 shows the spectral distribution of the fast and slow components in the visible region of the spectrum. It is clear that the processes are practically equal in amplitude but opposite in sense at all wavelengths.

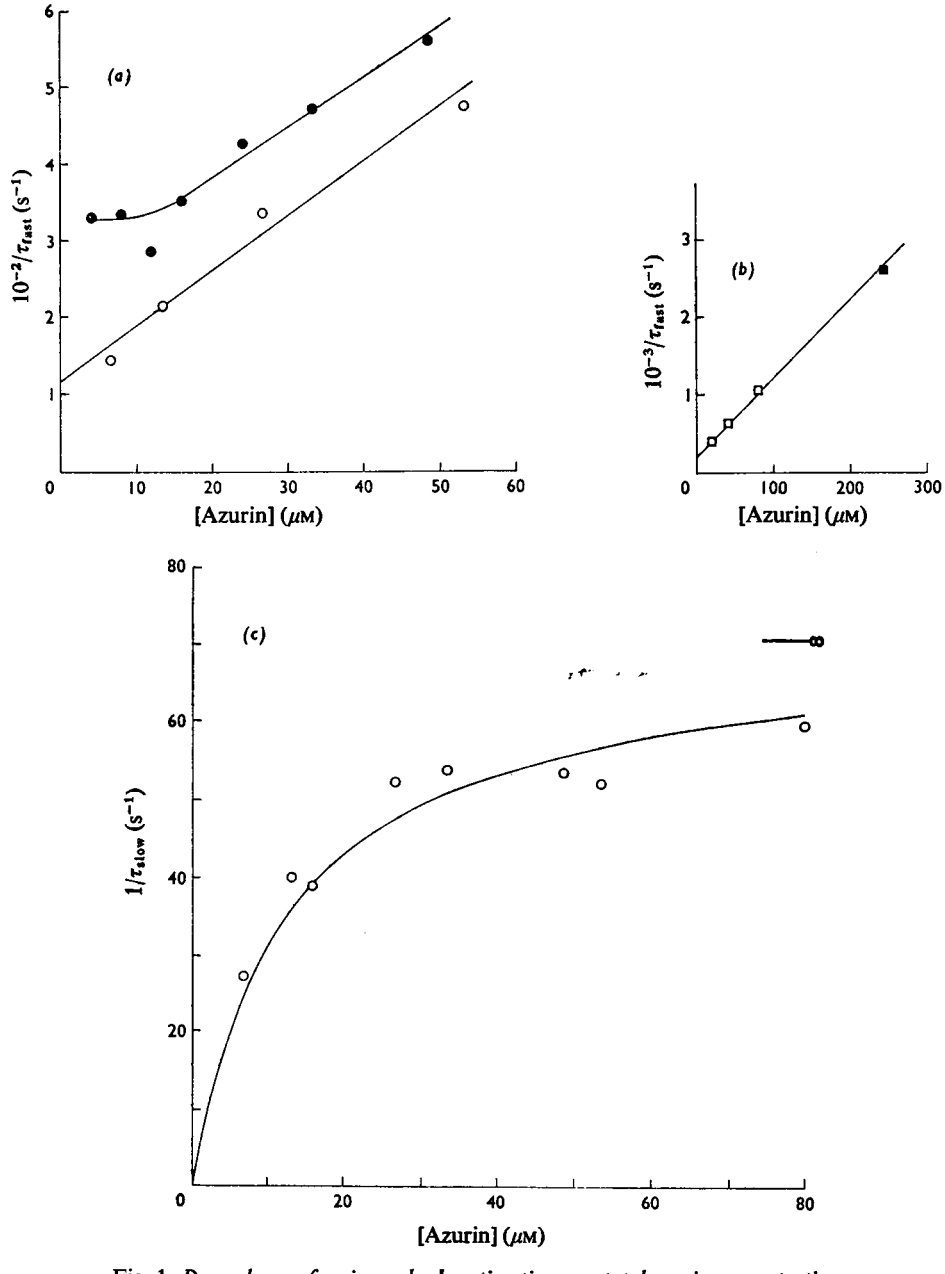

Fig. 1. *Dependence of reciprocal relaxation times on total azurin concentration*

