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# The Reduction of *Pseudomonas* Cytochrome $c_{551}$ Oxidase by Chromous Ions

By DONALD BARBER, STEPHEN R. PARR and COLIN GREENWOOD
*School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, Norfolk, U.K.*

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The reduction of cytochrome $c_{551}$ oxidase from *Pseudomonas aeruginosa* by $Cr^{2+}$ ions was followed in the stopped-flow apparatus at a number of wavelengths. The *c*-haem reduction proceeded in a biphasic fashion with second-order rate constants of $2.6\times10^5\,M^{-1}\cdot s^{-1}$ and $4.8\times10^4\,M^{-1}\cdot s^{-1}$ at 25°C, whereas the biphasic reduction of the $d_1$-haem appeared to be independent of reductant concentration with rate constants of approx. $1.0\,s^{-1}$ and $0.25\,s^{-1}$ respectively. The kinetically determined difference spectra (reduced minus oxidized) for the *c*- and $d_1$-haems are presented.

One of the features of *Pseudomonas* cytochrome $c_{551}$ oxidase (EC 1.9.3.2) that greatly facilitates studies on the enzyme is the presence in the protein of two different haems (*c* and $d_1$) (Kuronen & Ellfolk, 1972), with differing visible spectral properties. However, so far, it has only been possible to broadly assign regions of the spectrum to one or other of the haem components because of the spectral overlap (Barber *et al.*, 1976), and no quantitative information has been reported on the relative contributions of the *c*-haem and $d_1$-haem of the intact enzyme throughout the visible spectral region.

$Cr^{2+}$ ions have been used in a number of studies concerning the reduction reactions of redox proteins (Brittain *et al.*, 1974; Brittain & Greenwood, 1975). These studies have shown that Cr(II) is a very efficient reductant and have not revealed any evidence for there being more than one reactive species at the pH used for this work. Furthermore there is some evidence (Kowalski, 1969; Grimes *et al.*, 1974) to suggest that, in mammalian cytochrome *c* at least, the $Cr^{2+}$ ion reacts with the protein at the site normally concerned with the interaction with the natural reductase.

This present paper reports the use of $Cr^{2+}$ ions in an attempt to resolve kinetically the two haem chromophores of *Pseudomonas* cytochrome oxidase and thereby record their characteristic difference spectra.

## Materials and Methods

*Pseudomonas* cytochrome $c_{551}$ oxidase was prepared as described by Parr *et al.* (1976). The ratios $A_{410}/A_{280}$ and $A_{640}/A_{520}$ for the oxidized protein were 1.18–1.2 and 1.15–1.2 respectively. The concentration of the enzyme was determined by using $\varepsilon_{410} = 149\times10^3$ litre·mol$^{-1}$·cm$^{-1}$ for the oxidized form (Horio *et al.*, 1961). $CrCl_2$ solutions were prepared and standardized as reported by Brittain *et al.* (1974) and all the experiments were conducted in cacodylate buffer under anaerobic conditions. All chemicals were from Fisons (Loughborough, Leics., U.K.) and were of A.R. grade. $O_2$-free $N_2$ was from British Oxygen Co., London S.W.19, U.K., and was dispensed from the cylinder and stored in glass vessels over an alkaline solution of anthraquinonesulphonate before use. Spectrophotometry was carried out on a Cary 118C instrument and stopped-flow experiments were performed in an apparatus identical with that described by Gibson & Milnes (1964).

## Results and Discussion

Fig. 1 shows the time courses and associated semilogarithmic analyses for the reduction of *Pseudomonas* cytochrome *c* oxidase when followed at two wavelengths after mixing anaerobically, in the stopped-flow apparatus with $Cr^{2+}$ ions in cacodylate buffer, pH 7.0. From Fig. 1 it is clear that the overall reaction is complex, but on the basis of published spectra (Horio *et al.*, 1961) for *Pseudomonas* cytochrome oxidase and its apo-protein (where the $d_1$-haem has been removed), the bulk of the change at 418 nm can be ascribed to *c*-haem, whereas at 460 nm much slower reactions dominate the absorbance change and these processes may be identified with the reduction of $d_1$-haem. The analysis shown in Figs. 1(*b*) and 1(*d*) indicates that both the *c*- and $d_1$-haems undergo reduction in a characteristically biphasic fashion. However, at wavelengths where the spectral contribution of a particular component is small, for example the slow change due to $d_1$-haem at 418 nm, separation of all four kinetic phases is not possible and the minor contributor analyses as an apparent single exponential. For this reason we have only been able to determine difference spectra that represent the sums of the amplitudes of the two fast (*c*-haem) and two slow ($d_1$-haem) processes rather than obtain

