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Cytochrome *c*-551 and azurin oxidation catalysed by *Pseudomonas aeruginosa* cytochrome oxidase

A steady-state kinetic study

Maria Gabriella TORDI,* Maria Chiara SILVESTRINI,* Alfredo COLOSIMO,†§ Lorenzo TUTTOBELLO‡ and Maurizio BRUNORI*

**Istituto di Chimica e Biochimica, Facoltà di Medicina e Centro di Biologia Molecolare del C.N.R., Università di Roma 'La Sapienza', 00185 Roma, Italy, †Dipartimento di Medicina Sperimentale e Scienze Biochimiche, Università di Roma 'Tor Vergata', Via Orazio Raimondo, 00173 Roma, Italy, and ‡Istituto Superiore di Sanità, 00185 Roma, Italy*

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The kinetics of oxidation of azurin and cytochrome *c*-551 catalysed by *Pseudomonas aeruginosa* cytochrome oxidase were re-investigated, and the steady-state parameters were evaluated by parametric and non-parametric methods. At low concentrations of substrates (e.g. $\leqslant 50\,\mu$M) the values obtained for K_m and catalytic-centre activity are respectively $15 \pm 3\,\mu$M and $77 \pm 6\,min^{-1}$ for azurin and $2.15 \pm 0.23\,\mu$M and $66 \pm 2\,min^{-1}$ for cytochrome *c*-551, in general accord with previous reports assigning to cytochrome *c*-551 the higher affinity for the enzyme and to azurin a slightly higher catalytic rate. However, when the cytochrome *c*-551 concentration was extended well beyond the value of K_m, the initial velocity increased, and eventually almost doubled at a substrate concentration $\geqslant 100\,\mu$M. This result suggests a 'half-hearted' behaviour, since at relatively low cytochrome *c*-551 concentrations only one of the two identical binding sites of the dimeric enzyme seems to be catalytically active, possibly because of unfavourable interactions influencing the stability of the Michaelis–Menten complex at the second site. When reduced azurin and cytochrome *c*-551 are simultaneously exposed to *Ps. aeruginosa* cytochrome oxidase, the observed steady-state oxidation kinetics are complex, as expected in view of the rapid electron transfer between cytochrome *c*-551 and azurin in the free state. In spite of this complexity, it seems likely that a mechanism involving a simple competition between the two substrates for the same active site on the enzyme is operative. Addition of a chemically modified and redox inactive form of azurin (Hg-azurin) had no effect on the initial rate of oxidation of either azurin and cytochrome *c*-551, but clearly altered the time course of the overall process by removing, at least partially, the product inhibition. The results lead to the following conclusions: (i) reduced azurin and cytochrome *c*-551 bind at the same site on the enzyme, and thus compete; (ii) Hg-azurin binds at a regulatory site, competing with the product rather than the substrate; (iii) the two binding sites on the dimeric enzyme, though intrinsically equivalent, display unfavourable interactions. Since water is the product of the reduction of oxygen, point (iii) has important implications for the reaction mechanism.

Pseudomonas cytochrome oxidase (EC 1.9.3.2) is extracted from the bacterium *Pseudomonas aeruginosa* in a water-soluble dimeric state (M_r 120000). The enzyme is able to reduce both oxygen to water and nitrite to nitric oxide, although much more efficiently in the latter case (Yamanaka *et al.*, 1961). Two types of haem groups are present in the molecule: a haem *c* pair, which is the site of entrance of electrons, and a haem *d* pair, reacting with oxygen and nitrite. Reduction of the enzyme

§ To whom correspondence should be addressed.

in vivo is probably due to azurin and/or cytochrome *c*-551 (Horio *et al.*, 1960, 1961), which are two metalloproteins quite similar in size (M_r 14000 and 9000 respectively) and isoelectric point (5.2 and 4.7 respectively) but very different in their prosthetic groups, since cytochrome *c*-551 contains a haem *c*, and azurin a type I or 'blue' copper. Electron transfer between the enzyme and the two reducing substrates, investigated by steady-state and pre-steady-state kinetics under different conditions (Horio *et al.*, 1961; Gudat *et al.*, 1973; Barber *et al.*, 1976; Blatt, 1981), indicates similarities and some differences in the overall behaviour of the two substrates. Other typical features of the system are (i) the presence of a very fast and reversible electron transfer between azurin and cytochrome *c*-551 (Antonini *et al.*, 1970) and (ii) the decrease of the substrate oxidation rate under steady-state conditions, observed with both azurin and cytochrome *c*-551 (Barber *et al.*, 1976). Rapid-mixing experiments carried out anaerobically in order to characterize the direct electron transfer between the two metalloproteins and the haem *c* of the oxidase have shown that cytochrome *c*-551 is more efficient in transferring electrons to the enzyme (Wharton *et al.*, 1973; Parr *et al.*, 1977; Silvestrini *et al.*, 1982). Discrimination between a sequential and a parallel pathway for electron flow in the *Pseudomonas* respiratory system has been prevented, however, by the limited direct information on the relative amounts of azurin and cytochrome *c*-551 in the cell. Moreover information on the binding sites of the three macromolecular components, which would be essential for the formulation of a mechanistic model, is at present not available, and there is no answer even to the basic question whether the two substrates interact with the enzyme at the same or at a different site.

