CO-binding *c*-type cytochromes and a high-potential cytochrome *c* in *Nitrosomonas europaea*

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The purification of two soluble CO-binding cytochromes *c* from *Nitrosomonas europaea* is described. Cytochrome $c_{\text{CO-550}}$ ran on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with an apparent M_r of 32000, whereas for cytochrome $c_{\text{CO-552}}$ the apparent M_r was 16000. Redox potentials ($E_{m,7}$) were determined as +140 and –50 mV respectively. Cytochrome $c_{\text{CO-550}}$ was co-purified with a cytochrome *c*-553, for which an unusually high redox potential of +450 mV was measured. These latter components were not resolved by gel-filtration chromatography or electrophoresis under denaturing conditions.

We have previously described the use of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis combined with spectrophotometry for resolution of the complex mixture of *c*-type cytochromes present in *Nitrosomonas europaea* (Miller & Wood, 1982). Four *c*-type cytochromes were identified, but some minor fluorescent bands on the electrophoretograms remained unassigned. In a separate study we considered the nature of the soluble cytochrome *c* oxidase and whether it could be equated with the CO-binding cytochrome originally observed by Rees & Nason (1965) and termed by them 'cytochrome *o*' (Miller & Wood, 1983). The conclusion was that the soluble oxidase was a copper protein, free of haem, and that there was no evidence for a cytochrome *o* (defined as a CO-binding *b*-type cytochrome), but instead one or more soluble CO-binding cytochromes *c* were present. The present paper draws these two lines of work together and reports the properties of two distinct CO-binding cytochromes *c*, and also of a cytochrome *c* with a very high redox potential.

column (5 cm × 1.5 cm); with a 400 ml linear gradient of 0–100 mM-NaCl, cytochrome *c*-552 was eluted before 50 mM-NaCl, whereas cytochromes $c_{\text{CO-550}}$ and *c*-553 were eluted together at about 70 mM-NaCl.

For purification of cytochrome $c_{\text{CO-552}}$, the pellet from the 75–90% satn.-(NH₄)₂SO₄ fraction described by Miller & Wood (1982) was used. The pellet was dissolved in 10 mM-Tris/HCl buffer, pH 8.0, and applied to a Sepacryl S-200 column (80 cm × 5 cm) equilibrated with the same buffer. A cytochrome that was eluted at M_r about 30000 was collected. Peak fractions were loaded directly on to a short DE-52 DEAE-cellulose column (3 cm × 2.2 cm) equilibrated with the same buffer. The column was washed with 30 ml of the buffer followed by a 400 ml linear gradient of 0–500 mM-NaCl in the buffer. A cytochrome that was eluted at about 75 mM-NaCl gave a reduced + CO-minus-reduced difference spectrum. Peak fractions were pooled, dialysed against the same buffer and concentrated by freeze-drying.

Experimental

Cell growth and harvesting, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and redox potentiometry were as described in Miller & Wood (1982).

Cytochromes $c_{\text{CO-550}}$ and *c*-553 were co-purified as described for cytochrome *c*-550 by Miller & Wood (1982). A separation from the abundant low-molecular-weight cytochrome *c*-552 was achieved on the second DE-52 DEAE-cellulose

Results

The two CO-binding cytochromes *c* are designated cytochrome $c_{\text{CO-550}}$ and $c_{\text{CO-552}}$ from the positions of the α -band troughs in reduced + CO-minus-reduced difference spectra, shown in Figs. 1(a) and 1(b). In each case the trough is at a wavelength similar to the α -band maximum for the reduced cytochrome [see Figs. 1(c) and 1(d)]. Cytochrome $c_{\text{CO-552}}$ gave an $E_{m,7}$ value of –50 mV (detailed results not shown), distinguishing it clearly from the cytochrome *c*-552 reported previously, which had $E_{m,7} = +230$ mV (Miller & Wood, 1982).

