Circular-dichroic properties and secondary structure of *Pseudomonas aeruginosa* soluble cytochrome *c* oxidase

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The c.d. spectra of *Pseudomonas aeruginosa* cytochrome *c* oxidase in the oxidized state and the reduced state are reported in the visible- and u.v. absorption regions. In the visible region the comparison between the spectra of reduced cytochrome *c* oxidase and ferrocycytochrome *c*-551 allows the identification of the c.d. bands mainly due to the d_1 haem chromophore in cytochrome *c* oxidase. In the near-u.v. region the assignment of some of the observed peaks to the haem groups and to the aromatic amino acid residues is proposed. A careful analysis of the data in the far-u.v. region leads to the determination of the relative amounts of α -helix and β -sheet in the enzyme, giving for the first time a picture of its secondary structure. A significant difference in this respect between the reduced and the oxidized species is observed and discussed in the light of similar conclusions reported by other workers.

Cytochrome cd_1 (EC 1.9.3.2) is a water-soluble protein involved in the electron-transport chain of the bacterium *Pseudomonas aeruginosa*. It can accept electrons from both azurin and cytochrome *c*-551 and transfer them either to oxygen, reducing it to water, or to nitrite, reducing it to NO (Yamanaka, 1964). The enzyme consists of two identical subunits of 61 000 Da, each containing one *c* and one d_1 haem group (Kuronen & Ellfolk, 1972; Silvestrini *et al.*, 1979).

The attention of several groups to its functional properties has been continuously increasing over the past decade (see the review by Meyer & Kamen, 1982); however, knowledge of the structure of the enzyme is still quite limited. The presently available information on the molecular architecture is, in fact, essentially restricted to: (i) the electron-microscopy investigations by Saraste *et al.* (1977), which confirmed the dimeric structure of the enzyme; (ii) the small-angle X-ray scattering studied by Berger & Wharton (1980), providing the linear dimensions of the molecule, and the preliminary characterization of tridimensional crystals by Akey *et al.* (1980), both indicating a difference in the tertiary structure between the fully reduced and the fully oxidized enzyme; (iii) the

fluorescence data reported by Mitra & Bersohn (1980), which identified the relative position of the haem groups within the protein moiety. The limited structural information on the system, together with the difficulty of obtaining suitable crystals for X-ray-diffraction analysis (Berger & Wharton, 1980) and the complex electronic absorption spectrum of the molecule in the range where the two haem groups are the only absorbing species, has stimulated a systematic investigation of the c.d. properties of the enzyme. The results are reported below.

Materials and methods

Cytochrome *c* oxidase, azurin and cytochrome *c*-551 were purified from *Pseudomonas aeruginosa* (strain N.C.T.C. 6750) as described by Parr *et al.* (1976). The proteins were more than 95% pure on the basis of visible- and u.v.-absorption spectroscopy (Parr *et al.*, 1976) and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969). The cytochrome *c* oxidase samples were always characterized by the following absorbance ratios: $A_{410}/A_{280} > 1.1$ and $A_{640}/A_{520} > 1.1$. Reduced cytochrome *c* oxidase was prepared by adding to a degassed concentrated enzyme solution a stoichiometric amount of

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sodium ascorbate, or $\text{Na}_2\text{S}_2\text{O}_4$ and diluting with degassed buffer after complete reduction. A spectroscopic check showed that no oxidation occurred after dilution nor in the time required to run the spectra. Reduced cytochrome *c*-551 was prepared by addition of $\text{Na}_2\text{S}_2\text{O}_4$, the excess being removed by a Sephadex G-25 column. Sperm-whale myoglobin (type II) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and was further purified before use by $(\text{NH}_4)_2\text{SO}_4$ fractionation by the procedure described elsewhere (Antonini & Brunori, 1971). Egg-white lysozyme (crystallized three times; grade I) was purchased from Sigma Chemical Co. and used without additional purification. Protein concentrations were determined on a Cary 219 spectrophotometer, by using the absorption coefficients given in Table 1. All the proteins were dissolved or diluted in 0.04M-sodium phosphate buffer, pH 7.0, and the solutions were centrifuged at 25000g for 80 min before use. C.d. measurements were made with a Jasco J500 spectropolarimeter equipped with a Jasco model DP 500 N data processor. Calibration of the instrument was performed with a dioxan solution of 0.05% androsterone, giving in a 1 cm light-path a c.d. absorption of -192×10^{-3} deg at 304 nm. All the measurements were made at 20°C, except for cytochrome *c*-551 and cytochrome *c* oxidase, in which cases the temperature was 10–12°C. In order to improve the signal-to-noise ratio, all the spectra in the region between 190 and 200 nm have been accumulated eight times, those in the region between 200 and 350 nm four times and those in the visible region twice. The data below 240 nm are expressed as mean residue molecular ellipticities ($\text{deg} \cdot \text{cm}^2 \cdot \text{perdmol}$ of amino acid residues) and those in the visible and near-u.v. regions as molecular ellipticities ($\text{deg} \cdot \text{cm}^2 \cdot \text{perdmol}$ of protein). The mean residue weight of each protein, calculated from its amino acid composition, is reported in Table 1.

