Characterization of the *c*-type cytochromes of *Nitrosomonas europaea* with the aid of fluorescent gels

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When a total soluble extract of *Nitrosomonas europaea* was denatured with dodecyl sulphate, subjected to dodecyl sulphate/polyacrylamide-gel electrophoresis and illuminated with near-u.v. light, eight bands of protein fluorescence were observed. All but one of these bands were red in colour, a property characteristic of *c*-type cytochromes. Standard techniques were used to purify soluble *c*-type cytochromes from this organism, and it was then possible to assign all but two very minor bands to specific *c*-type cytochromes, namely hydroxylamine oxidase, cytochrome *c*-554, cytochrome *c*-552 and a cytochrome *c*-550 not previously described. The eighth band had fluorescence peaking in the green region of the spectrum, probably caused by covalently bound flavin, and co-purified with hydroxylamine oxidase. The following physical properties were determined for these components: isoelectric point, molecular weights according to gel filtration and mobility on dodecyl sulphate/polyacrylamide gels, and α -band spectra at room temperature and 77 K. Redox potentials were measured as follows: cytochrome *c*-554, $E_{m,7} = +20$ mV; cytochrome *c*-552, $E_{m,7} = +230$ mV; cytochrome *c*-550, $E_{m,7} = +140$ mV. When washed membranes were applied to dodecyl sulphate/polyacrylamide gels in the same way, a number of fluorescent bands were observed that could be matched by soluble proteins. In addition, there was one band that could not be detected in supernatants, migrating with an apparent molecular weight of 24 000. This species is probably coincident with a *c*-type cytochrome having $E_{m,7} = +170$ mV found in redox titration of these membranes. In future studies, gel fluorescence should form a useful complement to spectroscopy for analysis of cytochrome composition in active cell-free preparations or semi-purified material.

Katan (1976) showed that mitochondrial *c*-type cytochromes gave bands of red fluorescence when subjected to SDS/polyacrylamide-gel electrophoresis and excited with near-u.v. light. Wood (1981) has described how this technique can be modified for use with bacterial material, including unpurified soluble and membrane fractions. By combining this technique with spectrophotometry for the much-studied system of photosynthetic electron transport in *Rhodospseudomonas sphaeroides*, it was possible to demonstrate the existence of a membrane cytochrome, comparable with mitochondrial cytochrome c_1 , that had previously gone undetected (Wood, 1980). As the first stage in a study of electron transport in *Nitrosomonas europaea*, we have applied similar methods to this organism, and the present paper reports the results. As explained below, a large number of

red-fluorescent bands have been observed, and with minor exceptions we have been able to identify which cytochrome is responsible for each of them. This information should be particularly valuable in future studies with membrane vesicles and other active cell-free preparations. During the present work we have also investigated various physical properties of these cytochromes.

N. europaea is the classical example of a nitrifying bacterium, having as its sole source of energy the oxidation of NH_4^+ ions to NO_2^- . There is good evidence that hydroxylamine is formed as an intermediate (Hofman & Lees, 1953). Its oxidation is catalysed by hydroxylamine oxidase (EC 1.7.3.4), a *c*-type cytochrome of exceptional complexity, which has been studied by several research groups. Two reports give molecular weights of about 200 000 and a content of 10–20 haem groups per molecule (Hooper *et al.*, 1978; Yamanaka *et al.*, 1979), but a recent paper states that the 200 000-

Abbreviation used: SDS, sodium dodecyl sulphate.

mol.wt. form is an oligomer, the main subunit having a molecular weight of about 65000 (Terry & Hooper, 1981). Part of its *c*-type haem has an α -band maximum at 559nm when reduced, making for confusion with *b*-type cytochromes, and it also contains the enigmatic chromophore *P*-460. A number of soluble *c*-type cytochromes have been reported in addition to hydroxylamine oxidase [e.g. cytochromes *c*-552, *c*-553 and *c*-554 (Tronson *et al.*, 1973; Yamanaka & Shinra, 1974)], and their properties and likely roles are discussed below.