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Fig. 1. Room-temperature spectra of purified cytochromes from *N. europaea*

(a) Cytochrome $c_{\text{CO-550}}$ CO-difference spectrum (dithionite-reduced + CO minus dithionite-reduced); (b) cytochrome $c_{\text{CO-552}}$ CO-difference spectrum (dithionite-reduced + CO minus dithionite-reduced); (c) cytochrome $c_{\text{CO-550}}$ difference spectrum (dithionite-reduced minus ferrocyanide-reduced); (d) cytochrome $c_{\text{CO-552}}$ difference spectrum (dithionite-reduced minus ferricyanide-oxidized); (e) cytochrome $c_{\text{CO-553}}$ difference spectrum (ferrocyanide-reduced minus ferricyanide-oxidized). For (a), (c) and (e) a preparation of cytochromes $c_{\text{CO-550}}$ plus $c_{\text{CO-553}}$ was diluted to $0.2 \mu\text{M}$ -haem with 20 mM -Mops (4-morpholinepropanesulphonic acid)/KOH buffer, pH 7.0, containing 100 mM -KCl. (b) and (d) used a preparation of cytochrome $c_{\text{CO-552}}$ diluted in the same way. In (a) the calibration bar represents $0.004 A$, not $0.01 A$ as for (b)–(e).

Fig. 2. Redox potentiometry of *N. europaea* cytochrome $c_{\text{CO-553}}$

The redox potential was determined in a stirred anaerobic cuvette, as described by Miller & Wood (1982), with diaminodurene ($15 \mu\text{M}$) as the mediator. The protein concentration was approx. $25 \mu\text{g/ml}$. The ordinate represents A_{553} relative to a line drawn between 536 and 565 nm, and the curve is a theoretical one-electron plot.

As explained below, cytochrome $c_{\text{CO-550}}$ can be identified with the cytochrome $c_{\text{CO-550}}$ reported by us previously, for which $E_{m,7} = +140 \text{ mV}$ was measured (Miller & Wood, 1982).

The cytochrome $c_{\text{CO-550}}$ preparation also contained a second cytochrome, cytochrome $c_{\text{CO-553}}$. This species had the unusual property of a redox potential at $+450 \text{ mV}$ (see Fig. 2). As a consequence, the use of ferrocyanide as a redox-poising agent enabled spectra to be obtained for each of the components in such mixtures [see Figs. 1(c) and 1(e)]. No reduced + CO-minus-reduced difference spectrum was observed with ferrocyanide as reductant, demonstrating that the high-potential cytochrome did not bind CO.

Despite the clear differences between cytochrome $c_{\text{CO-550}}$ and cytochrome $c_{\text{CO-553}}$ in CO-binding ability, α -band position and redox potential, mixtures of the two were not resolved by chromatography on Sephacryl S-200 or Sephadex G-75. Moreover they gave only a single fluorescent band on sodium dodecyl sulphate/polyacrylamide-gel electrophoretograms (see Fig. 3). This band was that numbered III in our earlier study, and assigned to cytochrome $c_{\text{CO-550}}$ (Miller & Wood, 1982). Its

Fig. 3. Fluorescent gel electrophoresis of cytochromes c_{CO} from *N. europaea*

The Figure shows densitometer scans of a photographic negative exposed to red fluorescence from sodium dodecyl sulphate/polyacrylamide-gel electrophoretograms. The gels were loaded as follows: (a) 235 μ g of dialysed 50–75%-satn.-(NH_4)₂SO₄ fraction; (b) 14 μ g of the purified cytochromes c_{CO-550} plus $c-553$ preparations; (c) 50 μ g of whole soluble preparation; (d) 2.5 μ g of purified cytochrome c_{CO-552} preparation. (a) and (b) were from one gel-electrophoresis experiment, and (c) and (d) from another. Band assignment I–VIII is as given in Miller & Wood (1982). E, gel ends; F, dye front (a and b). The cytochrome c_{CO-552} preparation gave weak bands corresponding to III ($c-553$ plus c_{CO-550}) and VI in addition to the major band (VII).

mobility on electrophoresis corresponded to an M_r of 32000, and the mobility on gel filtration implied an M_r of 37000 (Miller & Wood, 1982). Fig. 3(d) shows that cytochrome c_{CO-552} ran on electrophoretic gels with the same mobility as a band left unidentified in the earlier study, band VII. Its apparent M_r on electrophoresis was 16000.

