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

By MAURIZIO BRUNORI

C.N.R. Centre of Molecular Biology, Institutes of Biochemistry and Chemistry, University of Rome, Rome 00185, Italy

and STEPHEN R. PARR and COLIN GREENWOOD

School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.

and MICHAEL T. WILSON

Department of Chemistry, University of Essex, Colchester CO4 3SQ, U.K.

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The electron-transfer reaction between azurin and the cytochrome oxidase from *Pseudomonas aeruginosa* was investigated by temperature-jump relaxation in the absence of O₂ and in the presence of CO. The results show that: (i) reduced azurin exists in two forms in equilibrium, only one of which is capable of exchanging electrons with the *Pseudomonas* cytochrome oxidase, in agreement with M. T. Wilson, C. Greenwood, M. Brunori & E. Antonini (1975) (*Biochem. J.* **145**, 449–457); (ii) the electron transfer between azurin and *Pseudomonas* cytochrome oxidase occurs within a molecular complex of the two proteins; this internal transfer becomes rate-limiting at high reagent concentrations.

As part of a study of the properties and reactions of various redox proteins from *Pseudomonas aeruginosa*, we have investigated the kinetics of electron transfer between *Pseudomonas* cytochrome *c*₅₅₁ oxidase (EC 1.9.3.2) and the *Pseudomonas* blue copper protein, azurin. This system has previously been investigated by Wharton *et al.* (1973) using stopped-flow methods. These authors reported that electron transfer between azurin and *Pseudomonas* cytochrome oxidase is rate-limited in both directions, indicating the formation of a molecular complex within which electron transfer occurs. In addition, they observed a slower process which was attributed to internal electron transfer between the haem *c* and haem *d* moieties of the *Pseudomonas* cytochrome oxidase.

The present paper reports the results of temperature-jump experiments performed anaerobically on this system in the presence of CO. Under the experimental conditions used, the haem *d* component of the *Pseudomonas* cytochrome oxidase is 'frozen' as the ferrous-CO complex and therefore should not participate in the electron-transfer reaction with azurin. These experiments were performed under conditions as far as possible analogous to those previously used to analyse the reaction between azurin and *Pseudomonas* cytochrome *c*₅₅₁ (Brunori *et al.*, 1974; Wilson *et al.*, 1975).

Materials and methods

Pseudomonas aeruginosa N.C.T.C. 6750 was grown and the *Pseudomonas* cytochrome oxidase and azurin were purified and tested as previously described (Parr *et al.*, 1974). Their concentrations

were determined by using the following values for the millimolar extinction coefficient: $\epsilon_{\text{MM}} = 3.5$ litre·mmol⁻¹·cm⁻¹ at 625 nm for oxidized azurin (Brill *et al.*, 1968), and $\epsilon_{\text{MM}} = 149$ litre·mmol⁻¹·cm⁻¹ at 410 nm for oxidized *Pseudomonas* cytochrome oxidase (Horio *et al.*, 1961).

Reduced azurin was prepared as previously described (Wilson *et al.*, 1975). In those experiments in which oxidized azurin was added to the reduced *Pseudomonas* cytochrome oxidase-CO complex, the latter was prepared in the temperature-jump cell by repeated outgassing and incubation under CO gas (101 kPa). Under these conditions autoreduction of the oxidase occurs in an analogous way to that which has been observed in mammalian cytochrome oxidase (Greenwood *et al.*, 1974).

Temperature-jump experiments were carried out by using the instrument built by Messanlagen at Göttingen (Germany), through the courtesy of Professor L. De Maeyer, and by using a cell with a 1 cm light-path and 6 ml total volume. A discharge of 30 kV, corresponding to a temperature increase of 4.5–5°C, was generally used. Appropriate amounts of azurin were added anaerobically to a known quantity of *Pseudomonas* cytochrome oxidase under CO, and the spectral properties of the mixture were recorded directly in the temperature-jump cell before and after each series of experiments, in a Cary 14 spectrophotometer.

Results and discussion

The relaxation behaviour of the system is complex, showing two phases associated with absorbance changes of opposite sign at $\lambda = 417$ nm. The time-

Fig. 1. Dependence on wavelength of the amplitude of the two relaxation phases observed in temperature-jump experiments

The reaction mixture contained $106\mu\text{M}$ -azurin and $14\mu\text{M}$ -*Pseudomonas* cytochrome oxidase in 0.1M -potassium phosphate buffer, pH7, and 25°C (final temperature). Amplitudes of the faster (Δ) and slower ($\circ$) relaxation processes are indicated, together with the overall amplitude ($\blacksquare$).

course of approach to equilibrium can be accurately analysed in terms of two relaxation times, hereafter referred to as τ_F (fast) and τ_S (slow).