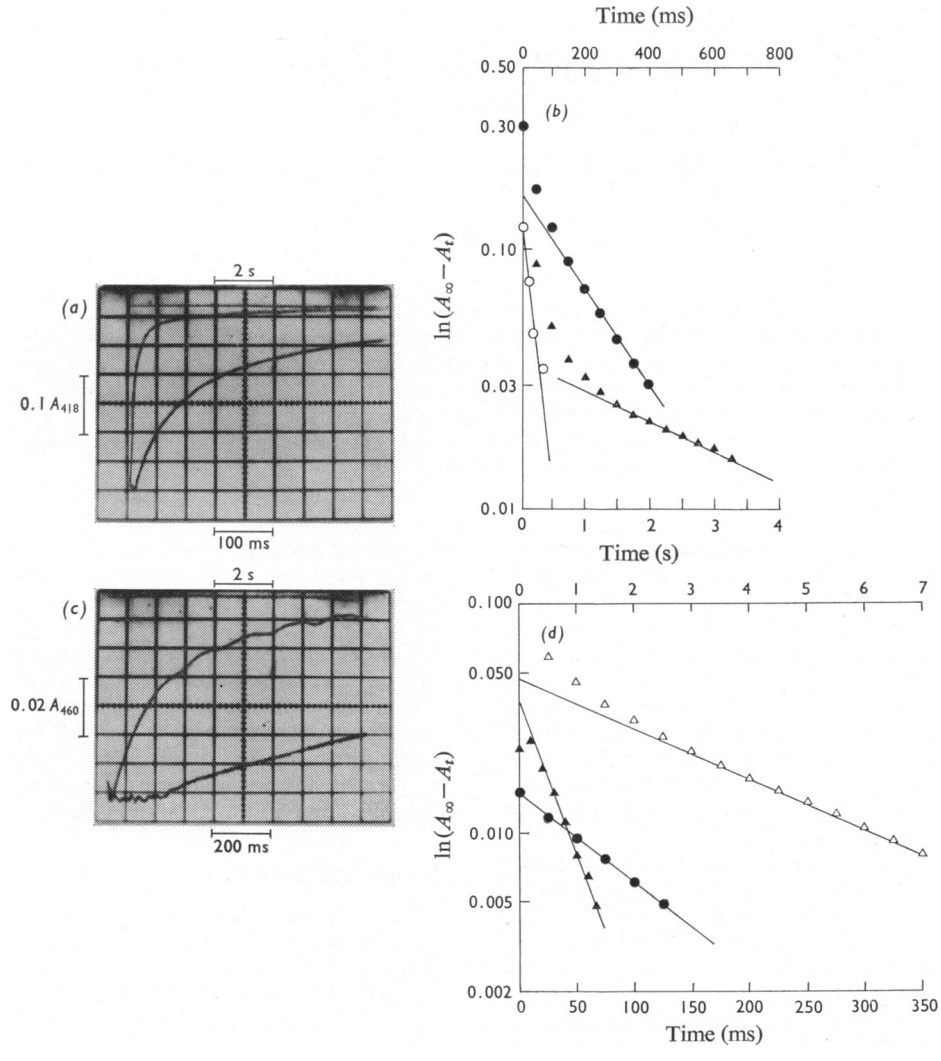


Fig. 1. *Reaction progress curves for the reduction of Pseudomonas cytochrome oxidase by $Cr^{2+}$ ions*
(*a*) An oscilloscope trace produced by mixing 4.4$\mu$M *Pseudomonas* cytochrome oxidase with 140$\mu$M-$Cr^{2+}$ in 0.1M-sodium cacodylate buffer, pH7.0. The reaction was observed at 418nm in a 2cm-path-length cell at a temperature of 25°C. The horizontal scales are 50ms and 1s per division for the lower and upper traces respectively. (*b*) Semi-logarithmic analysis of (*a*). The two fast phases (○, ●) are plotted by using the time scale on the upper abscissa; the apparent (see the text) single slow phase (▲) has been plotted with reference to the lower abscissa. (*c*) An oscilloscope trace produced by mixing 4.4$\mu$M *Pseudomonas* cytochrome oxidase with 310$\mu$M-$Cr^{2+}$. The conditions were as described in (*a*) except that the reaction was observed at 460nm. The horizontal scales represent sweep times of 100ms and 1s per division for the lower and upper traces respectively. (*d*) Semi-logarithmic analysis of (*b*). The two slow phases (△, ▲) were plotted by using the time scale on the upper abscissa; the apparent single fast phase (●) has been plotted with reference to the lower abscissa.

