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Short Communications

A Temperature-Jump Study of the Reaction between Azurin and Cytochrome *c*-551 from *Pseudomonas aeruginosa*

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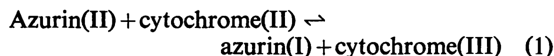
Temperature-jump studies on the electron-transfer reaction between azurin and cytochrome *c*-551 clearly reveal two chemical relaxations. The amplitudes of these relaxation processes have identical spectral distributions, but the relaxation times show different dependences on the reactant concentrations. These findings are discussed in terms of possible models.

Stopped-flow kinetic studies on the reaction between azurin and cytochrome *c*-551 (Antonini *et al.*, 1970) have suggested that electron transfer takes place within a complex or complexes which form between the two proteins. Both azurin and cytochrome *c*-551 are low-molecular-weight proteins (16000 and 9000 daltons respectively) and, judged from the very high rate of electron transfer between them (Antonini *et al.*, 1970), exhibit a high degree of biological specificity for each other. The suggestion, therefore, that electron exchange between these relatively small proteins is complex may have wider significance for biological redox processes in general.

A temperature-jump study on the reaction between azurin and cytochrome *c*-551 has been undertaken, with the idea of gaining direct information on the existence and the properties of a possible complex. Thus for a simple electron transfer between two partners:



$$\text{Azurin(II)} + \text{cytochrome(II)} \rightleftharpoons \text{azurin(I)} + \text{cytochrome(III)} \quad (1)$$

only one relaxation time would be expected (Eigen & De Maeyer, 1963). If, however, this simple equilibrium is complicated by the coupling of electron transfer with other processes, involving either complex-formation between the redox partners or other events before the transfer, a more complex relaxation pattern would be expected.

Materials and methods

Azurin was a kind gift from Dr. S. Vinogradov and cytochrome *c*-551 was isolated from *Pseudomonas aeruginosa* essentially by the procedure developed by Ambler (1963). Their concentrations were determined by using the following extinction coefficients: azurin, $\epsilon_{625} = 3500$ litre·mol^{-1}·cm^{-1} (Brill *et al.*, 1968); cytochrome *c*-551 (reduced), $\epsilon_{551} = 28300$ litre·mol^{-1}·cm^{-1} (Horio *et al.*, 1960). Ferrocytochrome *c*-551 was obtained by careful reduction of ferricytochrome with a minute excess of sodium dithionite, which was afterwards removed by passage through a Sephadex G-25 column. The reduced protein was stored at 4°C and was stable against oxidation for several days. For most experiments 0.1M-potassium phosphate buffer, pH7.0, containing 10μM-EDTA was used.

Temperature-jump experiments were carried out by using an instrument built by Messanlagen Gesellschaft (Göttingen, Germany). Experiments were performed with a temperature-jump cell of 7ml capacity and 1cm light-path. A discharge of 30kV, corresponding to a temperature increase of 4.5–5°C, was generally used.

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

Results and discussion

Static redox titrations were carried out spectrophotometrically by making small additions of a concentrated azurin (Cu^{2+}) solution to a solution of reduced cytochrome *c*-551. The spectra of the mixture, taken between 390 and 450nm, after each azurin addition, exhibit good isosbestic points at 410nm and 434nm.

To a good approximation the data exhibit simple

behaviour and allow us to determine a unique equilibrium constant according to the equation:

$$K = \frac{[\text{cytochrome(III)}][\text{azurin(I)}]}{[\text{cytochrome(II)}][\text{azurin(II)}]} \quad (2)$$

The value of $K = 3.7$ obtained from such experiments is close to that reported by Antonini *et al.* (1970). It appears therefore that, to a first approximation, eqn. (1) may be applied to the equilibrium behaviour.

Temperature-jump experiments on equilibrium mixtures of the two proteins clearly show that the attainment of equilibrium reflects a more compli-

