The Soluble Cytochrome Oxidase of *Nitrosomonas europaea*

By DAVID J. MILLER AND PAUL M. WOOD*

Department of Biochemistry, University of Bristol Medical School, University Walk,
Bristol BS8 1TD, U.K.

(Received 25 October 1982)

The soluble cytochrome oxidase of *Nitrosomonas europaea* has been highly purified and shown to be a copper protein devoid of haem, not a cytochrome *o* as was previously assumed. The native molecular weight was 120 000 and the subunit molecular weight 35 000. Soluble cytochrome oxidase activity co-purified with nitrite reductase activity; both activities were almost certainly associated with the same protein. The possible physiological role of the nitrite reductase activity is discussed.

INTRODUCTION

Nitrosomonas europaea contains both soluble and membrane-bound cytochrome *c* oxidase activities (Rees & Nason, 1965; Rees, 1968; Hooper *et al.*, 1972; Erickson *et al.*, 1972). The membrane oxidase is an *a*-type cytochrome (Erickson *et al.*, 1972); it has been termed cytochrome *a*₁ but, as with certain other bacterial cytochromes *a*, the α -band maximum at 598 nm lies between conventional definitions of *a*₁ and *aa*₃ (Ingledew, 1978). The soluble cytochrome *c* oxidase was first described by Rees & Nason (1965) who also reported the presence of a soluble cytochrome *o*. The cytochrome *o* was identified as such on the basis of CO-difference spectra, and was assumed to be responsible for the soluble oxidase activity. The soluble oxidase seems much less specific than the membrane oxidase; Hooper *et al.* (1972) found that, while 80% of the total cytochrome *c* oxidase activity was membrane-bound, activity with artificial donors such as *p*-phenylene diamine and hydroquinone was almost exclusively soluble. A partial purification was described by Erickson & Hooper (1968); using hydroquinone as the donor, they achieved only a partial separation from hydroxylamine oxidase (EC 1.7.3.4). Rees (1968) showed that cytochrome *c* oxidase and hydroxylamine oxidase could be separated on Sephadex G-200 or on sucrose density gradients. He established a molecular weight of 128 000 by the latter method but made no spectroscopic measurements. Nothing further has been published; several reviews have referred to the soluble cytochrome *o* without comment, despite the fact that other cytochromes *o* are almost exclusively membrane-bound.

Hooper (1968) partially purified nitrite reductase, assayed with hydroxylamine or NADH plus pyocyanin as donor, and commented on its apparent association with soluble oxidase activity. His product contained haem but the inhibition pattern led him to draw analogies with the copper-type nitrite reductase of *Pseudomonas denitrificans* [since renamed *Alcaligenes* sp. (Iwasaki *et al.*, 1975)]. Ritchie & Nicholas (1974) purified nitrite reductase further, without testing for oxidase activity. Separation from hydroxylamine oxidase was difficult but was achieved in very low yield. Their findings with inhibitors were similar to Hooper (1968). The product was essentially free from haem and had a small absorption maximum near 590 nm, similar to the *P. denitrificans* copper-containing nitrite reductase (Iwasaki & Matsubara, 1972).

Abbreviations: DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; DCPIP, 2,6-dichloro-indophenol; DEDTC, diethyldithiocarbamic acid; PMS, phenazine methosulphate (*N*-methyl-2-methylpyrazine methylsulphate).

In the last few years, this nitrite reductase system has acquired environmental interest, since nitrifying bacteria such as *N. europaea* are believed to constitute the largest global source of nitrous oxide (Lipschultz *et al.*, 1981), one of the agents implicated in the destruction of the ozone layer of the stratosphere.

METHODS

Organism and culture methods. A culture of *Nitrosomonas europaea* ATCC 19178 was kindly supplied by Dr N. Walker (Rothamsted Experimental Station, Herts., U.K.). The growth medium and cell culture methods were as described in Miller & Wood (1982).

Assay methods. Cytochrome *c* oxidase activity was measured spectroscopically by following the oxidation of reduced cytochrome *c* at 550 to 552 nm. Assay cuvettes contained 20 μ M-reduced cytochrome *c* in 50 mM-sodium phosphate buffer, pH 7.5. The reaction was initiated by addition of the protein solution. *Nitrosomonas europaea* cytochrome *c* 552 was purified as previously described (Miller & Wood, 1982). Both horse heart and *N. europaea* cytochromes *c* were reduced with an excess of ascorbate in 50 mM-sodium phosphate buffer, pH 7.5, and then separated from excess reductant by gel filtration on Sephadex G-25.

p-Phenylene diamine oxidase activity was assayed as described in Rees (1968) and Hooper *et al.* (1972), except that the buffer used was sodium phosphate (50 mM, pH 7.5).