Experimental

Nitrosomonas europaea A.T.C.C. 19178 was kindly supplied by Dr. N. Walker (Rothamsted Experimental Station, Harpenden, Herts., U.K.). It was grown at 28°C in semi-batch culture in a 10-litre fermenter fitted with pH-stat control (LH Engineering, Stoke Poges, Bucks., U.K.). The growth medium was based on that described by Skinner & Walker (1961) and contained, per litre, 3.3 g of $(\text{NH}_4)_2\text{SO}_4$, 0.53 g of KH_2PO_4 , 67 mg of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 67 mg of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ plus 0.67 mg of Fe added as an equimolar mixture of FeSO_4 and EDTA. The pH was adjusted to 7.8 before inoculation and maintained at this value by addition of autoclaved 5% (w/v) Na_2CO_3 . These conditions supported a doubling time of about 12 h, and gave a yield of about 0.25 g wet wt./litre. As a routine up to two-thirds of the total volume was run off for harvesting and replaced with fresh medium. Cells were harvested by centrifugation at 4°C (28000 *g* for 40 min) followed by resuspension in 50 mM-sodium phosphate buffer, pH 7.5, and re-centrifugation (38000 *g* for 20 min). They were stored as frozen pellets at -10°C until required.

For preparation of cell-free extracts the pellets were suspended in 50 mM-sodium phosphate buffer, pH 7.5, containing 10 mM-2-mercaptoethanol, at 0.5 g wet wt./ml. They were disrupted by three cycles of alternate rapid freezing in solid CO_2 and thawing in room-temperature water. A small amount (250 $\mu\text{g}/\text{ml}$) of deoxyribonuclease was then added. The material was left to stand for 5 min in ice, thoroughly mixed with a hand-operated homogenizer, and centrifuged (25000 *g* for 20 min). The clear red supernatant consisted of the soluble extract, and the pellet formed a crude preparation of cell membranes.

Cytochrome purification from the soluble extract started with the preparation of a series of $(\text{NH}_4)_2\text{SO}_4$ fractions: 0–50%-satn., 50–75%-satn. and 75–90%-satn. precipitates and 90%-satn. supernatant. For purification of cytochrome *c*-554 the 90%-satn.- $(\text{NH}_4)_2\text{SO}_4$ supernatant was used, since it contained at least 80% of this very hydrophilic protein (Yamanaka & Shinra, 1974). A 72 ml volume of the

90%-satn.- $(\text{NH}_4)_2\text{SO}_4$ supernatant was desalted by dialysis against 10 mM-sodium phosphate buffer, pH 7.5. It was concentrated by freeze-drying to 2.4 ml and applied to a Sephacryl S-200 column (35 cm $\times$ 2.6 cm) equilibrated with 10 mM-sodium phosphate buffer, pH 7.5. The fractions corresponding to the cytochrome band were pooled and concentrated. The 50–75%-satn.- $(\text{NH}_4)_2\text{SO}_4$ precipitate contained most of the other cytochromes. It was resuspended in 10 mM-Tris/HCl buffer, pH 8.0, and equilibrated with this buffer by dialysis. The material was then applied to a DEAE-cellulose column (11 cm $\times$ 2 cm) equilibrated with the same buffer. The column was washed with 100 ml of starting buffer and then developed with a 150 ml linear gradient of 0–200 mM-NaCl in the same buffer. A strong cytochrome band was eluted at about 40 mM-NaCl; this material was concentrated to 2 ml and passed down a Sephacryl S-200 column (45 cm $\times$ 2.2 cm) previously equilibrated with 10 mM-Tris/HCl buffer, pH 8.0. Two cytochromes were eluted, a minor component (cytochrome *c*-550) followed by the much more abundant cytochrome *c*-552. Hydroxylamine oxidase was eluted from the 0–200 mM-NaCl gradient at about 100 mM-NaCl, a value rather lower than that reported by Yamanaka & Shinra (1974). It was identified by the 460 nm-absorption peak on reduction by dithionite, but was not assayed. Some preparations gave a very minor further band at 150 mM-NaCl, which on SDS/polyacrylamide-gel electrophoresis ran with the same mobility as the main hydroxylamine oxidase fraction.

c-type cytochromes were detected on SDS/13.5%-polyacrylamide rod gels by their red fluorescence in near-u.v. light, with the same methods for sample preparation, electrophoresis and photography as described in Wood (1981). Dansylated marker proteins, fluorescing green, were used as molecular-weight standards (Wood, 1981). Their mobilities did not differ significantly from those of untreated proteins.