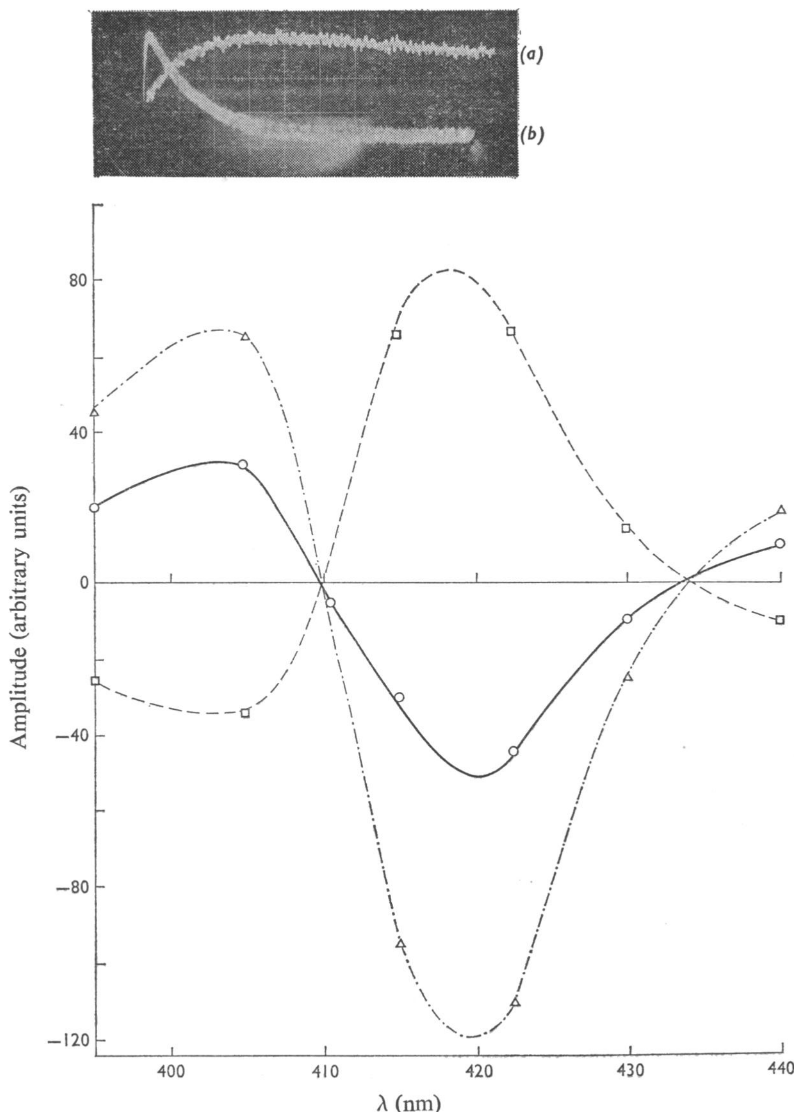


Fig. 1. ***Wavelength-dependence of the amplitude of the relaxation processes observed in temperature-jump experiments***

The reaction mixture contained 13.5 μM-cytochrome *c*-551 and 27 μM-azurin in 0.1 M-potassium phosphate buffer, pH 7.0, at 24°C (final temperature). □, Amplitude of fast relaxation; △, amplitude of slow relaxation; ○, amplitude of overall change. The static difference spectrum between ferri- and ferro-cytochrome *c*-551, normalized to the kinetic difference spectrum for the overall change (○), is shown as a bold solid line (——). The insert shows a temperature-jump relaxation spectrum of the reaction between 38 μM-cytochrome *c*-551 and 76 μM-azurin in 0.1 M-potassium phosphate, pH 7.0. The monitoring wavelength was 550 nm, the initial temperature 19.5°C, and $\Delta E = 0.0017$. Abscissa scale: 1 ms/cm (*a*); 20 ms/cm (*b*).

cated situation than that summarized by eqn. (1). As shown in the oscilloscope record of Fig. 1, two chemical relaxations are clearly resolved in the ms time range. The absorbance changes associated with these two relaxation processes are of opposite sign, and each one of the two processes corresponds to a single exponential, characterized by a single time-constant (τ). The overall absorbance change is therefore very small and is in favour of the formation of oxidized cytochrome *c*-551, indicating that the enthalpy in terms of eqn. (2) is positive.

The overall amplitude observed in a temperature-jump experiment, resulting from the opposed absorbance changes of the two relaxations, varies as the azurin concentration is increased, going through a maximum. However, it was found that at constant azurin and cytochrome concentrations the relative amplitudes of the slow and fast relaxation times change dramatically as the equilibrium temperature is varied. For example, in a set of experiments in which the cytochrome *c*-551 concentration was 5.4μM and that of the azurin 10.8μM in a 0.1M-potassium phosphate buffer, pH7.0, it was found that at 32°C (final) the two relaxations had almost equal amplitudes ($\Delta E_{fast} = 8.6\times10^{-4}$, $\Delta E_{slow} = 12\times10^{-4}$), whereas at 15°C (final) the slow process increased in amplitude and dominated ($\Delta E_{fast} = 2.9\times10^{-4}$, $\Delta E_{slow} = 22\times10^{-4}$).