Nitrite reductase activity was assayed as described in Ritchie & Nicholas (1974), estimating the residual nitrite by a modified Griess-Ilosvay method after incubation of enzyme solutions under O₂-free N₂ with 3 μ mol NADH, 0.1 μ mol PMS and 2 μ mol sodium nitrite in 0.25 M-potassium phosphate buffer, pH 6.0 for 3–10 min (total assay volume = 2 ml).

Protein was determined by the method of Meijbaum-Katzenellenbogen (1955), or (in column fractions only) the method of Warburg & Christian (1941). In both cases, the protein standard was bovine serum albumin (Fraction V, Sigma). Copper was determined in acidified preparations on a Varian Techtron AA6 atomic absorption spectrophotometer fitted with a Varian CRA-90 carbon rod atomizer. Haem was assayed as the pyridine haemochrome by standard methods.

Purification of the soluble cytochrome oxidase. A four-stage purification was developed (Table 1). Approximately 13 g frozen cell pellets was frozen and thawed three times (using solid CO₂ and running cold water) in 10 mM-Tris/HCl, pH 8.0, with addition of a few crystals of DNAase prior to the final freeze-thaw cycle. The DNAase-treated material was centrifuged at 20000 *g* for 20 min at 4 °C; the clear red supernatant was then carefully decanted and subjected to ammonium sulphate fractionation. The fraction precipitating between 75 and 90% saturation was redissolved in 5 ml of the Tris/HCl buffer and desalted simultaneously with fractionation on a large column of Sephacryl S-200 (4.5 $\times$ 45 cm). At this stage, the terminal oxidase was still heavily contaminated with cytochromes, mostly hydroxylamine oxidase. The peak fractions from the S-200 column were applied directly to a short column of DEAE-cellulose (DE-52) in the same buffer (column dimensions 1.5 $\times$ 11.5 cm), the column washed with 33 ml 10 mM-Tris/HCl, pH 8.0, and then eluted with a linear gradient (0–0.5 M) of NaCl in starting buffer (gradient volume 400 ml). Spectra of the fractions containing maximal oxidase activity revealed a degree of contamination by hydroxylamine oxidase. Peak fractions from the first DE-52 column were dialysed overnight against 1 l of the Tris/HCl buffer containing 20 μ M-CuSO₄ at 4 °C (previous experience had shown that significant loss of activity occurred if copper was omitted from the dialysis buffer). The activity of the dialysate was checked, and it was then applied directly to a small (1.5 $\times$ 4.5 cm) column of DE-52, washed with 66 ml of starting buffer, and then eluted with a 400 ml linear gradient of 0 to 0.25 M-NaCl in starting buffer. Oxidase activity coincided with the major protein peak eluting with about 0.2 M-NaCl.

RESULTS

Spectra of whole cell-free supernatants

CO-binding to a reduced cytochrome causes, among other effects, a broadening of the α -band. Thus in difference spectra (reduced plus CO minus reduced) a trough is observed at the original α -band position. If this is close to the α -band maximum for a *b*-type cytochrome (557 to 561 nm) the cytochrome is denoted cytochrome *o*. A trough at a lower wavelength, 550–554 nm, is expected for a CO-binding cytochrome *c*, denoted cytochrome *c*_{CO}. Figure 1 shows the CO-binding spectrum of an unfractionated cell-free supernatant. The spectral features are essentially the same as those reported by Rees & Nason (1965). Notably, the α -band trough is at 550.5 nm with no sign of any shoulder at longer wavelengths. In 1965, no CO-binding cytochrome *c* had been described, and consequently Rees & Nason attributed this feature to a cytochrome *o*. Subsequently, similar cytochromes were observed in many other bacteria

Table 1. Summary of purification scheme

Fraction	Protein (mg ml ⁻¹)	Volume (ml)	Specific activity (arbitrary oxidase units mg ⁻¹)	Yield (%)
Whole extract	13	78	16.5	100
75–90% (NH ₄) ₂ SO ₄ fraction	11.5	5.2	255	91.1
S-200 column (pooled peak fractions)	0.48	20	1740	99.8
DE-52 column (pooled peak fractions)	0.29	26.4	1860	85.1
DE-52 column (pooled peak fractions)	0.034	39.6	4630	37.3

Fig. 1. CO-difference spectrum of whole cell-free supernatant. The supernatant from the initial centrifugation step following freeze-thaw treatment was diluted 20-fold in 50 mM-HEPES/NaOH + 100 mM-NaCl, pH 7.7, and reduced by addition of a few crystals of sodium dithionite. Equal volumes of the reduced preparation were then dispensed into two stoppered glass cuvettes of 1 cm light path, and the baseline spectrum (a) recorded on a Pye-Unicam SP8-200 spectrophotometer. The sample cuvette was then bubbled with CO for 2 min, stoppered, and the resulting difference spectrum (b) recorded immediately.