To assess the secondary structure, the spectra in the region between 190 and 240 nm have been analysed by the procedure of Provencher & Glöckner (1981).

Results and discussion

Visible-absorption region

Fig. 1 shows the c.d. spectra of oxidized and ascorbate-reduced *Ps. aeruginosa* cytochrome *c* oxidase in the energy region where the *c* and *d*₁ haem groups are the only chromophores. The spectra of oxidized and reduced cytochrome *c*-551 run under identical conditions are also shown in

Fig. 1. C.d. spectra of *Ps. aeruginosa* cytochrome *c* oxidase in the visible region

Panels (a) and (b) refer to the oxidized (—) and to the ascorbate-reduced (---) protein respectively., Spectra of oxidized and reduced cytochrome *c*-551 reported on the same panels for comparison. All the spectra were run in a 1 cm-light-path quartz cuvette.

Table 1. Critical parameters used for the normalization of the c.d. spectra in the present work

For details see the Materials and methods section. Key to references: ^aSilvestrini *et al.* (1979); ^bSilvestrini *et al.* (1983); ^cBrill *et al.* (1968); ^dTang *et al.* (1968); ^eRyden & Lundgren (1976); ^fHorio *et al.* (1960); ^gDayhoff (1972); ^hAntonini & Brunori (1971); ⁱEdmundson (1965); ^jSteiner (1964); ^kJollès (1960); ^lJollès *et al.* (1963).

Protein	$10^{-3} \times \epsilon$ ($\text{M}^{-1} \cdot \text{cm}^{-1}$)	M_r	Mean residue M_r
<i>Ps. aeruginosa</i> cytochrome <i>c</i> oxidase (oxidized)	282 ^a (411 nm)	122000 ^a	110 ^b
Azurin (oxidized)	3.5 ^c (625 nm)	16400 ^d	109 ^e
Cytochrome <i>c</i> -551 (reduced)	28.3 ^f (551 nm)	9000 ^g	109.8 ^g
Myoglobin (reduced)	11.8 ^h (556 nm)	17800 ⁱ	113.1 ⁱ
Lysozyme	39 ^j (280 nm)	14600 ^k	112 ^l

Fig. 1, to give a hint in the assignment of the various bands. Whereas the latter spectra are essentially the same as those published by Vinogradov & Zand (1968), the cytochrome *c* oxidase spectra are rather complex and share very little common features with most of the available haemoprotein spectra (Myer & Pande, 1978). The sign and the position of the maxima in the Soret region are similar to those reported previously for *Ps. aeruginosa* cytochrome *c* oxidase by Oriei *et al.* (1977), although the intensities are rather different, possibly because of the use of different values for the absorption coefficient and molecular mass.

Cytochrome *c*-551 proved in the past to be a useful 'reference' compound for singling out the spectral contributions of the two haem groups of *Ps. aeruginosa* cytochrome *c* oxidase in the cases of magnetic-c.d., e.p.r. (Walsh *et al.*, 1979) and Raman (Ching *et al.*, 1982) spectroscopy. In order to make the spectra of the two proteins directly comparable, the c.d. absorption data are expressed as molecular ellipticities, with for *Ps. aeruginosa* cytochrome *c* oxidase the use of the M_r value of a subunit containing one *c* and one d_1 haem group, namely 61 000 (Silvestrini *et al.*, 1979).

Fig. 1(b) clearly shows the almost complete coincidence of the c.d. spectra of the reduced proteins in the region between 510 and 560 nm, which also in absorption spectroscopy is associated only to the *c* haem group of the oxidase. It is noteworthy that this coincidence disappears when the spectrum of the oxidase is compared with those of various eukaryotic cytochromes *c* (Vinogradov & Zand, 1968).