Fig. 1 shows the wavelength-dependence of the observed amplitudes and compares them with the static difference spectrum between ferricytochrome *c*-551 and ferrocytochrome *c*-551. It should be noted that azurin makes very little spectral contribution in this wavelength region. It is clear that both the fast and slow processes have the same kinetic difference spectrum (but have opposite senses), and that in shape and position of isosbestic points they correspond to the static difference spectrum. The spectroscopic changes accompanying the electron-transfer reaction are therefore acting as an indicator for both the fast and slow processes.

The dependence of the reciprocal relaxation time for the fast and slow processes on the total azurin concentration is shown in Fig. 2. Over the concentration range examined it is observed that τ_{fast}^{-1} depends linearly on azurin concentration, whereas τ_{slow}^{-1} is virtually independent of azurin concentration. This implies that the faster relaxation process reflects a bimolecular reaction associated with the electron transfer.

The temperature-jump experiments reported above, albeit preliminary, give some pertinent information on the reaction between azurin and cytochrome *c*-551 from *Pseudomonas aeruginosa*. In agreement with stopped-flow experiments of Antonini *et al.* (1970), they show unequivocally that electron transfer between the two redox proteins cannot be described by eqn. (1). The presence of two clearly resolved relaxation events demands that at least two elementary steps are involved in the overall reaction between azurin and cytochrome *c*-551. In addition it was shown that the faster relaxation time is dependent on the concentration of the reaction partners, whereas the slower one is concentration-independent over the range explored. These results are in broad agreement with those given by Pecht & Rosen (1973), who have used a similar approach with *Pseudomonas fluorescens* proteins.

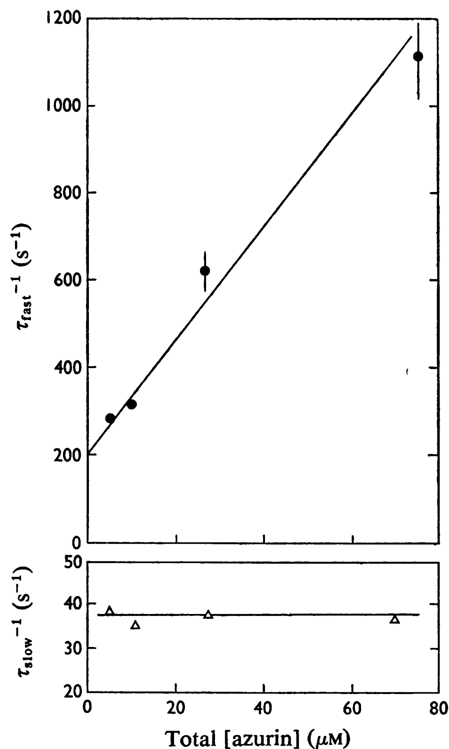


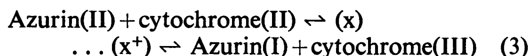
Fig. 2. *Dependence of the reciprocal times on total azurin concentration*

The cytochrome *c*-551 concentration was always half that of the azurin. The experiment was carried out in 0.1M-potassium phosphate buffer, pH7.0, at 19.5°C (initial temperature). ●, Reciprocal relaxation time for the faster process; △, reciprocal relaxation time for the slower process.

A crucial result, which imposes a constraint on the possible mechanism describing the reaction under study, is represented by the comparison of the static and kinetic difference spectra given in Fig. 1. The identity of the spectral features (maxima and isosbestic points) for both the fast and slow processes with the overall difference spectrum between ferri- and ferro-cytochrome *c*-551 is unequivocally established. This implies that both relaxation times are observable

through spectral changes involving the same chromophore, i.e. the haem group of cytochrome *c*-551, and the observed spectral changes correspond to a change in the redox state of the haem iron. Therefore the faster relaxation process must reflect a reaction step which involves electron transfer between the two redox partners.

The model that has been proposed on the basis of the stopped-flow data by Antonini *et al.* (1970) involves the formation of a complex (or complexes) within which electron transfer occurs:



$$\text{Azurin(II)} + \text{cytochrome(II)} \rightleftharpoons (x) \ldots (x^{+}) \rightleftharpoons \text{Azurin(I)} + \text{cytochrome(III)} \quad (3)$$

where (x) and (x^{+}) represent different possible complexes. The present data do not allow us to propose a unique mechanism consistent with or alternative to the one represented in eqn. (3); however, they seem to exclude models such as that proposed by Pecht & Rosen (1973), in which the protein-concentration-dependent step occurs before, and therefore without, electron transfer.

The system is obviously more complex than it seemed on the surface, and this is shown, among other things, by the large dependence of the relative amplitude of the two relaxation times on temperature.

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