Redox potentials were determined in a stirred anaerobic cuvette (Dutton, 1978) with a Pt-Ag/AgCl combination electrode (Russell pH, Auchtermuchty, Fife, Scotland, U.K.). The potential of the reference electrode (Ag/AgCl/satd. KCl) was taken as $E_h = +198 \text{ mV}$ (Bates, 1954). The medium contained 100 mM-KCl in 20 mM-Mops (4-morpholinopropanesulphonic acid)/NaOH buffer, pH 7.0. The following mediators were added to the sample cuvette: 15 μM -diaminodurene, 15 μM -*N*-methylphenazonium methosulphate, 50 μM -duroquinone, 50 μM -2-hydroxy-1,4-naphthoquinone, 12 μM -anthraquinone, 12 μM -anthraquinone-2-sulphonic acid and 12 μM -anthraquinone-2,6-disulphonic acid.

Protein was assayed by the method of Meibaum-Katzenellenbogen (1955), with bovine serum

albumin (fraction V; Sigma Chemical Co., Poole, Dorset, U.K.) as standard. Protein in column fractions was measured by the method of Warburg & Christian (1941). Difference spectra were recorded with a Pye–Unicam SP.8-200 split-beam spectrophotometer (Pye–Unicam, Cambridge, U.K.), with wavelength calibration provided by a standard solution of holmium perchlorate (BDH Chemicals, Poole, Dorset, U.K.). Molecular-weight determinations by gel-filtration chromatography on Sephacryl S-200 or Sephadex G-75 were conducted as described by Andrews (1965), with columns calibrated with Blue Dextran (Pharmacia, Uppsala, Sweden) and the following marker proteins: horse heart cytochrome *c* (mol.wt. 11 700), sperm-whale myoglobin (mol.wt. 17 000), trypsinogen (mol.wt. 24 400), ovalbumin (mol.wt. 45 000) and bovine serum albumin (mol.wt. 68 000).

Results

Purification and physical properties

The course of cytochrome purification was monitored both spectroscopically (α -band position and magnitude) and by gel fluorescence (relative intensities of different bands), and in this way the purification scheme described above was developed. The designation of the cytochromes as *c*-550, *c*-552 and *c*-554 is based on the absorbance maxima of their α -bands. With the exception of cytochrome *c*-550 preparations all the purified cytochromes gave single bands of red fluorescence on SDS/polyacrylamide-gel electrophoresis, indicating that they were free from contamination with other cytochromes. Absorbance ratios giving a measure of absolute purity are shown in Table 1, with literature values for comparison. This Table also gives estimated isoelectric points.

Published spectra cover all except cytochrome *c*-550 at room temperature (Yamanaka & Shinra, 1974; Hooper *et al.*, 1978; Yamanaka *et al.*, 1979), but only hydroxylamine oxidase at 77 K (Hooper *et al.*, 1978). In view of this, Fig. 1 shows α -band profiles at 77 K. There is a definite asymmetry for cytochromes *c*-552 and *c*-554 and a separate peak at 556 nm for the long-wavelength absorption of hydroxylamine oxidase. It should be noted that an asymmetric α -band at 77 K is not an indication of impurity; see, for instance, the spectrum of horse heart cytochrome *c* reported by Estabrook (1957) or Wood (1980).

We have also investigated their behaviour in redox titrations, previously only reported for cytochrome *c*-552 (Tronson *et al.*, 1973; Yamanaka & Shinra, 1974). Cytochrome *c*-552 gave a plot fitting closely to a one-electron transfer with $E_{m,7} = +230$ mV. Similar experiments with cytochromes *c*-550 and *c*-554 also gave good profiles for one-electron transfer, with $E_{m,7}$ at +140 and +20 mV respectively. Thus the reported stoichiometry of two haem groups per molecule for cytochrome *c*-554 did not affect its redox behaviour noticeably (Yamanaka & Shinra, 1974).

Assignment of fluorescent bands and molecular-weight determinations

Fig. 2 shows densitometer scans of a photographic negative for fluorescence from SDS/polyacrylamide-rod-gel electrophoretograms. Trace (a) is for a gel loaded with supernatant from centrifugation of frozen-and-thawed cells. As can be seen, such complete soluble extracts gave at least eight distinct fluorescent bands, numbered I–VIII in order of decreasing molecular weight. Some variation in relative intensity was observed, but the same major bands at high, intermediate and very low molecular

Table 1. Absorbance ratios and isoelectric points of *c*-type cytochromes of *N. europaea*

Isoelectric focusing was conducted in thin polyacrylamide slab gels (0.2 mm), with LKB Ampholines 1809–101 (pH 3.5–10), 1809–106 (pH 2.5–4) and 1809–121 (pH 5–7) each at 3% (v/v) (LKB Produkter, Bromma, Sweden). Isoelectric points above pH 4.7 were assayed by comparison of mobilities with those of coloured proteins of known pI, supplied as a marker kit (BDH Chemicals). The marker kit extended down only to pH 4.7, so for very acidic pI values the pH was measured after diffusion of ampholyte from 2 mm gel sections into distilled water. Key to references: ^a Yamanaka & Shinra (1974); ^b Yamanaka *et al.* (1979); ^c Hooper *et al.* (1978).