(*a*) Dependence of the fast relaxation time on total azurin concentration; ○, experiments where a constant ratio of azurin to cytochrome *c* was maintained at 2.08; ●, experiments at a constant cytochrome *c* concentration of 16.1 μM. A 30kV discharge was applied to the 1cm path-length temperature-jump cell resulting in a temperature rise of 4.5°C from the initial value of 20°C. The monitoring wavelength was 417nm, and the experiments were conducted in 0.1M-potassium phosphate buffer, pH7.0. (*b*) Dependence of the fast relaxation time on azurin concentration over a wider range. □, Experiments at 417nm at a constant ratio of cytochrome to azurin of 2.0; ■, experiments monitored at 550nm at an azurin to cytochrome *c*-551 ratio of 2.4. A 20kV discharge was applied to a 0.5cm path-length cell resulting in a temperature rise of 6.5°C from the initial value of 20°C. All the experiments were performed in 0.1M-potassium phosphate buffer, pH7.0. (*c*) Dependence of the slow relaxation process on total azurin concentration. All conditions are as for Fig. 1(*a*). ——, Theoretical curve calculated as described in the text and approaching the line marked ∞.

Both phases exhibit, to a first approximation, the spectral properties of the difference spectrum between reduced and oxidized cytochrome *c*. However, as expected from the fact that the contribution of azurin to the distribution is not negligible, the observed isosbestic points do not correspond exactly to those expected for oxidized and reduced cytochrome *c*. These results extend the findings previously reported for the Soret region (Brunori *et al.*, 1974), from which it was concluded that both relaxation processes are observed through electron transfer between azurin and cytochrome *c*-551, thus excluding the possibility that the fast relaxation reflects the formation of a complex between azurin and cytochrome *c*-551 and the slow relaxation represents electron transfer within such a complex. On the other hand, the type of model shown in eqn. (1), in which reduced azurin exists in two forms, complies with the experimentally found restriction that there is only one type of spectral change, i.e. that associated with electron transfer, since both A_r and A_r' are colourless throughout the wavelength range explored. The two chemical relaxations found could result from the presence of the two equilibria, the fast process being associated with the electron-transfer reaction, characterized by the equilibrium constant K_{12}, and the slow process with the equilibrium characterized by K_{23}. This latter relaxation would be signalled by the fast electron-transfer reaction to which it is coupled, and would therefore have the same spectral distribution as the fast relaxation (Brunori *et al.*, 1974).

Further evidence that the slow relaxation process does not reflect slow electron transfer within a complex is given in Table 1, which shows the relative amplitudes of the two phases as a function of absolute protein concentration. It is clear that the ratio of the amplitudes is unaffected by protein concentration over the range 10–350 μM, i.e. a 35-fold change. This result conflicts with the type of scheme shown in eqn. (2).

$$C_r + A_o \overset{K_1}{\rightleftharpoons} [C_rA_o] \rightleftharpoons [C_oA_r] \overset{K_2}{\rightleftharpoons} C_o + A_r \qquad (2)$$

The symbols have the same meaning as for eqn. (1) and the terms in brackets refer to complexes within

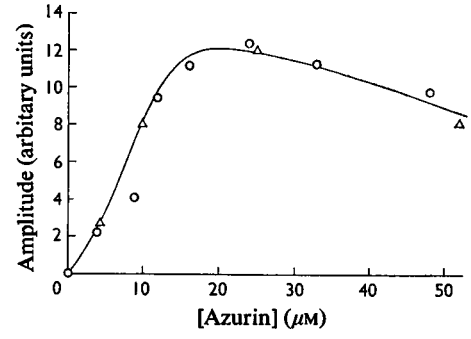

Fig. 2. *Amplitude of the relaxation processes as a function of total azurin concentration*

A 30kV discharge was applied to a 1cm path-length temperature-jump cell resulting in a temperature rise of 4.5°C from the initial value of 20°C. The cytochrome *c* concentration was 16 μM. ○, Fast relaxation process; △, total change, observed at 417nm in 0.1M-potassium phosphate buffer, pH7.0; ——, theoretical curve calculated as described in the text. Both sets of experimental points have been normalized to the line and the ordinate is thus in arbitrary units.

Table 1. *Dependence of the observed amplitude in temperature-jump experiments on protein concentration*

The experimental conditions were the same as those described in the legend to Fig. 1.