the difference spectrum of each of the four phases separately. The rates of the two processes attributed to *c*-haem reduction were found to depend linearly on $Cr^{2+}$ ion concentration over the range 70–310$\mu$M with second-order rate constants of $2.6\times10^5$M$^{-1}\cdot$s$^{-1}$ and $4.8\times10^4$M$^{-1}\cdot$s$^{-1}$. The relative proportions of these two phases remained constant over the entire concentration range studied, with the faster phase comprising 30–35% of the total *c*-haem reaction. We have also obtained such biphasic reduction behaviour of the *c*-haem in experiments in which the *Pseudomonas* cytochrome oxidase was reduced by azurin

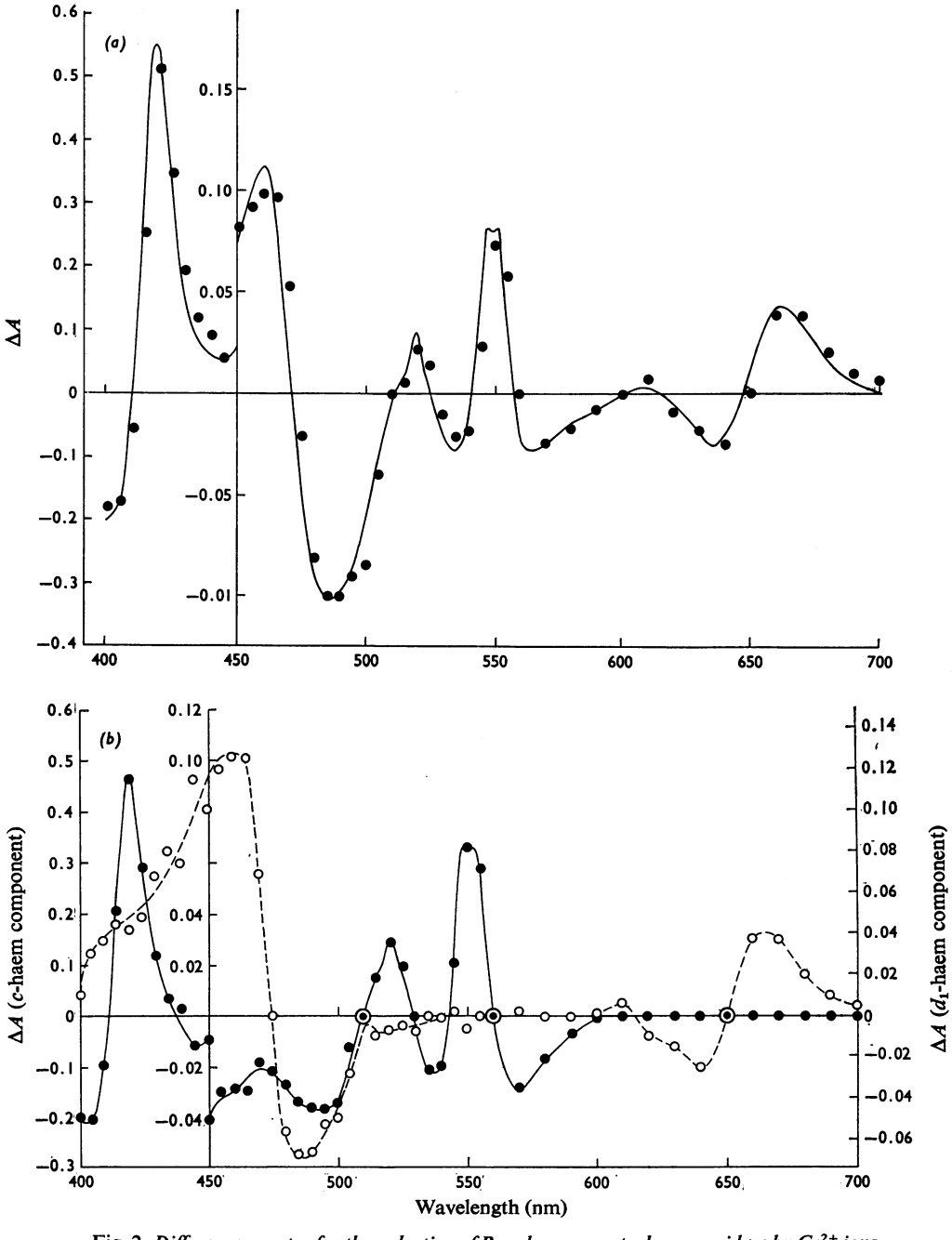


Fig. 2. *Difference spectra for the reduction of Pseudomonas cytochrome oxidase by $Cr^{2+}$ ions*