Component	Absorbance relative to A_{280}		Isoelectric point (pI)	
	Present work	Published values	Present work	Published values
Cytochrome <i>c</i> -552	$A_{552}^{\text{red}} = 0.6$	1.0 ^a	3.7	3.7 ^a
Cytochrome <i>c</i> -554	$A_{554}^{\text{red}} = 1.0$	1.0 ^a	10*	10.7 ^a
Cytochrome <i>c</i> -550	$A_{550}^{\text{red}} = 0.4$	None	4.2	None
Hydroxylamine oxidase	$A_{410}^{\text{ox}} = 3$	3.0 ^{b,c}	5.3	4.7 ^b , 5.3 ^c

* Cytochrome *c*-554 migrated off the end of the focusing gel with a mobility similar to horse heart cytochrome *c* (pI 10.6).

Fig. 1. Difference spectra of *c*-type cytochromes of *N. europaea* at 77 K

The specially designed cuvette as described by Bendall *et al.* (1971) was used. In each case the cytochrome was present at about $0.5 \mu\text{M}$, as determined from the pyridine haemochrome (Wood, 1980), in 50 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH buffer, pH 7.7, containing 1 M-sucrose. $\text{K}_3\text{Fe}(\text{CN})_6$ (final concn. $130 \mu\text{M}$) was added to the reference cuvette and a few crystals of $\text{Na}_2\text{S}_2\text{O}_4$ to the sample. Trace (a), cytochrome *c*-550; trace (b), hydroxylamine oxidase; trace (c), cytochrome *c*-552; trace (d), cytochrome *c*-554.

weight were always present. Traces (b)–(e) show how these bands could be matched with purified cytochromes. Hydroxylamine oxidase gave the major band I and minor bands II and VI (see trace b); band VI, unlike the others, had a yellowish-green colour as observed, and is discussed below; the possible significance of band II is considered in the Discussion section. The cytochrome *c*-550 preparation gave a major band coinciding with band III (trace c), although band VII was present as a contaminant. Band IV could be matched with purified cytochrome *c*-554 (trace d), and band VIII coincided with purified cytochrome *c*-552 (trace e). Thus only bands V and VII, both minor components, remain unassigned.

The distinct character of the fluorescence from band VI is brought out by densitometry at different wavelengths after colour photography, as shown in Fig. 3. Its mobility corresponded to a molecular

Fig. 2. Red-fluorescent bands of *N. europaea* preparations and their assignment

The Figure shows densitometer scans of a photographic negative exposed to red fluorescence from SDS/polyacrylamide-rod-gel electrophoretograms. The gels were loaded as follows: trace (a), whole soluble extract ($150 \mu\text{g}$ of protein); trace (b), hydroxylamine oxidase ($5 \mu\text{g}$); trace (c), cytochrome *c*-550 as prepared ($10 \mu\text{g}$); trace (d), cytochrome *c*-554 ($2 \mu\text{g}$); trace (e), cytochrome *c*-552 ($4 \mu\text{g}$). The assignment of the fluorescent bands I–VIII is explained in the text. S, start of stacking gel; R, start of resolving gel; F, dye front.

weight of about 18000. Katan (1976) remarked on similar fluorescence after electrophoresis of sub-

Fig. 3. Distinct character of fluorescence from band VI of *N. europaea* extract

An SDS/polyacrylamide rod gel was loaded with whole soluble extract (150 μ g of protein) and subjected to electrophoresis. Its fluorescence was photographed with Ektachrome 64 film through a K2 Hoya yellow filter and a Hoyarex UV/haze filter (Hoya, Tokyo, Japan) and developed in accordance with Kodak process E6. Densitometer scans were taken with the light-beam set at different wavelengths: trace (a), 650 nm; trace (b), 600 nm; trace (c), 550 nm. Band assignments I–VIII are as indicated in Fig. 2.