The present work was undertaken to investigate the mutual effect of the two substrates by measuring, at steady state, the oxidation of each substrate either alone or in the presence of the other component; in addition, the effects of apoazurin and Hg-azurin on the enzymic reactions were also examined. The results obtained provide novel information on the system, especially with reference to the mechanism of oxidation. The statistical methods of non-parametric analysis for kinetic data introduced by Cornish-Bowden & Eisenthal (1974) and improved by others (Porter & Trager, 1977) were exploited.

Materials and methods

Sample preparation

Cytochrome oxidase, cytochrome *c*-551 and azurin from *Ps. aeruginosa* N.C.T.C. 6750 were purified by the method of Parr *et al.* (1976). The proteins were more than 90% pure as judged by spectroscopic and electrophoretic analysis. Cytochrome oxidase was characterized by the following spectroscopic ratios: $A^{ox.}_{410}/A^{ox.}_{280} \geqslant 1.1:1$ and $A^{ox.}_{640}/A^{ox.}_{520} \geqslant 1.1:1$. Concentrations were determined by using the following absorption coefficients: for oxidized cytochrome oxidase $\varepsilon_{411} = 282000\,M^{-1}\cdot cm^{-1}$, expressed per mole of dimeric enzyme containing 4 iron atoms/molecule (Silvestrini *et al.*, 1979); for cytochrome *c* $\Delta\varepsilon^{red.-ox.}_{551} = 19700\,M^{-1}\cdot cm^{-1}$ (Horio *et al.*, 1960); for azurin $\Delta\varepsilon^{ox.-red.}_{625} = 3500\,M^{-1}\cdot cm^{-1}$ (Brill *et al.*, 1968).

Apo-azurin was prepared by the method of Yamanaka *et al.* (1963). Hg-substituted azurin was prepared by adding to apo-azurin mercuric acetate in a 1:2 stoichiometric ratio; after about 1 h, the reactant in excess was removed by overnight dialysis. The concentrations of both apo- and Hg-azurin were determined from the absorbance at 280 nm by using the same absorption coefficient as that of the holo-enzyme. The integrity of both species was tested by reconstitution experiments with $CuSO_4$ in water (Finazzi-Agrò *et al.*, 1970): in the case of apo-azurin, stoichiometric amounts of $CuSO_4$ restored the characteristic absorption of the native species, whereas with Hg-azurin the blue colour failed to appear even when $CuSO_4$ was added in great excess.

Oxygen titration experiments

A cuvette was sealed with a vaccine cap, degassed and completely filled with an anaerobic solution containing 150 μM-cytochrome *c*-551 and catalytic amounts of cytochrome oxidase in 0.1 M-potassium phosphate buffer, pH 7. After complete reduction by a stoichiometric amount of ascorbate, the solution was titrated with oxygen-containing buffer ($[O_2] = 270\,\mu M$) and the absorbance change monitored at 620 nm ($\Delta\varepsilon^{red.-ox.}_{620} = 1\,mM^{-1}\cdot cm^{-1}$). This wavelength of relatively small absorption for the chromophore was chosen because of the high concentration of reduced cytochrome *c*-551, necessary in order to minimize the errors due to possible oxygen leaks.

Kinetic measurements

All the steady-state experiments were carried out, with a Cary 219 spectrophotometer, in 0.04 M-potassium phosphate buffer or in Hepes/KOH buffer at the same ionic strength, both at pH 7 and containing 1 mM-EDTA at 25°C. The reduced substrates were prepared immediately before each experiment, by using minute amounts of $Na_2S_2O_4$ as a reductant and removing its excess with a Sephadex G-25 column equilibrated with the assay buffer.