[Cytochrome *c*] (μM)	[Azurin] (μM)	Amplitude slow relaxation / Amplitude fast relaxation	λ (nm)	Temp (°C)
98	245	1.12	550	26.6
40	80	1.06	417	26.6
20	40	1.08	417	26.6
10	20	1.06	417	26.6
25.7	53.5	1.08	417	24.5
12.9	26.7	1.1	417	24.5
6.45	13.5	1.2	417	24.5
3.22	6.67	1.18	417	24.5
16.1	4	1.12	417	24.5
16.1	8	1.16	417	24.5
16.1	12	1.3	417	24.5
16.1	16.1	1.17	417	24.5
16.1	24.4	1.14	417	24.5
16.1	33	1.14	417	24.5
16.1	48	1.09	417	24.5

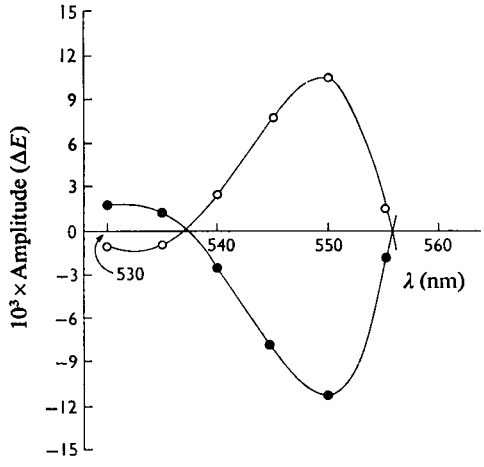

Fig. 3. *Spectral distribution of the amplitudes of the fast and slow relaxation processes in the visible region*

The 0.5cm path-length cell contained a mixture of 245 μM-azurin and 98 μM-cytochrome *c*-551 prepared by mixing reduced azurin with oxidized cytochrome *c*-551. The proteins were dissolved in 0.1 M-potassium phosphate buffer, pH 7.0, and the 20kV discharge raised the temperature by 6.5°C from the initial value of 20°C. ○, Fast relaxation; ●, slow relaxation.

which electron transfer takes place. If the slow relaxation reflects electron transfer between the partners in the complexes, application of the Law of Mass Action implies that the weight fraction of the complexes should change with the absolute concentration of the proteins. Therefore one would expect the ratio of the amplitudes of the two relaxation processes to be similarly sensitive to protein concentration. For example, assuming the two equilibrium constants for complex formation are the same (i.e. $K_1 \cdot K_2 = 1$), then under conditions in which the total concentration of azurin is equal to the total concentration of cytochrome *c*-551, the following conclusions may be drawn. (*a*) The equilibrium constants for complex-formation must be between $10^7 M^{-1}$ and $10^3 M^{-1}$, since if they were greater than $10^7 M^{-1}$ almost all the proteins would exist as complexes, whereas if the constants were less than $10^3 M^{-1}$ no complex would be present at μM protein concentration. (*b*) Between these limiting values the proportion of azurin and cytochrome *c* present as complexes depends on the absolute protein concentration, as shown in Table 2.

In our experiments the concentration of cytochrome *c* was varied between about 5 and 100 μM. If the slow relaxation was signalling electron transfer within the complexes of azurin and cytochrome, then Table 2 makes it clear that, as the proportion of protein present as complex changes over this concentration range, we should see a change in the relative amplitudes of the two relaxations. Alternatively, the model in eqn. (1) predicts no such dependence of the relative amplitudes on absolute protein concentrations, as observed (see Table 1).

Table 2. *Protein-concentration-dependence of complex concentration calculated on the basis of eqn.* (2)

See the text for details. $[X] = [C_rA_o] + [C_oA_r]$. K is the equilibrium constant for complex formation.

	Concn. of cytochomre *c*-551 (μM)		
[X] (μM)	$K = 10^6 M^{-1}$	$K = 10^5 M^{-1}$	$K = 10^4 M^{-1}$
1	3	7.3	21
10	16	30	73
100	140	160	300

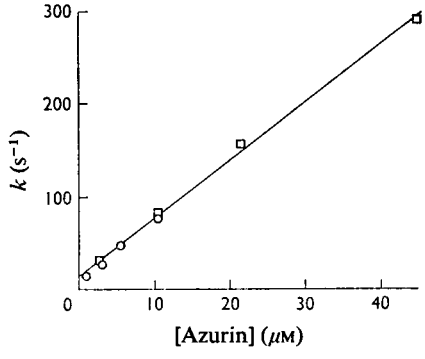

Fig. 4. *Effect of increasing azurin concentration of the reaction between oxidized azurin and reduced cytochrome c-551*

The reaction was followed at 417nm in the stopped-flow apparatus when 1 μM-ferrocytochrome *c*-551 was mixed with various concentrations of oxidized azurin. ○, Measurements performed in a 2cm path-length cell having a dead time of approx 3.5ms; □, experiments using a 2mm path-length cell with a nominal dead time of 1.5ms. All the experiments were carried out in the presence of 0.1 M-potassium phosphate buffer, pH 7.0, at 20°C.