Thus we can reasonably assume the *c* haem contribution to the c.d. absorption to be similar in *Ps. aeruginosa* cytochrome *c* oxidase and in cytochrome *c*-551, as supported by the close homology between the primary structures of *Ps. aeruginosa* cytochrome *c*-551 and of the fragment carrying the *c* haem group in *Ps. aeruginosa* cytochrome *c* oxidase, outlined by Meyer & Kamen (1982). When the contribution of the *c* haem group is subtracted from the total c.d. spectrum, the c.d. bands that can be attributed to the d_1 haem group are those centred at 640, 455 and 432 nm in the reduced state and at 635 and 428 nm in the oxidized state.

The c.d. bands at 635 nm in the oxidized species and those at 640 and 455 nm in the reduced one are almost coincident with those observed in the absorption spectrum and already assigned to the d_1 haem group (Barber *et al.*, 1977), with a small shift towards shorter wavelengths.

In contrast, the c.d. bands at 428 nm in the oxidized state and 432 nm in the reduced one are not at all observed in absorption spectroscopy, where in this region there is in both cases only one band, centred at wavelengths typical of *c* haem groups.

It is noteworthy that in the absorption spectrum of the oxidized protein the bands of d_1 and *c* haem groups centred at 640 and 520 nm have almost the same absorption coefficient, whereas the *c* haem group appears to be essentially optically inactive in c.d. in the same region.

Near-u.v.-absorption region

This is a region of strong c.d. absorption for *Ps. aeruginosa* cytochrome *c* oxidase as shown in Fig. 2, where the c.d. spectra of both oxidized and reduced derivatives are reported side by side. In this region, the large number of tryptophan, phenylalanine and tyrosine residues present in the molecule (12, 16 and 19 per monomer respectively; Silvestrini *et al.*, 1983) as well as the haem groups clearly contribute to the c.d. values (Myer & Pande, 1978). In the absence of independent information, however, unequivocal assignment of the various peaks to specific residues is impossible, since the overall absorption pattern of the three groups is extremely variable. A careful analysis on a huge number of model compounds has indicated that the relative positions of the peaks strongly depend not only on the solvent composition but also on the stereochemical environment of the chromophores (Strickland, 1974). In spite of this the absorption centred at 298–299, 288–289 and 280 nm may be tentatively assigned to the $\pi \rightarrow \pi^*$ aromatic 1L_a and 1L_b transitions of tryptophan and to the 1L_b transition of tyrosine respectively (Strickland, 1974). The absolute ellipticity values of these bands are in reasonable agreement with the values calculated on the basis of the relative abundance of the three residues given above and their $[\theta]$ reported by Strickland (1974). Such calculation, however, assumes the same $[\theta]$ for all

Fig. 2. C.d. spectra of *Ps. aeruginosa* cytochrome *c* oxidase in the near-u.v. region

The spectra of oxidized (—) and ascorbate-reduced (---) protein were run in a 0.5 cm-light-path quartz cuvette.

tyrosine, tryptophan and phenylalanine residues, thus implying an identical environment for each amino acid residue, which is certainly not the case. Actually, heterogeneity in fluorescence emission behaviour for tryptophan residues in *Ps. aeruginosa* cytochrome *c* oxidase, and hence in their environment, has been demonstrated by Mitra & Bersohn (1980). As a consequence it is more likely that the agreement between the measured and calculated $[\theta]$ values in this region is somewhat fortuitous and that the high absolute value of the c.d. absorption is partly due to a broad haem-group contribution, on top of which the specific bands of aromatic residues are superimposed.

The spectra shown in Fig. 2 indicate differences between the oxidized and the reduced enzyme, on the two sides of an isodichroic area at approx. 298–299 nm. At lower energies, i.e. for wavelengths in the range 320–340 nm a similar difference has been observed in many other cytochromes (Myer & Pande, 1978) and is due to the exclusive contribution of the haem group in the two oxidation states.

At higher energies, for wavelengths in the range 270–295 nm, the situation is more complex, since the decrease in the negative absorption at 288 and 280 nm observed on reduction may be due to some overlapping positive haem-group contribution. If the presence of such contribution could be excluded by independent spectroscopic evidence, the observed decrease in c.d. absorption would be diagnostic of an increased degree of freedom of the aromatic side chains. This would lead to the conclusion that a more mobile and less ordered conformation is associated to the reduced state, in agreement with findings obtained by Mitra *et al.* (1981), who reported a decreased thermal stability of the reduced enzyme with respect to the oxidized one.