(*a*) The static difference spectrum (reduced minus oxidized) of 7.5 $\mu$M *Pseudomonas* cytochrome oxidase (——) determined by use of the Cary 118C spectrophotometer and the total kinetic difference spectrum (●), representing the change in absorbance from 3 ms, after mixing the protein with 440 $\mu$M-$Cr^{2+}$ in the stopped-flow apparatus to the final value. The reactions were carried out in 0.1 M-sodium cacodylate, pH 7.0, at 25°C in a 2 cm-path-length cell. (*b*) The reduced-minus-oxidized difference spectra produced on analysis of the total kinetic difference spectrum in (*a*) into *c*- and $d_1$-haem phases. The two left-hand ordinates (400–450 nm and 450–700 nm) refer to the *c*-haem component (●); the right-hand ordinate refers to the $d_1$-haem (○).

[Cu(I)], one of its physiological substrates (S. R. Parr, D. Barber & C. Greenwood, unpublished work). The dependence of the rates, on $Cr^{2+}$ ion concentration, of either of the processes attributed to the reduction of the $d_1$-haem component of the enzyme was in no sense so clearly marked. The rate constant of the faster of the two phases varied between 0.8 and $1.8\,s^{-1}$ and for the slower process was about $0.25\,s^{-1}$, a value identical with that attributed by S. R. Parr, D. Barber & C. Greenwood (unpublished work) to the internal electron transfer from *c*- to $d_1$-haem. We cannot at this stage exclude the possibility that the faster phase of $d_1$-haem reduction represents a direct attack by $Cr^{2+}$. Because of the apparent lack of any strong dependence of $d_1$-haem reduction on $Cr^{2+}$ ion concentration is has been possible to select conditions that permit a clear kinetic separation of the reactions of the two contributing chromophores and thus generate their reduced-minus-oxidized difference spectra.

Fig. 2(*a*) compares the overall difference spectrum determined kinetically with that obtained statically for the oxidized and reduced forms of the enzyme, and it is evident from the close correlation of these two difference spectra that no events are being overlooked during the 'dead time' of the stopped-flow apparatus. Fig. 2(*b*) presents the kinetically resolved difference spectra (reduced minus oxidized) for the *c*- and $d_1$-haem components of the intact enzyme. It can be seen from Fig. 2(*b*) that the solid line, which represents the sum of the two fast $Cr^{2+}$-dependent phases, is typical of the difference spectrum of a *c*-type cytochrome with extrema at 405, 420, 445, 520, 535, 550 and 570nm. The difference spectrum of the $d_1$-haem shows a Soret difference of much lower extinction with extrema at 460 and 490nm and a broad $\alpha$-band difference over the wavelength range 615–700nm with extrema at 640 and 665nm. The effect of any absorbance changes associated with the oxidation of $Cr^{2+}$ ions has been ignored. Spectrophotometric studies of the chromium(II) and chromium(III) cacodylate complexes have shown that any absorbance changes due to this process would be comparatively small; the maximal change occurs at 435nm with a $\Delta\varepsilon$ of 30.8 $litre \cdot mol^{-1} \cdot cm^{-1}$, which corresponds, at worst, to a $\Delta A$ of less than 0.001. Static spectra of reduced *Pseudomonas* cytochrome oxidase were found to be indistinguishable from those produced by using ascorbate as the reductant, provided that the concentration of $Cr^{2+}$ ions added was close to the stoicheiometric amount.

In summary, a definitive interpretation of the complicated mechanism implicit in these kinetic observations on the reduction of *Pseudomonas* cytochrome oxidase by $Cr^{2+}$ does not appear to be justified. The inhomogeneous nature of the reaction of both haem moieties offers no obvious relationship either between the two haems or in terms of a possible dimeric model for the structure of the molecule (Kuronen & Ellfolk, 1972). The fact that the relative proportions of the fast and slow reactions of both haem moieties did not appear to change with $Cr^{2+}$ concentration would, however, imply that under the conditions of the experiment two different species of each haem were present and that if any interconversion is possible from one form into another then it must be a very slow process. In spite of this obvious complexity we have achieved a satisfactory separation of the contributions of the two haem moieties to the overall difference spectrum of *Pseudomonas* cytochrome $c_{551}$ oxidase and this should be of particular value in assessing spectrophotometric results in future studies.

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