Stopped-flow experiments

Stopped-flow experiments in which reduced cytochrome *c* was mixed with oxidized azurin yielded monophasic progress curves which corresponded spectrally to the production of oxidized cytochrome *c*. The rate of this process was linearly dependent on the azurin concentration up to high rates (Fig. 4). The second-order rate constant (k_{12} of eqn. 1) was found to be $6.25 \times 10^6 M^{-1} \cdot s^{-1}$, close to the value of $3 \times 10^6 M^{-1} \cdot s^{-1}$ found by Antonini *et al.*

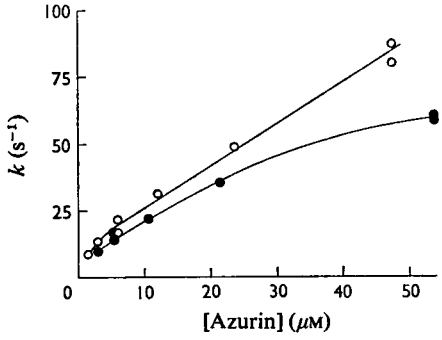

Fig. 5. *Effect of azurin concentration on the reaction between reduced azurin and ferricytochrome c-551*

The reaction was followed at 417nm in the 2cm path-length cell of the stopped-flow apparatus when 0.5μM-ferricytochrome *c*-551 was mixed with various concentrations of reduced azurin; all concentrations are after mixing. ○, In the presence of 0.1M-phosphate buffer, pH7.0; ●, in 0.1M-Tris–HCl, pH7.4. The temperature was 20°C.

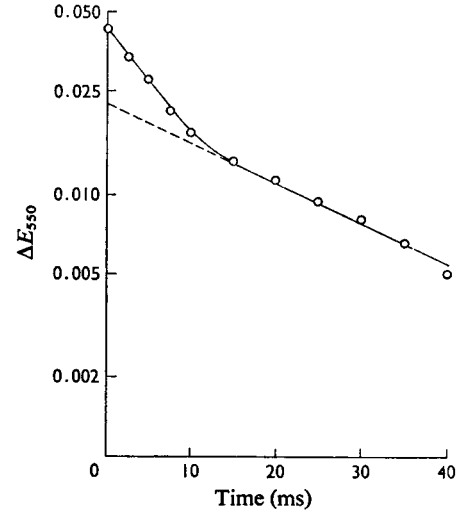

Fig. 6. *Logarithmic plot to show the biphasic nature of the reaction between ferricytochrome c-551 and reduced azurin*

A log plot of data obtained during a flow experiment in which 12μM-azurin was mixed with 52μM-ferricytochrome *c*-551 in the 2cm-path-length cell of the stopped-flow apparatus. The reaction was followed at 550nm in the presence of 0.1M-potassium phosphate buffer, pH7.0, and at 20°C.

(1970) for the same protein system but extracted from *Ps. fluorescens*.

This second-order behaviour, with no tendency to reach a rate limit, is in agreement with predictions for the reaction between C_r and A_o as described in eqn. (1). However, eqn. (2) would predict that, as interconversions within the complexes are, in this scheme, thought to account for the slow relaxation observed in temperature-jump experiments, rates measured from stopped-flow experiments should also approach a rate limit at high azurin concentrations.

Stopped-flow experiments in which reduced azurin was mixed with oxidized cytochrome *c* were performed under two different concentration regimes.