Far-u.v.-absorption region

Fig. 3 shows the results obtained on the oxidized and the reduced derivatives of *Ps. aeruginosa* cyto-

Fig. 3. C.d. spectra of oxidized and reduced *Ps. aeruginosa* cytochrome *c* oxidase in the far-u.v. region

The spectra of oxidized (—) and dithionite-reduced (---) protein were run in a 0.2-cm- or 0.1 cm-light-path quartz cuvette. The bars represent the maximum deviation observed for four spectra in the case of the oxidized sample and for two spectra in the case of the reduced sample, obtained on different enzyme preparations. The inset shows an actual spectrum of the oxidized enzyme after a number of accumulations (see the Materials and methods section); the light-path was 0.2 cm between 200 and 250 nm and 0.1 cm between 190 and 210; the protein concentration was 1.1 μ M.

Table 2. Percentages of α -helix and β -sheet obtained by crystallography (when known) and those obtained by the c.d. spectra in the far-u.v. region analysed by the procedure of Provencher & Glöckner (1981)

The standard deviations shown with these estimates do not account for systematic errors, which probably increase the standard deviations to about 6%. Key to references: ^aBlake *et al.* (1965); ^bKendrew *et al.* (1960); ^cAdman *et al.* (1978); ^dAlmassy *et al.* (1978).

Protein	α -Helix (c.d.)	α -Helix (X-ray)	β -Sheet (c.d.)	β -Sheet (X-ray)
Lysozyme	35 $\pm$ 2	41 ^a	29 $\pm$ 2	16 ^a
Myoglobin (oxidized)	78 $\pm$ 1	79 ^b	0	0 ^b
Azurin (oxidized)	4 $\pm$ 1	6–8 ^c	55 $\pm$ 1	55 ^c
Cytochrome <i>c</i> -551 (oxidized)	55 $\pm$ 4	55 ^d	14 $\pm$ 5	0 ^d
<i>Ps. aeruginosa</i> cytochrome <i>c</i> oxidase (oxidized)	16 $\pm$ 1	—	48 $\pm$ 2	—
<i>Ps. aeruginosa</i> cytochrome <i>c</i> oxidase (reduced)	16 $\pm$ 1	—	35 $\pm$ 1	—

chrome *c* oxidase in the region between 190 and 250 nm, where the c.d. absorption of the peptide bond depends on the secondary structure of the protein (Greenfield & Fasman, 1969). The signal-to-noise ratio and the reproducibility of the results are satisfactory, except at or below 200 nm, where with the reduced species the absorbance due to the reductant prevented the measurements.

Among the various methods proposed to extract information on the secondary structure from c.d. spectra, of particular interest is the one proposed by Provencher & Glöckner (1981), which has been very satisfactorily tested up to now in studies on proteins with known X-ray-crystallographic structures. This method is based on the use of known protein structures as internal standards in the fitting procedure of data on unknown systems.

The results of this analysis applied to *Ps. aeruginosa* cytochrome *c* oxidase spectra are reported in Table 2, together with the results of the parallel analysis carried out on known macromolecules, whose c.d. spectra were run under identical conditions.

The agreement with the X-ray-crystallographic results is excellent for myoglobin and azurin and acceptable for lysozyme and cytochrome *c*-551.

In the case of *Ps. aeruginosa* cytochrome *c* oxidase it is noteworthy that the proportion of α -helix is very low as compared with the β -sheet contribution. If, as many indications suggest, the *c*-haem-binding region of the protein is similar to cytochrome *c*-551, which in contrast has a rather high α -helical content, we can speculate that the α -helix array in *Ps. aeruginosa* cytochrome *c* oxidase is mainly concentrated in the *c*-haem-binding region. It may be recalled at this stage that by controlled subtilisin proteolysis (Horowitz *et al.*, 1982) of *Ps. aeruginosa* cytochrome *c* oxidase it is possible to obtain a *c*-haem-containing moiety of approx. 11000 Da. Thus it is not unreasonable to propose a model in which the *c*-haem-binding region in the oxidase is a compact globular domain, rather rich in α -helical structure.

Another interesting element stemming from Table 2 is that there is a significant albeit small difference between the secondary structure of the oxidized and the reduced enzymes. This is in line with a number of independent pieces of evidence pointing to the same conclusion (Berger & Wharton, 1980; Mitra *et al.*, 1981) and reflecting, at different levels of resolution, the macroscopic phenomenon of crystals breaking on reduction (Akey *et al.*, 1980).

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