The oxidation of cytochrome *c*-551 by catalytic amounts of cytochrome oxidase in the absence and

in the presence of different azurin derivatives was monitored at 551 or 416nm; at these wavelengths the spectral contribution of oxidized azurin is negligible. The oxidation of azurin by catalytic amounts of oxidase was monitored at 625nm in the absence and in the presence of Hg-azurin or reduced cytochrome *c*-551. In the latter case the spectral contribution to the total absorbance change due to the oxidation of reduced cytochrome *c*-551 is almost comparable with that due to the oxidation of azurin, and a correction of the apparent reaction rate is necessary: thus parallel experiments were carried out under identical conditions with monitoring at 551nm of the oxidation rate of cytochrome *c*-551, which was subtracted from the apparent rate of 625nm, giving the net oxidation rate of azurin.

Data analysis

All computations were carried out on an HP model 87 desk-top computer, with programs written by one of the authors (A. C.) and available on request. To avoid the pitfalls of the kinetic analysis based on linear transformation of the *v*-versus-[S] plots (Eisenthal & Cornish-Bowden, 1974), as a standard procedure the data were directly fitted to the Michaelis–Menten model by using the simple computer program for non-linear regression provided by Duggleby (1981), or, in the case of more than two variables, the one provided by Barisas & Gill (1979). The efficiency of these procedures, however, of the least-squares type, is strongly dependent on the corrected 'weighting' of the data, and in our case it is difficult to provide a non-empirical weighting based on non-standard errors for each single experimental condition. Thus the graphical procedure of Eisenthal & Cornish-Bowden (1974), based on a non-parametric method and essentially independent of outliers, was used as an independent check. In no case did discrepancies between values obtained by the two methods exceed 50%.

Results

Oxidation of single substrates

Determination of steady-state parameters for the cytochrome oxidase-catalysed oxidation of azurin or cytochrome *c*-551 by molecular oxygen is complicated by the continuous decrease in the rate of the reaction under saturating conditions of the enzyme. This has been interpreted in terms of competitive product inhibition (Barber *et al.*, 1976) and implies that the correct calculation of K_m and $V_{max.}$ depends critically on the determination of the true initial velocity. In the present paper the range of substrate concentrations explored has been extended (by almost a factor of 10-fold) as compared with previous reports and the calculation of the initial velocities carefully standardized in order to minimize errors related to product inhibition. Moreover, errors introduced by the common linearization procedures have been avoided by application of parametric and non-parametric analysis.

Figs. 1(*a*) and 1(*b*) report the results obtained for azurin and cytochrome *c*-551 respectively, with the oxidation kinetics being monitored over the substrate concentration range commonly explored by others (0–20μM). The K_m and $V_{max.}$ values, calculated by both a parametric and a non-parametric method, based on a Michaelis–Menten model, are shown in Table 1 and compared with those reported by other authors. Differences with previous reports can only partially be explained, in our opinion, by the different experimental conditions. It seems more realistic to take into account the presence of more than one population of enzyme molecules in some older purification procedures (see also Barber *et al.*, 1976), or difficulties in determining consistently initial rates of the reaction, because of the substantial inhibitory effects exerted by both products. In spite of this, the higher affinity of the enzyme for cytochrome *c*-551 has been confirmed, and the catalytic-centre activities for the two substrates are similar (with a slightly higher value for azurin).

Fig. 1(*c*) shows the results obtained exploring a much wider range of cytochrome *c*-551 concentrations (up to 140μM). In the low-concentration region the data overlap with those reported in Fig. 1(*b*); it is clear, however, that on increasing the concentration of reduced cytochrome *c*-551 v increases well above the value of $V_{max.}$ obtained from low-substrate concentration measurements and given in Table 1. The v obtained at a cytochrome *c*-551 concentration of 140μM is almost twice the value obtained from the results in Fig. 1(*b*). The observed trend is not related to the presence of phosphate anions since identical results (within the errors) were obtained in the presence of Hepes buffer (see Fig. 1*c*).

A possible explanation of the complete set of kinetic data given in Figs 1(*b*) and 1(*c*) is that, although the two substrate-binding sites of the dimeric cytochrome oxidase are intrinsically identical, since the two polypeptides are identical, binding of the first cytochrome *c*-551 molecule affects unfavourably the stability of the Michaelis–Menten complex for the second substrate molecule. In the simplest possible hypothesis these unfavourable interactions only affect the stability of the Michaelis–Menten complex for the completely saturated dimer, though $V_{max.}$ is the same for the two half-molecules once the complex has formed. Thus substrate oxidation by the dimeric