Fig. 4. Reduced + CO-minus-reduced difference spectrum of whole *N. europaea* cells at 77 K

Cells were harvested, washed and resuspended at 0.1 g/ml as described by Miller & Wood (1982). They were diluted 15-fold in 20 mM-Mops/KOH buffer, pH 7.0, containing 0.1 M-KCl and 1 M-sucrose, and reduced with a few crystals of $Na_2S_2O_4$. The sample cuvette was gassed with CO for 60 s. Both cuvettes were then frozen and scanned as described previously (Miller & Wood, 1982).

Fig. 4 shows a difference spectrum for whole cells at 77 K, both cuvettes being reduced with dithionite and the sample bubbled with CO. The double α -band trough at 546 and 549 nm can be attributed to the presence of two CO-binding cytochromes c as described above. The slight shoulder at 557 nm is caused by peroxidase (Anderson *et al.*, 1968).

Discussion

In our earlier paper we commented that we had been unable to find the cytochrome $c-553$ reported by Tronson *et al.* (1973), described as having an α -band maximum at 553 nm, a redox potential at pH 7.5 of +460 or +500 mV, and an M_r on Sephadex G-75 of 52000 (their fraction III). They also mentioned briefly that it bound CO. Yamanaka & Shinra (1974) commented likewise that they had detected a cytochrome, having this M_r value, that bound CO. In our earlier study this component escaped notice through being inseparable from cytochrome c_{CO-550} on purification and electrophoresis. The M_r was different from that reported for cytochrome $c-553$, and we had not thought it necessary to continue the redox titration above

$E_h = +400$ mV. The results reported in the present paper agree with Tronson *et al.* (1973) for the α -band position and unusually high redox potential, but give a rather lower M_r value. Moreover, they show that cytochrome *c*-553 does not bind CO, but that instead CO binding is associated with a cytochrome that is co-purified with it, namely cytochrome *c*_{CO}-550. It may be that the two proteins run together on columns and gels because they are two forms of the same protein, differing in the sixth ligand to the haem group. The second CO-binding cytochrome *c* reported in the present paper has not been described before and accounts for one of the two previously unassigned bands of fluorescence on sodium dodecyl sulphate/polyacrylamide-gel electrophoretograms (Miller & Wood, 1982). This leaves only one band unaccounted for, namely band V; preliminary studies suggest that it may be a third CO-binding cytochrome *c*.

CO binding to the reduced form is an unusual property of certain *c*-type cytochromes frequently considered to be indicative of a function as a terminal oxidase. However, relief of CO-inhibited respiration by light corresponding to the cytochrome *c*_{CO} absorption peak has been demonstrated for very few cytochromes *c*_{CO} (Weston & Knowles, 1974). Peroxidase is a well-known example of a haem protein that is capable of binding CO when reduced but is without oxidase activity. We have found that dithionite-reduced cytochrome *c*_{CO}-550 also shows spectral changes with NO or NO₂⁻, the latter being reduced to NO by the dithionite (Rich *et al.*, 1978). This points to the cytochrome being able to bind NO, as is the case with most CO-binding

haem proteins. As discussed elsewhere, it is likely that hydroxylamine oxidase does not oxidize its substrate all the way to nitrite (Hooper, 1978; Miller & Wood, 1982). If this view is correct, separate machinery will be required to complete the process. It is tempting to hypothesize that the function of the cytochromes *c*_{CO} in *N. europaea* lies in binding NO resultant from hydroxylamine oxidation and in bringing about its conversion into nitrite. The presence of more than one such cytochrome might be desirable for reasons similar to those behind the presence of multiple terminal oxidases in many bacteria.

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