(Weston & Knowles, 1973). We have found evidence for two distinct cytochromes c_{CO} (D. J. Miller & P. M. Wood, unpublished results). Rees & Nason (1965), followed by others (see Introduction), assumed that this cytochrome *o* was responsible for the cytochrome *c* oxidase activity. We have found the soluble oxidase to be readily separable from the bulk of the CO-binding cytochromes by ammonium sulphate fractionation; the fraction precipitating between 50 and 75% saturation contained the cytochrome c_{CO} , while the soluble oxidase was recovered in the fraction precipitating between 75 and 90% saturation. Such fractionation revealed a further minor component giving a trough in CO-difference spectra at 557 nm. This was identified as peroxidase, a protein purified from *N. europaea* and characterized by Anderson *et al.* (1968).

Spectra of purified soluble cytochrome oxidase and molecular weight determinations

Highly purified preparations of the soluble cytochrome oxidase showed only a single peak (at 280 nm) between 250 and 900 nm. However, the concentrations of protein used were low (50 $\mu\text{g ml}^{-1}$) so that minor spectral features may have been overlooked. The native molecular weight of the protein, determined by gel-filtration on Sephacryl S-200, was approximately 120 000. The following proteins were used to calibrate the column: *N. europaea* hydroxylamine oxidase (mol. wt 200 000), bovine serum albumin (mol. wt 68 000), ovalbumin (mol. wt 45 000), trypsinogen (mol. wt 24 400) and horse heart cytochrome *c* (mol. wt 11 700).

Table 2. *Comparison of inhibitor profiles for the soluble cytochrome oxidase and nitrite reductase*

O₂ uptake assays with *p*-phenylene diamine as electron acceptor were conducted as described in Methods using approx. 5 µg purified oxidase preparation in a total assay volume of 1.5 ml. The rate of O₂ uptake in the absence of inhibitor was determined over 5 min, then the inhibitor was added as 5 to 10 µl of neutralized solution and the rate measured over a further 5–10 min. The effect of DEDTC on oxidase activity was determined in 0.1 M-potassium phosphate buffer, pH 6.0, under the same conditions.

Inhibitor	O ₂ uptake		NO ₂ ⁻ removal		Reference
	Inhibitor concn (mM)	Inhibition (%)	Inhibitor concn (mM)	Inhibition (%)	
KCN	1	62	0.5	77	Hooper (1968)
	2	89	0.1	0	Ritchie & Nicholas (1974)
NaN ₃	0.5	34	1.5	57	Hooper (1968)
	1	51			
DEDTC	0.1	0	6 × 10 ⁻³	100	Hooper (1968)
			2 × 10 ⁻³	55	Hooper (1968)
			0.1	100	Present study
EDTA	5	0			
NaNO ₂	10	0			

The sub-unit molecular weight, as determined by SDS-PAGE on 13.5% slab gels using a Laemmli-type gel system (Laemmli, 1970), was approximately 35000. Protein staining of SDS-slab gels showed that the major component accounted for at least 90% of the total protein present, though a trace of protein running at about 28000 mol. wt (possibly a proteolytic fragment) was also present in these highly purified preparations. The soluble cytochrome oxidase therefore probably consists of three or (more likely) four identical sub-units.

Copper content

The highly purified soluble cytochrome oxidase was devoid of haem but contained 3.72 ± 0.2 mol of copper per mol of protein (mean of four determinations) assuming a molecular weight of 120000. The protein therefore probably bears one copper atom per sub-unit.

Nitrite reductase activity of cytochrome oxidase preparations

The soluble cytochrome oxidase activity always co-purified with nitrite reductase activity, the final oxidase preparations always giving specific activities of 2 to 10 µmol min⁻¹ mg⁻¹, at least an order of magnitude higher than those of the most highly purified preparations of Ritchie & Nicholas (1972, 1974). The elution behaviour from ion-exchange columns and mol. wt on gel filtration also agree well with those reported for nitrite reductase activity by earlier authors.