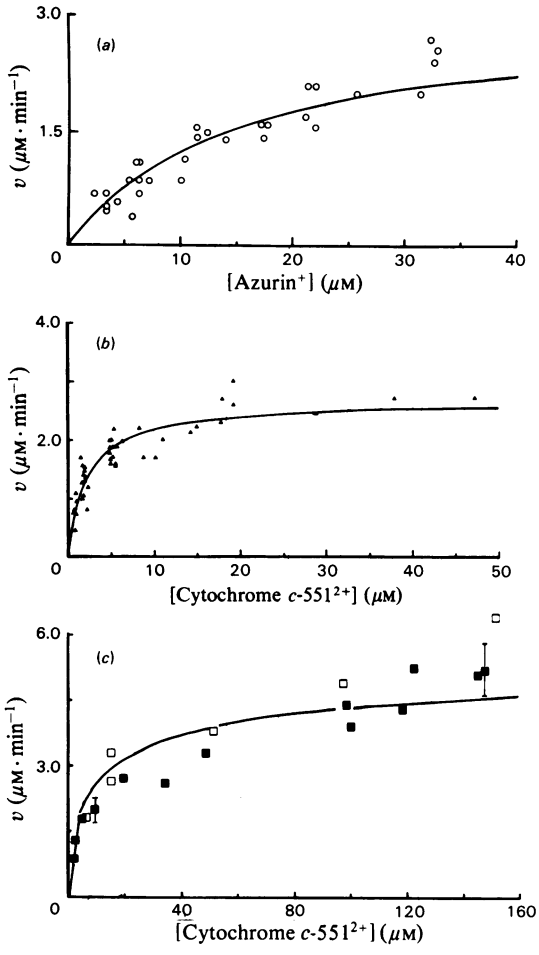


Fig. 1. *Initial velocity of the oxidation of azurin and cytochrome c-551 catalysed by Ps. aeruginosa cytochrome oxidase as a function of substrate concentration*

The rates of oxidation of azurin (*a*) and cytochrome *c*-551 (*b*) were measured spectrophotometrically. The buffer was 0.04M-potassium phosphate, pH 7.0, containing 1 mM-EDTA. The temperature was 25°C and the enzyme concentration was 20nM. The continuous lines in (*a*) and (*b*) represent the result of fitting the data to a simple Michaelis–Menten model by a non-linear least-squares method (Duggleby, 1981). The best-fit kinetic parameters are listed in Table 1. (*c*) gives the initial velocity (v) for the oxidation of cytochrome *c*-551 observed over a much broader range of substrate concentration [some of the points in (*c*) are obtained from (*b*)]. □ symbols refer to experiments carried out in Hepes buffer under identical conditions. Each point is the average for at least three independent determinations and the bars indicate the maximum error. The continuous line is the result of fitting the data by eqn. (1) (see the text for explanations) by using a non-linear least-squares method (Barisas & Gill, 1979). The best-fit parameters in eqn. (1) are listed in Table 2.

enzyme may be described by the following equation:

$$v = V_{max.} \cdot \frac{[S]}{K_{m1} + [S]} + V_{max.} \cdot \frac{[S]}{K_{m2} + [S]} \quad (1)$$

where the overall initial velocity is contributed by two Michaelian terms containing different values for K_m ($K_{m1} \neq K_{m2}$) and identical values for $V_{max.}$. At relatively low cytochrome *c*-551 concentrations only a single site on the enzyme, characterized by the higher substrate affinity, is effectively active, and the data shown in Fig. 1(*b*), as well as the parameters reported in Table 1, refer to such a situation. The continuous line in Fig. 1(*c*) is the best fit to the data of eqn. (1) by a non-linear least-squares method (Barisas & Gill, 1979), with the use of as initial estimates for K_m and $V_{max.}$ the values reported in Table 1. The best-fit parameters obtained are reported in Table 2 and are commented on in the Discussion section.

Determination of the product of oxygen reduction

Although it is generally accepted that the product of oxygen reduction by cytochrome oxidase under our conditions is water (Yamanaka *et al.*, 1961; Timkovich & Robinson, 1979), owing to the critical relevance of the issue in the context of the present work it seemed useful to verify it. In Fig. 2 a typical spectrophotometric titration of reduced cytochrome *c*-551 with oxygen in the presence of cytochrome oxidase (see the Materials and methods section) is shown. The determined value of the stoichiometry between oxidized cytochrome *c*-551 and added oxygen derived from seven independent experiments is 3.88 ± 0.12:1,

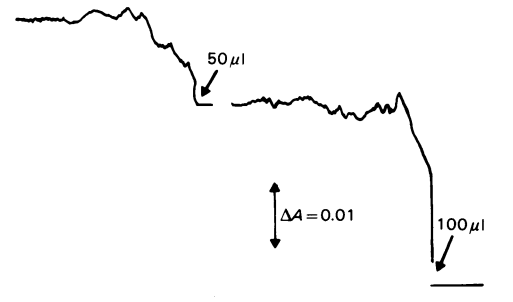


Fig. 2. *Titration of reduced cytochrome c-551 by molecular oxygen*

Two different air-equilibrated buffer volumes ($[O_2] = 270\mu M$) were added to a cuvette of total volume 3.7ml containing reduced 150μM-cytochrome *c*-551 and 3.7μM-cytochrome oxidase in 0.1M-phosphate buffer, pH 7, and the changes in absorbance were recorded at 620nm.

