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# Cytochrome $a_1$ of *Nitrosomonas europaea* resembles $aa_3$-type cytochrome *c* oxidase in many respects

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**Cytochrome *c* oxidase of *Nitrosomonas europaea* has been called cytochrome $a_1$ by Erickson et al. (Erickson, R.H., Hooper, A.B. and Terry, K.R. (1972) Biochim. Biophys. Acta 283, 155–166) because the reduced form of their preparation had the α peak at 595 nm. In the present studies, the enzyme was purified to an electrophoretically almost homogeneous state and some of its properties were studied. The enzyme much resembled cytochrome $aa_3$-type oxidase although its reduced form showed the α peak at 597 nm. (1) The absorption spectra of the CO compound of the reduced enzyme and $CN^-$ compounds of the oxidized and reduced enzyme were similar to those of the respective compounds of cytochrome $aa_3$, as well as the absorption spectrum of the intact enzyme resembled that of the cytochrome. (2) The enzyme possessed two molecules of haem *a* and 1–2 atoms of copper in the molecule. (3) The enzyme molecule was composed of two kinds of subunits of $M_r$ 50000 and 33000, respectively, as are other bacterial cytochromes $aa_3$. Although the enzyme resembled other bacterial cytochromes $aa_3$ in many properties, it differed greatly in two properties; its CO compound was easily dissociated into the oxidized enzyme and CO in air, and 50% inhibition of its activity by $CN^-$ required approx. 100 μM of the reagent. The enzyme oxidized 0.57, 1.6 and 1.8 mol horse, *Candida krusei* and *N. europaea* ferrocytochromes *c* per s per mol haem *a*, respectively, in 10 mM phosphate buffer, pH 6.0. The turnover numbers with eukaryotic ferrocytochromes *c* were increased to 32 and 14, respectively, by addition of cardiolipin (14 $\mu g \cdot ml^{-1}$).**

## Introduction

The cytochrome *a*-type terminal oxidases are classified into two groups, cytochrome $aa_3$-type (including cytochrome $c_1aa_3$-type) and cytochrome $a_1$-type [1]. Some of the bacterial cytochrome $aa_3$-type oxidases have been highly purified and characterized; they resemble the mitochondrial oxidase except for the subunit structures [2–4]. As cytochrome $a_1$-type terminal oxidase has not been extensively purified, its molecular properties are not known [5]. Although, recently, cytochrome $a_1$ has been highly purified as a cytochrome $a_1c_1$ complex from *Nitrobacter agilis*, it is a 'nitrite dehydrogenase' but not a terminal oxidase [6]. Currently, the criterion for designation of cytochrome $a_1$ seems to be based only on spectral properties; there is a tendency to designate a cytochrome with the α band at 585–595 nm as cytochrome $a_1$ without checking the prosthetic group. Indeed, it has been recently indicated that the 'cytochrome $a_1$' of *Escherichia coli* may not have haem *a* [7]. Therefore, it seems necessary to know detailed molecular and enzymatic properties of a cytochrome with the α band around 590 nm before the cytochrome is designated as cytochrome $a_1$.

An *a* type cytochrome *c* oxidase of *Nitro-*

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*somonas europaea* has been partially purified and characterized to some extent [8]. As the oxidase preparation contained haem *a* and showed its $\alpha$ band at 595 nm, it has been termed cytochrome $a_1$. Our present investigation showed that cytochrome $a_1$ of the bacterium resembled cytochrome $aa_3$ in many respects. In the present study, cytochrome *c* oxidase was purified from *N. europaea* to an electrophoretically almost homogeneous state. It showed spectral, molecular and enzymatic properties quite similar to those of other bacterial cytochrome $aa_3$-type terminal oxidases in spite of its $\alpha$ peak at 597 nm.