Fig. 4. Red-fluorescent bands for washed *N. europaea* membranes

The membranes pelleted by centrifugation subsequent to the freeze/thaw process were washed three times in 50 mM-sodium phosphate buffer, pH 7.5, with resuspension each time with both a glass/Teflon homogenizer and an ultrasonic water bath. After resuspension in the same buffer (50 ml for 5 g wet wt. of cells), the membranes were passed twice through a French press (0.12 GPa). This was followed by centrifugation (20 000 *g* for 20 min) and re-centrifugation of the supernatant (78 000 *g* for 2 h). The high-speed pellet was resuspended in the same buffer containing 5% NaCl, stirred overnight at 4°C and re-centrifuged (78 000 *g* for 2 h). Finally the pellet was resuspended in 50 mM-sodium phosphate buffer, pH 7.5. The Figure shows densitometer scans of a photographic negative as indicated in Fig. 2 legend. The gels were loaded as follows: trace (a), membranes (600 μ g of protein); trace (b), membranes as in (a) plus purified cytochromes *c*-554 and *c*-550 (0.5 and 2.5 μ g of protein respectively). M, a fluorescent band that could not be matched in soluble fractions; other labelling is as in Fig. 2.

mitochondrial particles and attributed it to covalently bound flavin on succinate dehydrogenase.

Gel-filtration chromatography yielded the following molecular weights: cytochrome *c*-552, 10 500; cytochrome *c*-554, 27 000; cytochrome *c*-550, 36 000 (Sephadex G-75) and 38 000 (Sephacryl S-200); hydroxylamine oxidase, about 200 000. These values can be compared with the mobilities on fluorescent gels. Cytochrome *c*-552 was found to run well in advance of horse heart cytochrome-*c* (mol.wt. 11 700), a result probably explained by the unreliability of SDS/polyacrylamide-gel electrophoresis for assessment of molecular weights below 15 000 (Weber *et al.*, 1972). Cytochrome *c*-554 ran with an apparent molecular weight of 28 000, in good agreement with the gel-filtration value, and

cytochrome *c*-550 gave an apparent molecular weight of 32 000, rather lower than the values above. Hydroxylamine oxidase ran as expected from its molecular weight on gel-filtration chromatography.

Detection of a distinct membrane cytochrome *c*

When the crude cell-membrane preparation resulting from breakage of cells by the freeze/thaw process was analysed for gel fluorescence it was found that fluorescent bands corresponding to all the soluble *c*-type cytochromes were still present. It was therefore treated to remove as much soluble protein as possible. The processed membranes were then subjected to SDS/polyacrylamide-gel electrophoresis, and Fig. 4 shows the fluorescence observed. Hydroxylamine oxidase was still clearly

Fig. 5. Redox titration for washed *N. europaea* membranes at pH 7

Membranes as in Fig. 4 were present at 800 µg of protein/ml. The ordinate shows A_{552} relative to a line through 538 and 571 nm. The sigmoid curve represents a theoretical one-electron plot (fraction oxidized = $0.5 + 0.5 \tanh [0.5 \log (E - E_{m,7})/59]$, for E in mV) fitted to the high-potential part of the titration.

visible at the high-molecular-weight end, but at the other end cytochrome *c*-552 could not be detected. There was a cluster of bands at apparent molecular weights of 24 000–30 000. Trace (b) shows that one of these could be matched with purified cytochrome *c*-554, but none coincided with cytochrome *c*-550. It seems likely that one of the remaining two bands can be matched by the unassigned band V of the supernatant. The other one, at 24 000 molecular weight, was absent from supernatants and thus appears to be a distinct membrane component. Fig. 5 shows results for a redox titration with the same membranes. In addition to low-potential contributions, almost certainly attributable to hydroxylamine oxidase and cytochrome *c*-554, a component titrating at +170 mV could be resolved. In the supernatant the only cytochromes having such high potentials were cytochromes *c*-552 and *c*-550, neither of which could be detected in the membranes by gel fluorescence. The implication is that the +170 mV species is a membrane cytochrome, probably corresponding to the band at 24 000 molecular weight. Its α -band peaked at 552 nm in room-temperature spectra.

Discussion

This study has shown that with the aid of fluorescent-gel techniques it has been possible to resolve the complex mixture of *c*-type cytochromes

in *Nitrosomonas europaea* to a greater extent than was possible before. Table 2 presents the results for components other than hydroxylamine oxidase in collected form, and compares them with data from published work. For cytochrome *c*-552 our results agree well with those reported by Yamanaka & Shinra (1974). Cytochrome *c*-552 interacts efficiently with the terminal oxidase, cytochrome a_1 (Yamanaka & Shinra, 1974). Judged by its molecular weight and redox potential, there seems little doubt that it belongs to the same cytochrome family as cytochrome *c*-550 of *Paracoccus denitrificans*, cytochrome c_2 of purple photosynthetic bacteria and mitochondrial cytochrome *c*.