Table 1. *Steady-state parameters for oxidation of cytochrome c-551 and azurin catalysed by Ps. aeruginosa cytochrome oxidase*

Reference	Experimental conditions	Azurin			Cytochrome *c*-551		
		K_m (μM)	C.C.A.* (min^{-1})	K_i (μM)	K_m (μM)	C.C.A.* (min^{-1})	K_i (μM)
Horio *et al.* (1961)	pH 7.5, 18°C	39	100	—	19	96	—
Barber *et al.* (1976)	pH 7.0, 30°C	49	192	4.9	5.6	168	1–2
Blatt (1981)	pH 7.0, 22°C	42	48	1.2	2.2	20.4	0.47
Present work	pH 7.0, 25°C	15 ± 3	77 ± 6	—	2.15 ± 0.2	66 ± 2	—
		8.75†	58.7†		1.55†	62†	

* The turnover numbers given in the original papers have been transformed in catalytic-centre activities (C.C.A.), assuming that the values reported by Horio *et al.* (1961) and Barber *et al.* (1976) refer to the monomeric form of the enzyme and that those of Blatt (1981) refer to the dimeric form.

† Values obtained from the non-parametric analysis (see the text).

Table 2. *Steady-state kinetics of cytochrome c-551 oxidation catalysed by Ps. aeruginosa cytochrome oxidase, fitted by Michaelis–Menten model*

Column A refers to the parameters of the simple Michaelis–Menten model obtained fitting data of Fig. 1(*b*) by a non-linear least-squares method assuming that both sites are identical. Column B refers to the results obtained by the same fitting procedure including the whole set of v (Figs. 1*b* and 1*c*) and applying eqn. (1), which depicts the two sites as having the same $V_{max.}$ but different K_m. The standard errors (S.E.) give a direct indication of the relative goodness of fit.

	A (S.E. = 0.35)	B (S.E. = 0.41)
$V_{max.}$ ($\mu M \cdot min^{-1}$)	2.64	2.64*
K_{m1} (μM)	2.15	2.13
K_{m2} (μM)	—	45

* Constrained value.

very close to the value of 4:1 expected if water is the only product of the reaction.

Oxidation of substrate mixtures

The oxidation of mixtures of both reduced metalloproteins was studied as a function of the concentration of one of them in the presence of fixed concentrations of the other. The results are reported in Fig. 3 in the form of Dixon plots, which provide, at least in ideal cases, a discrimination between different mechanisms of inhibition and a relatively unbiased estimate of the inhibition constants (Dixon, 1953). A plot of $1/v$ versus different effector concentrations and at constant concentration of substrate yields a straight line with a slope = 0 when the reaction is insensitive to the presence of the effector and a slope > 0 when an inhibition is present. In the two limiting cases (completely competitive and non-competitive inhibition), the plots are always straight, approaching slope = 0 at high substrate concentration, and intercepting in the second quadrant in the former and on the negative portion of the abscissa in the latter (Webb, 1963).

In the present situation the trend appearing in Fig. 3(*a*), where cytochrome *c*-551 and azurin play the roles of the effector and substrate respectively, suggests the existence of a competition between the two substrates for the same active site. The data relative to the opposite situation, however (Fig. 3*b*) show a 'levelling off' at high cytochrome *c*-551 concentrations, and reflect a more complex situation whose phenomenological appearance is somewhat similar to that of 'partial inhibition' (Webb, 1963). Given the relative affinities of the enzyme for azurin and cytochrome *c*-551 (see Table 1), the difference in the mutual effects between the two substrates may be more apparent than real. It should be noticed, in fact, that (i) oxidation of azurin is more and more difficult to monitor spectroscopically at high cytochrome *c*-551 concentrations and (ii) the understanding and the analysis of these results is made even more complex by the fast redox equilibrium between the two substrates in the free state that is well established (Brunori *et al.*, 1975; Rosen & Pecht, 1976).