(i) In experiments in which the azurin was in excess over the cytochrome, simple progress curves were again observed. The dependence of the rate on reduced azurin concentration is shown in Fig. 5. In phosphate buffer, the dependence is linear over the concentration range explored, yielding a second-order rate constant, $k_{apparent}$, of $1.6\times10^6 M^{-1}\cdot s^{-1}$, in agreement with Antonini *et al.* (1970). For the model in eqn. (2), a rate-limiting step reflecting electron transfer within complexes may be expected. As the rates measured at high azurin concentrations in stopped-flow experiments exceed the rate limit for slow phase seen in temperature-jump studies, the model in eqn. (2) appears unlikely. For the model of eqn. (1), provided that the total concentration of azurin is such that the concentration of A_r greatly exceeds the concentration of C_o, simple monophasic progress curves and apparent second-order behaviour such as were found experimentally, are to be expected. The dependence of the rate of electron transfer on total azurin concentration would not, however, directly yield k_{21}. The second-order rate constant, $k_{apparent}$, should in fact be related to k_{21} via the equilibrium constant K_{23} (see eqn. 4). When this type of experiment was repeated in Tris buffer the results presented in Fig. 5 show a marked deviation from linearity. This behaviour may be accounted for in terms of the model in eqn. (1) if the value of k_{21} is unaffected but the equilibrium, K_{23}, between the two forms of reduced azurin is being perturbed by the ionic environment. In fact this explanation demands that the equilibrium is shifted in favour of A_r' in Tris–HCl buffer, pH7.4, as compared with potassium phosphate, pH7.0. In view of the limited number of flow and temperature-jump experiments performed in Tris–HCl buffer this explanation demands experimental verification.

(ii) Where the concentration of C_o exceeds that of A_r and where the rate of electron transfer is greater than the interconversion rate k_{23}, eqn. (1) predicts deviation from simple monophasic progress curves. Once A_r has been rapidly converted into A_o by reaction with excess of C_o, then one should observe, via the electron-transfer reaction, the ensuing interconversion of A_r' into A_r. Stopped-flow experiments,

in which the concentration of ferricytochrome *c*-551 was high and in excess of the azurin concentration, clearly showed departures from simple monophasic behaviour. Fig. 6 shows a logarithmic plot of a typical experiment in which a fast phase is followed by a slower phase. The form of the progress curve was dependent on the protein concentration, the two phases merging as the concentrations were decreased. This conforms to the predictions of eqn. (1). From Fig. 6 we may assign (*a*) a value for k_{32} not exceeding $40\,s^{-1}$ from the slow phase, and (*b*) a value of K_{23} of approx. 1, from the ratio of the amplitudes of the fast and slow processes (proportional to the amounts of A_r and A_r' present at zero time).

Discussion

The above results are in opposition to the formulation of the electron-transfer mechanism shown in eqn. (2), but are in general agreement with the formulation of eqn. (1). However, in order to have confidence in the existence of this proposed mechanism we should be able to assign values to all the rate constants and equilibrium constants in eqn. (1) and show that these are self-consistent and capable of predicting experimental results. The kinetic and equilibrium constants describing the model represented in eqn. (1) are given in Table 3. Some of the constants have been measured and/or calculated and some of the relevant points are discussed below.

The overall equilibrium constant (K') measured by redox titration for the scheme in eqn. (1) is related to K_{12} and K_{23} by the following relationship:

$$K' = K_{12}(K_{23}+1) \qquad (3)$$

K' has been measured previously and found to be 3.7 (Brunori *et al.*, 1974). If we now take K_{23} to be equal to 1 (from the experiment shown in Fig. 6) we may assign a value of 1.85 to K_{12}.

The rate constant k_{12} may be unambiguously assigned the value of $6.25\times10^6\,M^{-1}\cdot s^{-1}$ from the stopped-flow data shown in Fig. 4. From our calculated value of K_{12} and the measured value for k_{12} we may calculate k_{21} to be $3.4\times10^6\,M^{-1}\cdot s^{-1}$. This value for k_{21} is reasonable on two counts.

(1) From Fig. 6 the initial part of the reaction yields a rate of approx. $180\,s^{-1}$. Assuming second-order behaviour this result yields a value of k_{21} equal to $3.6\times10^5\,M^{-1}\cdot s^{-1}$.

(2) In stopped-flow experiments where the total reduced azurin is greatly in excess of the oxidized cytochrome *c* with which it is mixed, the rate measured is related to k_{21} by the eqn. (4).

$$k_{apparent} = \frac{k_{21}}{K_{23}+1} = \frac{3.4\times10^6\,M^{-1}\cdot s^{-1}}{2} = 1.7\times10^6\,M^{-1}\cdot s^{-1} \qquad (4)$$

This value is in good agreement with the value of $k_{apparent}$ found, which was $1.6\times10^6\,M^{-1}\cdot s^{-1}$ (see Fig. 5).