Fig. 1 depicts the spectral distribution of the absorbance changes associated with the two relaxation times, as well as the overall difference spectrum, in the region 370–435 nm. The presence of isosbestic points and the shape of the difference spectrum common to both phases show that: (i) both relaxation events are associated with a single chromophore and (ii) the observed changes correspond to a change in oxidation state of the haem *c* moiety of the *Pseudomonas* cytochrome oxidase. No contribution, due to either the haem *d* of the oxidase or the azurin, was observed in this wavelength region.

Fig. 2 shows the dependence of the reciprocal relaxation times ($1/\tau_F$ and $1/\tau_S$) on total concentration of azurin. Both relaxation times are concentration-dependent only in the lower concentration range, and tend to approach limiting values of about 70s^{-1} for $1/\tau_S$, and about 280s^{-1} for $1/\tau_F$.

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

(a) shows the behaviour of the faster and (b) the slower relaxation times. Experiments were carried out in 0.1M -potassium phosphate buffer, pH7, and 25°C (final temperature). Total concentrations of *Pseudomonas* cytochrome oxidase were from $7\mu\text{M}$ to $14\mu\text{M}$. $\lambda = 417\text{nm}$.

Any model describing a system of this type should incorporate the reaction between the free partners and the transfer of electrons between them [see, for instance, Antonini *et al.* (1970) and Pecht & Rosen (1973)]. The following scheme is therefore proposed to describe all the events associated with the reaction between azurin (indicated by A) and the haem *c* moiety of the *Pseudomonas* cytochrome oxidase (indicated by C):

where X_1 and X_2 represent forms of a molecular complex between azurin and *Pseudomonas* cytochrome oxidase, e.g. $(\text{A}^{1+} \cdot \text{C}^{3+})$ and $(\text{A}^{2+} \cdot \text{C}^{2+})$ respectively. (The haem *d* of the oxidase is omitted since spectral evidence indicated it to be always in the ferrous-CO form under the conditions used.)

The slowest observed relaxation time is taken to reflect the pre-equilibrium between the two forms of

reduced azurin, A^{1+} and $(A_1^+)^*$, which is itself spectrally silent, but is monitored through changes occurring at the level of the haem *c*. This is fully consistent with previous work (Wilson *et al.*, 1975), which has shown that reduced azurin exists in two forms, only one of which is capable of exchanging electrons with *Pseudomonas* cytochrome c_{551} . The concentration-dependence of τ_s (Fig. 2) is, of course, expected on the basis of coupling between the isomerization process and the faster bimolecular events. The limiting values for $1/\tau_s$, i.e. 0 and 70 s^{-1} when the concentration of azurin is respectively 0 and $\geq 300\text{ }\mu\text{M}$, are qualitatively consistent with expectations from scheme (1) [see also Wilson *et al.* (1975) for a similar situation]. The limiting value of $1/\tau_s$ at high azurin and cytochrome concentrations should approach the sum of the two rate constants, k_{+4} and k_{-4} , for the conformational change between the two forms of reduced azurin, which were previously found to be approximately equal, with a value of 40 s^{-1} (i.e. $k_{+4} + k_{-4} = 80\text{ s}^{-1}$). The observed value of 70 s^{-1} obtained with this system is indeed very close to expectations. From scheme (1), the analytical expression for the slowest relaxation time would be extremely complex since it should contain rate constants and equilibrium concentrations for all the faster steps, i.e. 1, 2 and 3 in scheme (1) (Eigen & De Maeyer, 1963). It is therefore impractical to deal with the actual shape of the concentration-dependence for τ_s shown in Fig. 2(b).

The concentration-dependence of the faster relaxation time deviates from the behaviour expected for a simple bimolecular process and approaches a limiting value at high concentrations of azurin (Fig. 2a). This is in marked contrast with the behaviour of the azurin-*Pseudomonas* cytochrome c_{551} system, for which the faster process was found to be strictly bimolecular up to very high rates (Brunori *et al.*, 1974; Wilson *et al.*, 1975).

In line with the proposal by Wharton *et al.* (1973), the faster relaxation time is taken to reflect electron transfer which occurs within a molecular complex between azurin and *Pseudomonas* cytochrome oxidase (indicated by X in scheme 1). To simplify the scheme, the bimolecular steps leading to complex-formation from either direction (steps 1 and 3 in scheme 1) are assumed to be: (i) spectrally silent, (ii) very fast in comparison with the observable fastest process (i.e. about 300 s^{-1} at azurin concentrations above $300\text{ }\mu\text{M}$); (iii) leading to equal values of the equilibrium constants for complex-formation, i.e. K_1 and K_3 . The two latter points were suggested by Wharton *et al.* (1973) on the basis of stopped-flow experiments performed in both directions. Given these conditions, and applying the necessary simplification of decoupling between electron transfer and the processes leading to complex-formation (Eigen & De Maeyer, 1963) and also ignoring the influence of

the slow pre-equilibrium in azurin, the expression for $1/\tau_F$ would be of the form:

$$\frac{1}{\tau_F} = \frac{k_{+2}(a+b) + k_{-2}(c+b)}{a+b+c+(k_{-1}k_{+3})} \quad (2)$$

$$\begin{aligned} \text{where } a &= k_{+1}k_{+3}(C^{3+} + A^{1+}); \\ b &= k_{+1}k_{-3}(C^{3+} + A^{1+})(C^{2+} + A^{2+}); \\ c &= k_{-1}k_{-3}(C^{2+} + A^{2+}) \end{aligned}$$

(see also Pecht & Rosen, 1973).