Oxidation of single substrate in the presence of redox-inactive azurin derivatives

In order to help the interpretation of the data reported in Fig. 3, steady-state experiments with both azurin and cytochrome *c*-551 were carried out in the presence of either apo- or Hg-azurin. Since these species, although redox-inactive, are structurally very similar to native azurin (Finazzi-Agrò *et al.*, 1973; Hill & Smith, 1979), they may interact

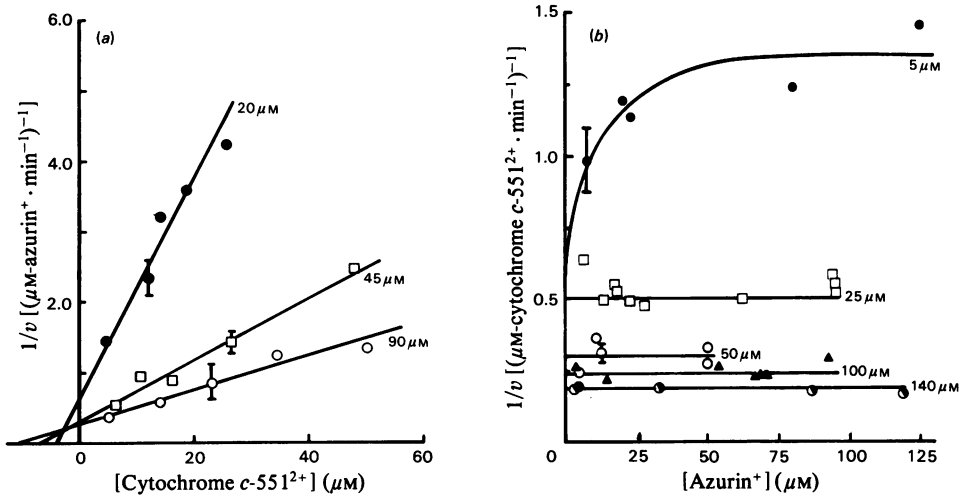


Fig. 3. *Initial velocity for the oxidation of mixtures of azurin and cytochrome c-551 catalysed by Ps. aeruginosa cytochrome oxidase*

The initial rates for oxidation of azurin in the presence of various concentrations (indicated on the Figure) of cytochrome *c*-551 (*a*) and of cytochrome *c*-551 in the presence of various amounts of azurin (*b*) were measured spectrophotometrically, and are reported in the form of Dixon plots. All other experimental conditions were as indicated in Fig. 1 legend.

with the enzyme at the azurin-binding site and hence behave as competitive inhibitors, at least with respect to azurin. Fig. 4 shows the initial rate for the oxidation of both substrates as a function of the concentration of the two modified azurins (apo- and Hg-azurin). Even at very high concentrations the initial rates are not affected, suggesting at first the absence of interactions between the enzyme and the two azurin derivatives. This possibility, however, can be excluded for Hg-azurin, as shown by the experiment shown in Fig. 5. Addition of Hg-azurin seems to have the effect of removing, at least partially, product inhibition and thus, although v appears to be unchanged, this result shows a competition between Hg-azurin and both products (oxidized cytochrome *c*-551 and azurin). This effect is not observed with apo-azurin, and thus cannot be interpreted as a non-specific interaction.

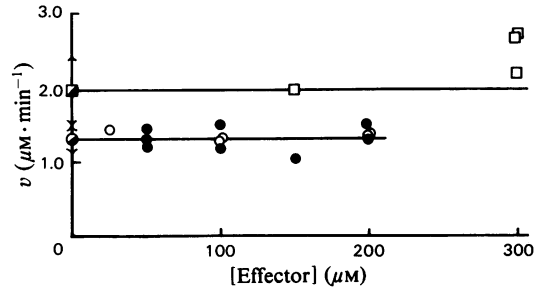


Fig. 4. *Effect of azurin derivatives on the initial velocity of the oxidation reactions catalysed by Ps. aeruginosa cytochrome oxidase*

The initial rates for the oxidation of cytochrome *c*-551 (● and ○) and azurin (□) were measured spectrophotometrically at 551 and 625nm respectively in the presence of various concentrations of Hg-azurin (●) and apo-azurin (○ and □). The controls (◔ and ◪) were repeated 17 and 8 times in the former and the latter case respectively, and the uncertainty bars indicate standard deviations. All other experimental conditions were as indicated in Fig. 1 legend.

Discussion

The steady-state kinetics of oxidation of either azurin or cytochrome *c*-551 catalysed by an oxidase extracted from the same micro-organism, namely *Ps. aeruginosa*, have been re-investigated experimentally, with the use of different procedures for data fitting and handling (Cornish-Bowden & Eisenthal, 1974; Barisas & Gill, 1979; Duggleby, 1981).