## Materials and Methods

*Culture of organism*

*N. europaea* (ATCC 19718), kindly supplied by Professor D.J.D. Nicholas (University of Adelaide, Australia), was used throughout this work. Mass cultivation of the organism was performed in 500 l inorganic culture medium [9] using a stainless steel fermenter of 600 l volume. The medium inoculated with the seed culture (50 l) was incubated at 28°C for 5 days with vigorous aeration and mechanical agitation, and then the cells were collected by a continuous-flow centrifuge. About 40 g wet cells (centrifugally packed state) were obtained. The cells were stored at −20°C until use.

*Reagents*

Horse cytochrome *c* (Type VI) was purchased from Sigma Chemical Company (U.S.A.). *Candida krusei* cytochrome *c* was kindly supplied by Sankyo Co., Ltd. (Tokyo). *N. europaea* cytochrome *c*-552 was purified by the method of Yamanaka and Shinra [10]. Azurin of *Pseudomonas aeruginosa* was prepared according to the methods of Yamanaka et al. [11].

*Physical and chemical measurements*

Spectrophotometric determinations were performed in a Hitachi 220A spectrophotometer, using 1 cm light path cuvettes. Haem *a* content was determined by the method of Morrison and Horie [12], assuming the millimolar extinction coefficient at 587 nm of the pyridine ferrohaemochrome *a* to be 26 [13]. Copper content was determined by atomic absorption, with a Varian AA-875 atomic absorption spectrophotometer. Molecular weight estimation by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) was performed by the method of Laemmli [14]. Amino-acid composition was analyzed with a high sensitivity amino-acid analyzer (Irica Instruments Inc., model A-3300, Kyoto, Japan) after the sample was hydrolysed with 6 M HCl for 24 h at 110°C in an evacuated sealed tube. Protein content was determined by the method of Lowry et al. [15] with slight modifications [16]. Oxygen consumption was determined by the use of a Clark-type oxygen electrode (Model 0260 oxygen analyser, Beckman, U.S.A.).

*Assay of enzymatic activity*

The enzymatic activity of cytochrome *c* oxidase was determined spectrophotometrically. The standard reaction mixture contained 10 mM sodium phosphate buffer (pH 6.0)/15 $\mu$M ferrocytochrome *c*/2–10 nM enzyme in a total volume of 1.0 ml. The reaction was started by addition of the enzyme and decrease in the absorbance at 550 nm (or 552 nm in the case of cytochrome *c*-552) was followed spectrophotometrically with time. When the activity was assayed in the presence of cardiolipin, 10 $\mu$l 0.14% (w/v) cardiolipin dissolved in ethanol was added to 1.0 ml of the reaction mixture immediately after addition of the enzyme.

## Results

*Purification*

Frozen cells of about 40 g were suspended in 200 ml 10 mM Tris-HCl buffer (pH 8.0), treated with a sonic oscillator (20 kHz, 150 W, Heat system, U.S.A.) for 30 min, and centrifuged at $3000 \times g$ for 15 min. The supernatant thus obtained was used as the cell-free extract. The difference spectrum, reduced with $Na_2S_2O_4$ minus oxidized with $(NH_4)_2S_2O_8$ of the extract showed the $\alpha$ peak of the *a*-type cytochrome as a small shoulder around 600 nm on a slope of a large absorption trough which was attributed to the presence of *b*- and *c*-type cytochromes. Next, the extract was 20% saturated with $(NH_4)_2SO_4$ and centrifuged at $30\,000 \times g$ for 20 min. The resulting red pellet was resuspended in 200 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 0.7 M KCl

and treated with the sonic oscillator for 30 min. The suspension thus treated was 20% saturated with $(NH_4)_2SO_4$ and centrifuged at 30000 × *g* for 20 min. The sonication-precipitation with $(NH_4)_2SO_4$ treatment as described above was repeated 6-times to remove the soluble proteins from the particulate fraction. The brown pellet thus obtained was suspended in 200 ml of 50 mM Tris-HCl buffer (pH 8.0), containing 0.25 M KCl. The resulting suspension was used as the membrane fraction. It showed an absorption peak at 597 nm in the difference spectrum. To the membrane fraction obtained above was added Triton X-100 to a final concentration of 1% (w/v), and the resulting mixture was treated with a French pressure cell at 1500 kg/cm$^2$. The mixture thus treated was stirred for 18 h at 4°C to solubilize cytochrome *c* oxidase, with 30 min treatment by sonic oscillation every few hours. Then, the suspension was 20% satuarated with $(NH_4)_2SO_4$ and centrifuged at 30000 × *g* for 20 min. The resulting supernatant contained solubilized cytochrome *c* oxidase.