Eqn. (2) should yield, in the limit of infinite azurin concentration, a value corresponding to the sum of the two rate constants for electron transfer within the complex. Thus, from the data in Fig. 2, $k_{+2} + k_{-2} = 280\text{ s}^{-1}$. This value should not be taken as inconsistent with that of 150 s^{-1} which was calculated from the flow data of Wharton *et al.* (1973) since (a) the two sets of results refer to different derivatives of the *Pseudomonas* cytochrome oxidase, (b) they were obtained under different experimental conditions, i.e. pH 6.6 and 20°C , instead of pH 7 and 25°C , and (c) our result is subject to the validity of the assumptions listed above. It can also be seen from eqn. (2) that, since our experiments were performed by adding fully oxidized azurin (A^{2+}) to fully reduced *Pseudomonas* cytochrome oxidase (C^{2+}) under CO, $1/\tau_F$ should approach, in the limit of $A^{2+} \rightarrow 0$, a value corresponding to:

$$\frac{k_{-2}}{1 + \left(\frac{K_3}{C^{2+}}\right)}$$

Therefore an independent estimate of K_3 may allow the determination of the value of k_{-2} and thus of both forward and reverse rate constants for electron transfer within the complex.

It appears from these preliminary observations that a complete quantitative description of the system is well within reach, even if it cannot be achieved at the present stage. In particular, it seems necessary to assign independent values to some of the parameters in scheme (1), and especially to evaluate both k_{+2} and k_{-2} for the CO derivative of *Pseudomonas* cytochrome oxidase. The system is being investigated, both in the presence of CO and anaerobically, by rapid mixing.

In summary, the experiments described above provide additional evidence for the presence of a conformational equilibrium in reduced azurin, which was found to account for previous kinetic data obtained with *Pseudomonas* cytochrome c_{551} (Wilson *et al.*, 1975). In addition, they provide strong support to the idea that a specific complex is formed between azurin and *Pseudomonas* cytochrome oxidase and that electron transfer occurs efficiently between the two partners in the complex.

This observation acquires special significance when compared with the situation found previously

for the electron exchange between azurin and cytochrome c_{551} isolated from the same organism. In this latter case the kinetic data indicated that any complex of the partners is likely to exist in only catalytic amounts at equilibrium and that no rate-limit of electron transfer within the complex was evident. It appears that, for electron transfer between azurin and the *Pseudomonas* cytochrome oxidase, the formation of a specific complex has greater kinetic significance since electron transfer within the complex becomes rate-limiting and this may have important implications at the physiological level. It may be that these findings offer a means of testing different possible pathways of electron transfer in the *Pseudomonas* system, i.e. sequential or alternative. However, a fuller understanding can only emerge after a complete analysis of the reaction kinetics of all the separate components.

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- Antonini, E., Finazzi-Agrò, A., Avigliano, L., Guerrieri, P., Rotilio, G. & Mondovì, B. (1970) *J. Biol. Chem.* **245**, 4847–4849
- Brill, A. S., Bryce, G. F. & Maria, H. (1968) *Biochim. Biophys. Acta* **154**, 342–351
- Brunori, M., Greenwood, C. & Wilson, M. T. (1974) *Biochem. J.* **137**, 113–116
- Eigen, M. & De Maeyer, L. (1963) in *Techniques of Organic Chemistry* (Freiss, S. L., Lewes, E. S. & Weissberger, A., eds.), pp. 895–1054, Interscience, New York and London
- Greenwood, C., Wilson, M. T. & Brunori, M. (1974) *Biochem. J.* **137**, 205–215
- Horio, T., Higashi, T., Yamanaka, T., Matsubara, H. & Okunuki, K. (1961) *J. Biol. Chem.* **236**, 944–951
- Parr, S. R., Wilson, M. T. & Greenwood, C. (1974) *Biochem. J.* **139**, 273–276
- Pecht, I. & Rosen, P. (1973) *Biochem. Biophys. Res. Commun.* **50**, 853–858
- Wharton, D. C., Gudat, J. C. & Gibson, Q. H. (1973) *Biochim. Biophys. Acta* **292**, 611–620
- Wilson, M. T., Greenwood, C., Brunori, M. & Antonini, E. (1975) *Biochem. J.* **145**, 449–457