In the case of cytochrome *c*-551 the data obtained in experiments exploring a very broad range of concentrations (up to 150μM) cannot be fitted satisfactorily by a simple Michaelis–Menten model (see Fig. 1*c*). In the primary plot of v against substrate concentration, in fact, data in the lower substrate concentration range (e.g. below 20μM, which is the only region explored by all other authors) may appear to reach a plateau; however, on extending the cytochrome *c*-551 concentration

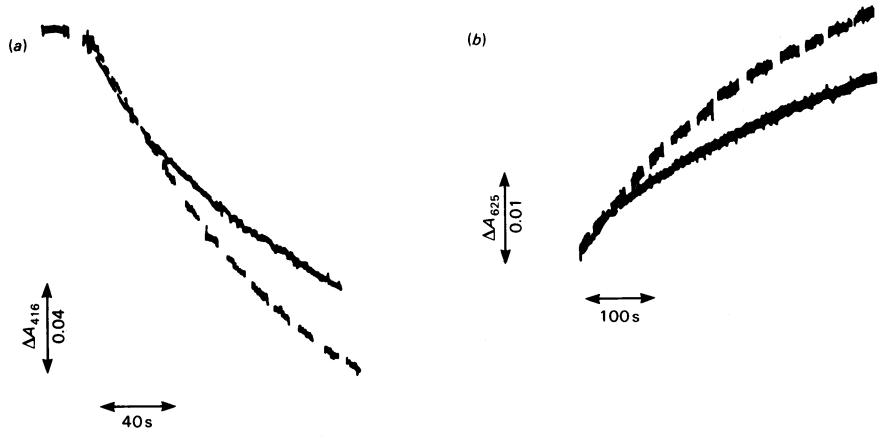


Fig. 5. *Influence of Hg-azurin on the time course of oxidation of cytochrome c-551 and azurin catalysed by Ps. aeruginosa cytochrome oxidase*

The time courses for cytochrome *c*-551 (*a*) and azurin (*b*) oxidation catalysed by *Ps. aeruginosa* cytochrome oxidase were monitored at 416 and 625nm respectively. At the end of the reactions the total absorbance changes observed were 0.86 in the former and 0.11 in the latter case. The continuous traces refer to the controls and the broken traces to the same experiments carried out in the presence of 200μM- and 150μM-Hg-azurin in (*a*) and (*b*) respectively. Substitution of Hg-azurin by apo-azurin did not alter the shape of the controls. All other conditions were as indicated in Fig. 1 legend.

range well above this value v increases considerably.

Table 2 (column B) shows the results of fitting all the data on cytochrome *c*-551 oxidation, reported in Figs. 1(*b*) and 1(*c*), to eqn. (1). This assumes that the two binding sites on the enzyme are characterized by different substrate affinities ($K_{m1} \neq K_{m2}$) and equal contribution to maximal velocity obtained at the highest substrate concentrations. Fixing $V_{max.}$ for both sites on the dimeric enzyme to the value obtained at relatively low substrate concentrations (2.64μM·min^{-1}), where only one site should be active ('or operative') at any time, the best fit yields $K_{m1} = 2.13\mu$M and $K_{m2} = 45\mu$M. The former, reflecting the properties of the first site on the dimer, is very close to the value reported in column A (2.15μM), whereas the latter indicates that complete saturation of dimeric oxidase can only be achieved at high substrate concentrations, largely exceeding those explored in other kinetic investigations, as well as the probable physiological concentration range

The presence of mutually unfavourable interactions between the two sites binding reduced cytochrome *c*-551 may tentatively be correlated to the limited structural information available on cytochrome oxidase, which is a symmetrical dimer where both the haem *c* pair and the haem *d* pair are located close to each other (Takano *et al.*, 1979). Moreover, fluorescence energy-transfer experiments have indicated that the four haem groups are segregated at one extreme of the dimeric structure and that two large protein domains in the dimer are quite distant from the active site (Mitra & Bershon, 1980). It is not at all unreasonable, therefore, to assume that binding of a reduced cytochrome *c*-551 molecule to one of the two subunits of the dimer may limit, by steric hindrance and/or electrostatic effects, binding to the other subunit.

There is no reason *a priori* why the same phenomenon should not occur with azurin as a reducing substrate. However, in the latter case, if the affinity ratio between the first and the second site is similar to that obtained for cytochrome *c*-551, (i.e. 21:1), saturation of the second site would only be significant at substrate concentrations around millimolar.