Finally we may assign values to k_{23} and k_{32}. As stated above, k_{32} may be taken to be close to $40\,s^{-1}$ and since K_{23} is 1 it follows that k_{23} equals $40\,s^{-1}$.

The values given in Table 3 form a self-consistent set which are compatible with the equilibrium and stopped-flow data and it now remains to be seen if eqn. (1), with the above values of the constants, is in agreement with the temperature-jump data.

Eqn. (1) predicts two relaxation processes with the following reciprocal relaxation times (Eigen & De Maeyer, 1963):

$$\frac{1}{\tau_I} = k_{12}(\bar{C}_r+\bar{A}_o)+k_{21}(\bar{A}_r+\bar{C}_o)+k_{23}+k_{32} \qquad (5)$$

$$\frac{1}{\tau_{II}} = \frac{\{[k_{12}(\bar{C}_r+\bar{A}_o)+k_{21}(\bar{A}_r+\bar{C}_o)](k_{23}+k_{32})\}-k_{23}k_{21}\bar{C}_o}{k_{12}(\bar{C}_r+\bar{A}_o)+k_{21}(\bar{A}_r+\bar{C}_o)+k_{23}+k_{32}} \qquad (6)$$

The rate constants are as depicted in eqn. (1) and the 'barred' symbols indicate the concentrations of the species at equilibrium.

These equations are complex, but fortunately can be simplified in some cases by taking advantage of the experimental design. For example, all equilibrium mixtures for temperature-jump studies were prepared by adding completely oxidized azurin to completely reduced cytochrome *c*. Under these conditions it is clear that at equilibrium the amount of oxidized cytochrome *c* must be equal to the total amount of reduced azurin (A_r+Ar'). Then we may express K' as follows:

$$K' = \frac{(A-\bar{C}_o)(C-\bar{C}_o)}{\bar{C}_o^2} = K_{12}(K_{23}+1) \qquad (7)$$

where A and C are the total concentrations of azurin and cytochrome *c*-551.

Table 3. *Rate and equilibrium constants for model described in eqn.* (1)

See the text for details.

k_{12}	$6.25\times10^6\,M^{-1}\cdot s^{-1}$	(Measured)
k_{21}	$3.38\times10^6\,M^{-1}\cdot s^{-1}$	(Calculated and measured)
K_{12}	1.85	(Calculated from k_{12}/k_{21})
k_{23}	$40\,s^{-1}$	(Calculated from K_{23} and k_{32})
k_{32}	$40\,s^{-1}$	(Measured)
K_{23}	1	(Measured)
K'	3.7	(Measured)
K'	3.7	[Calculated from $K_{12}(K_{23}+1)$]

Further, for most experiments the ratio of the concentration of azurin to cytochrome *c* was constant, i.e. $C = 0.5A$.

Under these conditions, eqn. (7) shows that when $K' = 3.7$, as measured, then $\bar{C}_o = 0.42A$. Taking account of the value of $K_{12} = k_{12}/k_{21} = 1.85$ and $K_{23} = 1$, we may now express $1/\tau_I$ as follows:

$$\frac{1}{\tau_I} = k_{12}\left[(C-\bar{C}_o)+(A-\bar{C}_o)+\frac{\frac{\bar{C}_o}{2}+\bar{C}_o}{1.85}\right]+(k_{23}+k_{32}) \quad (8)$$

$$\frac{1}{\tau_I} = k_{12}\,[A+C-1.2\bar{C}_o]+(k_{23}+k_{32}) \quad (9)$$

If we consider the experiments in which $C = 0.5A$ and therefore $\bar{C}_o = 0.42A$ then eqn. (9) becomes

$$\frac{1}{\tau_I} = k_{12}\,[A+0.5A-0.48A]+(k_{23}+k_{32}) \simeq k_{12}\,[A]+(k_{23}+k_{32}) \quad (10)$$

We expect therefore a linear dependence of $1/\tau_I$ on the total azurin concentration, the slope being k_{12} and the intercept $(k_{23}+k_{32})$. Fig. 1(*a*) shows that this expectation is fulfilled, the value of k_{12} being $7.2\times 10^6\,M^{-1}\cdot s^{-1}$ and $(k_{23}+k_{32}) = 115\,s^{-1}$. These values are in fair agreement with the values of $6.25\times 10^6\,M^{-1}\cdot s^{-1}$ and $80\,s^{-1}$ derived from the stopped-flow experiments (see also Table 3).