For cytochrome *c*-554 we find a slightly higher molecular weight than did Yamanaka & Shinra (1974), and report a value for the redox potential for the first time. This cytochrome is reported to mediate electron flow from hydroxylamine oxidase (Yamanaka & Shinra, 1974; Yamanaka *et al.*, 1979; Suzuki & Kwok, 1981). Its redox potential, $E_{m,7} = +20$ mV, lends weight to the view that the oxidase oxidizes hydroxylamine to only an intermediate level and not all the way to NO_2^- (Hooper, 1978). This is because the $\text{NH}_2\text{OH}/\text{NO}_2^-$ couple has $E_{m,7} = +60$ mV, whereas for NO as product the value is significantly lower, $E_{m,7}(\text{NH}_2\text{OH}/\text{NO}) = -35$ mV. [$E_{m,7}$ values were calculated with data from Wagman *et al.* (1968), Charlott *et al.* (1958) and Sillén & Martell (1964)]. Cytochrome *c*-554 is usually regarded as passing electrons directly to cytochrome *c*-552 (Yamanaka & Shinra, 1974; Suzuki & Kwok, 1981); this seems energetically wasteful, and we consider that the reported reconstitutions do not rule out the possibility of part of the membrane electron-transport chain mediating *in vivo*. We were unable to deplete the membrane fraction effectively of either cytochrome *c*-554 or hydroxylamine oxidase, perhaps because they became trapped inside small vesicles.

Cytochrome *c*-550 is present in smaller quantities than cytochromes *c*-552 and *c*-554. It does not seem to have been previously reported, unless it corresponds to the cytochrome *c*-553 described by Tronson *et al.* (1973). We were unable to find cytochrome *c*-553, described as having a molecular weight of 52 000 and an abnormally high redox potential, $E_{m,7} = +460$ mV. Yamanaka & Shinra (1974) commented briefly that they had found a cytochrome having this molecular weight and that it bound CO. Cytochrome *c*-550 does bind CO (D. J. Miller & P. M. Wood, unpublished work), but with respect to α -band position and $E_{m,7}$ it has very different properties from those reported for cytochrome *c*-553. It may be that its CO-binding ability reflects a physiological role in NO oxidation, but this will have to await further study.

The distinct membrane cytochrome *c*-552 has not

Table 2. Determinations of molecular weight and redox potential for c-type cytochromes other than hydroxylamine oxidase
Abbreviations: GF, gel filtration; SDS, mobility on SDS/polyacrylamide-gel electrophoresis; H, haem analysis.

Component	Reference	Molecular weight	$E_{m,7}$ (mV)
Cytochrome c-552	Tronson <i>et al.</i> (1973)	12 600 (GF)	+240
	Yamanaka & Shinra (1974)	10 000 (GF)	+250
	Present work	10 500 (GF)	+230
Cytochrome c-554	Yamanaka & Shinra (1974)	21 500 (GF)	
		25 000 (SDS)	
		10 700 (H)	
	Present work	27 000 (GF) 28 000 (SDS)	+20
Cytochrome c-550	Present work	37 000 (GF)	+140
		32 000 (SDS)	
Membrane cytochrome c (c-552)	Present work	24 000 (SDS)	+170

been reported before, and it could scarcely have been distinguished from the other cytochromes without the use of fluorescent gels. It is tempting to see it as an analogue of mitochondrial cytochrome c_1 [cf. the study mentioned above with a photosynthetic bacterium (Wood, 1980)].

Our results for hydroxylamine oxidoreductase can be compared with the complex behaviour reported by Terry & Hooper (1981). They found that brief boiling in SDS gave a major band on gels at an apparent molecular weight of 195 000, as we did. They also reported that prior treatment with *o*-nitrophenylsulphonyl chloride to remove haem led to a single strong protein band at molecular weight 62 700, implying that some of the haem thioether linkages form peptide-peptide bridges. Thus the minor fluorescent band observed by us at about 60 000 molecular weight may be the true monomer. In addition, they detected a small subunit with c-type haem at an apparent molecular weight of 11 000, present at a variable stoichiometry of up to 4 mol/mol of large polypeptide. We observed no low-molecular-weight red-fluorescent material from the purified protein. However, all our preparations gave a green-fluorescent band at molecular weight 18 000 (see Fig. 2), tentatively ascribed to covalent flavin.

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