The physical model underlying eqn. (1) is in general accord with several features of the reactions of *Ps. aeruginosa* cytochrome oxidase, such as (1) the heterogeneity of the fast bimolecular process observed in transient kinetic studies of the reaction between the oxidase and azurin (Parr *et al.*, 1977) and (2) the haem–haem interaction effects, either positive or negative, postulated to account for some features of the redox titrations (Blatt & Pecht, 1979) and the CO-binding isotherm (Parr *et al.*, 1975). Finally, if this model is correct, and considering that the final product of the oxygen reduction is water (see Fig. 2; Yamanaka *et al.*, 1961; Timkovich & Robinson, 1979), the view

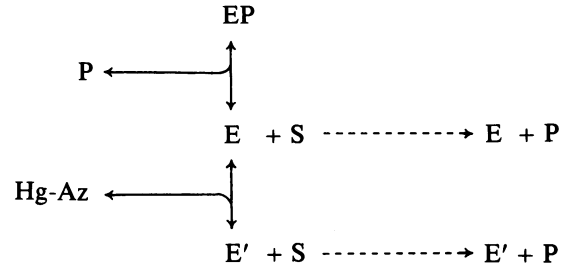
presented above may predict the existence of rather long-lived oxygen intermediates bound to the cytochrome oxidase.

The mechanistic implications of the 'half-hearted' model are not in contrast with the complete reduction of the enzyme by reduced azurin observed in rapid-mixing experiments (Parr *et al.*, 1977). If the unfavourable site–site interactions postulated above affect the stability of the Michaelis–Menten complex, it is conceivable that they become fully amenable to observation under steady-state conditions only.

The complex and apparently asymmetric kinetic pattern appearing when reduced azurin and cytochrome *c*-551 are simultaneously exposed to the action of the enzyme could lead to the conclusion that different interactions with the oxidase may be involved in the case of the two substrates. A simpler hypothesis, however, is that the differences seen in Figs. 3(*a*) and 3(*b*) depend on the difference between the two substrates in the stability of the Michaelis–Menten complex and the difficulties in using higher concentrations of cytochrome *c*-551 in view of spectroscopic limitations (see the Materials and methods section). Hence, if higher cytochrome *c*-551(II) concentrations had been experimentally accessible, the trend observed in Fig. 3(*a*) would also have appeared in Fig. 3(*b*). Thus the levelling off of the Dixon plots at high inhibitor concentrations (observed in Fig. 2*a*) may be accounted for by the fact that the two substrates undergo a fast and direct electron re-equilibration, as characterized in several previous reports (Antonini *et al.*, 1970; Brunori *et al.*, 1975; Silvestrini *et al.*, 1981).

Given the above complexities, we believe, especially on the basis of the results in Fig. 3(*b*), that reduced azurin and cytochrome *c*-551 indeed compete for the same site on the cytochrome oxidase, in agreement with (i) the linear dependence of $1/v$ on the inhibitor concentration under appropriate concentration conditions and (ii) the independence of $1/v$ on the inhibitor concentration at high substrate concentrations.

Another important point emerging from the data reported in the present work is that the Hg-azurin is able to modify the time course of oxidation of both native azurin and cytochrome *c*-551, but not the initial rate (Figs. 4 and 5). The results in Fig. 4 are in agreement with those reported by Blatt (1981) for apo-, Co-, Zn- and Ni-azurin. On this basis, Blatt (1981) assumed the absence of a binding site on the enzyme for the azurin derivatives as well as for the oxidized substrates, and interpreted the inhibitory effect of product, on thermodynamic grounds, as an accumulation of the products. In our case, however, the interpretation must be different, since Fig. 5 indicates a clear interaction between Hg-azurin and the enzyme molecule. This is not completely surprising, since Hg-azurin is thought to be more similar than apo-azurin to the native molecule (Finazzi-Agrò *et al.*, 1973). A minimum scheme accounting for the observed behaviour of the system in the presence of Hg-azurin is as follows:



where E and E′ are two conformational states of the enzyme, the latter being stabilized by the interaction with Hg-azurin (Hg-Az) and insensitive to the inhibitory effect of the product. This implies the presence of a binding site for Hg-azurin well distinct from the site for the substrates, although it cannot be excluded that it may partially overlap with the binding site for the products.

A final comment is merited on the problem of understanding the actual physiological role of azurin and cytochrome *c*-551 in the respiratory chain of *Ps. aeruginosa*. The previous suggestion assigning to cytochrome *c*-551 a pre-eminent role as an electron donor to cytochrome oxidase (Silvestrini *et al.*, 1982), on the basis of pre-steady-state experiments, has been confirmed by the steady-state kinetic studies reported in the present paper [K_m(Az)/K_m(cyt. *c*-551) = 7; C.C.A.(Az)/C.C.A.(cyt. *c*-551) = 1.3]. Whether azurin and cytochrome *c*-551 are interchangeable electron donors for the cytochrome oxidase, or the principal reason for the presence of one of them must be looked for at a different metabolic step, may only be understood after purifying and studying the substrate specificity of other oxidases present in the cell (Azoulay & Couchoud-Beaumont, 1965; Matsushita *et al.*, 1982).

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