Under conditions where the cytochrome concentration is fixed and titrated with azurin the situation is more complex. However, we may see from eqn. (9) that when the azurin concentration is much greater than the cytochrome concentration then, as oxidized azurin was always added to reduced cytochrome *c*, the total cytochrome *c* concentration is approximately the $\bar{C}_o$ concentration, i.e. $C \approx \bar{C}_o$ in eqn. (9). To a first approximation, $1/\tau_I$ therefore becomes proportional to the total azurin concentration and yields $k_{12} = 6.7\times 10^6\,M^{-1}\cdot s^{-1}$ from the upper part of the curve in Fig. 1(*a*). When the azurin concentration is lowered and becomes smaller than that of the cytochrome, then both A and C_o ($=\bar{C}_o$) in eqn. (1) may be neglected, and therefore $1/\tau_I$ becomes independent of the total azurin concentration, as observed.

For those experiments in which $C = 0.5A$ and therefore $\bar{C}_o = 0.42A$, we may also predict the dependence of $1/\tau_{II}$ on the total azurin concentration. Substituting the values of the constants from Table 3 into eqn. (6), we arrive at the following relationship:

$$\frac{1}{\tau_{II}} = \frac{444\,A}{6.25\,A+80} \quad (11)$$

where A here is expressed in μM. The calculated dependence of $1/\tau_{II}$ on A is shown in Fig. 1(*c*) and is in good agreement with the experimental data points.

It is apparent therefore that the model in eqn. (1) is able to accommodate the equilibrium, stopped-flow and temperature-jump data in a self-consistent manner. It is, of course, realized that we cannot exclude the possibility that other complexities of the system, such as multiple forms of cytochrome *c*-551, may be present, although they are not apparent under the conditions used here.

Given the fact that reduced azurin exists in two forms, we may expect that complex kinetics will be observed in the reaction of azurin with other redox systems. Such complex kinetics have in fact been observed, both in stopped-flow experiments (Wharton *et al.*, 1973) and in temperature-jump experiments (M. Brunori, S. R. Parr, C. Greenwood & M. T. Wilson, unpublished work), in the reaction of azurin with the *Pseudomonas* cytochrome oxidase.

In general the results reported above are in agreement with previous observations on the system (Antonini *et al.*, 1970; Pecht & Rosen, 1973; Brunori *et al.*, 1974) insofar as they show very high rates for electron transfer and complex kinetics. It is found that the rates of electron transfer between azurin and cytochrome *c*-551 are indeed very high and within the same order of magnitude in both directions. As already pointed out (Antonini *et al.*, 1970), these rate constants are very much higher than those obtained for electron transfer between either of the *Pseudomonas* redox proteins and redox proteins from other species (e.g. mammalian cytochrome *c*) or small molecules (e.g. ferrocyanide). This may indicate that, when dealing with redox proteins from the same species, electron transfer occurs through an optimal fit between the partners. The formulation of a molecular complex stabilized by specific interactions has been postulated to account for the observed efficiency of electron transfer (Antonini *et al.*, 1970). Indeed it probably is the case that such a complex exists in catalytic amounts, but, as shown by the above discussion, it is not necessary to invoke electron transfer within a complex to explain the kinetic observations. Over and above this, the experimental results seem to exclude the presence of significant amounts of such a complex at equilibrium.

On the contrary, the picture that emerges from the quantitative analysis of the results indicates that the origin of the complex kinetics resides in the presence of multiple forms of one of the partners, in this case reduced azurin. The existence of two forms of oxidized cytochrome *c*, only one of which can accept electrons from ascorbate, has also been reported (Greenwood & Palmer, 1965). The presence of an 'active' and an 'inactive' form of redox proteins in equilibrium, and the possibility of perturbing such an equilibrium by specific interactions, may constitute a regulatory mechanism for electron-transfer systems, and therefore a meaningful physiological role for the presence of multiple components may be suggested,

if one of the reactive components may be specifically removed from the system.

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