Electron donor specificity and inhibition profile

As described by previous authors (Hooper *et al.*, 1972) the soluble cytochrome oxidase activity has a broad specificity, accepting electrons from DCPIP, DAD, ascorbate and *p*-phenylene diamine in addition to cytochrome *c*-552 from *N. europaea* and horse heart cytochrome *c*. The *K_m* for horse heart cytochrome *c* was very high (200 µM), but the corresponding figure for cytochrome *c*-552 was 10 µM.

Table 2 summarizes results of inhibition experiments with soluble cytochrome oxidase preparations and compares these with published and present data for the nitrite reductase activity. The only major difference in the inhibition profiles is in the effect of DEDTC, which did not affect oxidase activity, but which inhibited nitrite reductase activity when present at very low levels.

DISCUSSION

The soluble cytochrome oxidase, previously described as cytochrome *o*, is a copper protein devoid of haem. It has a molecular weight of 120000 and probably consists of four identical subunits each having a single copper atom. It is almost certainly also responsible for the nitrite reductase activity of *N. europaea*. Copper-type nitrite reductases have been reported for *Alcaligenes* sp. (formerly called *P. denitrificans*), *Alcaligenes faecalis*, *Achromobacter cycloclastes* and *Rhodopseudomonas sphaeroides* f. *denitrificans* (Iwasaki & Matsubara, 1972; Iwasaki *et al.*, 1975; Sawada *et al.*, 1978; Kakutani *et al.*, 1981). The three cases tested also possess cytochrome oxidase activity, and this is true also for the cytochrome *cd₁* type of nitrite reductase (Yamanaka & Okunuki, 1963; Miyata & Mori, 1969; Iwasaki *et al.*, 1975; Sawada *et al.*, 1978). This duality may reflect similarities between nitrite reduction to N₂O and O₂ reduction to H₂O, both being four electron processes with $E_{m,7}$ close to +800 mV. The copper-type nitrite reductases vary considerably in molecular weight and apparent subunit structure; that of *Alcaligenes faecalis* is closest to the figures reported here, having a tetrameric structure with one copper atom per subunit molecular weight of 30000 (Kakutani *et al.*, 1981). The inhibition profiles of copper-containing nitrite reductases have been reported (Iwasaki & Matsubara, 1972; Iwasaki *et al.*, 1963; Miyata & Mori, 1968; Kakutani *et al.*, 1981; Sawada *et al.*, 1978), but no one has mentioned the effect of inhibitors on oxidase activity. Generally this class of protein is strongly inhibited by KCN or DEDTC (0.1 to 1.0 mM) and relatively insensitive to azide at these levels. By contrast, the *N. europaea* protein is sensitive to 1 mM-azide, while the absence of inhibition of the oxidase activity by 0.1 mM-DEDTC is also noteworthy. Hooper *et al.* (1977) found that addition of DEDTC to aerobic assays for hydroxylamine oxidase activity led to its oxidation, so it cannot be assumed to act solely as a metal-chelating agent.

Why should *N. europaea* contain a soluble cytochrome oxidase/nitrite reductase? The dissimilatory nitrite reductase is normally associated with facultative anaerobes, for which it allows nitrite to be used as an alternative electron acceptor when oxygen is depleted. Yet *N. europaea* is an obligate aerobe, and requires oxygen in particular for the monooxygenase reaction that converts ammonia to hydroxylamine. The use of this protein as an alternative to the membrane-bound cytochrome *a* seems wasteful. Membrane cytochrome oxidases can take the protons required for O₂ reduction from the cytoplasm, thereby contributing to the protonmotive force. By contrast, the soluble oxidases/nitrite reductases are periplasmic for all cases where this has been determined (Wood, 1978; Sawada & Satoh, 1980), so the protons would have to come from outside. Measurement of relative K_m values for O₂ would help establish whether the oxidase activity has any physiological significance. With the nitrite reductase activity the position is rather different. It is well known that nitrifiers such as *N. europaea* produce a small amount of N₂O, with the yield rising markedly as the oxygen tension falls (Lipschultz *et al.*, 1981). Nitrite will normally be available to the cells, and nitrite reductase activity could be useful by enabling a sparing of oxygen when only limited amounts are available.

This research was supported by a grant from the Science and Engineering Research Council. The technical assistance of Dr S. Hopkin is gratefully acknowledged.

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