To purify the solubilized enzyme, the extract obtained above was 40% saturated with $(NH_4)_2SO_4$, the resulting mixture was stirred for about 1 h, and centrifuged at 30000 × *g*. The red oily floating material appeared on the top of the aqueous phase in the centrifugal tube was collected by removing the aqueous phase with careful suction. The oily floating material thus obtained was dissolved in 100 ml of 50 mM Tris-HCl buffer (pH 8.0). The resulting solution was fractionated by $(NH_4)_2SO_4$. The precipitate appearing between 24 and 30% saturation was collected by centrifugation, dissolved in a minimal volume of 50 mM Tris-HCl buffer (pH 8.0), containing 1.5% Triton X-100 and the resulting solution was dialyzed for a few hours against the same buffer as used for the dissolution mentioned above. The light green solution thus obtained contained cytochrome *c* oxidase and *c*-type cytochromes, and was subjected to chromatography on a DEAE-cellulose column (4.0 × 5.0 cm) which had been equilibrated with the same buffer. Cytochrome *c* oxidase passed through the column, while some of the *c*-type cytochrome was adsorbed on the column. The eluate which contained about equal amounts of *a*- and *c*-type cytochromes, was dialysed against 10 mM Tris-HCl buffer (pH 8.0), containing 1.5% Triton X-100 and subjected to chromatography with a DEAE-cellulose column (4.0 × 20 cm). The enzyme adsorbed on the column was eluted with a linear gradient solution produced from 200 ml each of 10 mM Tris-HCl buffer (pH 8.0) containing 1.5% Triton X-100 and the buffer containing 1.5% Triton X-100 and 0.2 M NaCl. The enzyme was eluted at an NaCl concentration of about 80 mM. The *c*-type cytochromes, which contaminated the preparation, were removed by this procedure. The eluted enzyme was concentrated by addition of $(NH_4)_2SO_4$ to 40% saturation and centrifuged at 30000 × *g* for 15 min. The green oily material thus obtained was dissolved in a minimal volume of 10 mM Tris-HCl buffer (pH 8.0) and subjected to gel filtration on a Sephacryl S-300 column (3.5 × 120 cm) in 10 mM Tris-HCl buffer (pH 8.0) containing 0.5% (w/v) Tween 20 and 0.25 M NaCl to remove micelles of Triton X-100. The enzyme was eluted earlier than micelles of Triton X-100. To obtain a concentrated solution of the enzyme, the enzyme was precipitated by $(NH_4)_2SO_4$, the precipitate was dissolved in 10 mM Tris-HCl buffer (pH 8.0) containing 0.5% Tween 20, and the resulting solution was dialysed against the same buffer. The solution thus obtained was used as the purified enzyme preparation. A summary of the purification of the enzyme is shown in Table I.

The enzyme preparation was almost pure as judged from the results of slab polyacrylamide gel electrophoresis; a single band was observed in the gel on staining with haem staining reagents [17] or the use of the Nadi reaction [18] (Fig. 1). Although some faint bands were found besides the main band when the gel was stained with Coomassie brilliant blue, the amount of the contaminant seemed to be less than 3% of that of the enzyme as judged from the tint of the stained bands.

In the case of the *N. europaea* enzyme, haem *a* was easily dissociable during the purification. Thus, in the second DEAE-cellulose column chromatography, a considerable amount of free haem *a* usually remained on the column after the holoenzyme was eluted, and in the gel filtration on Sephacryl S-300, free haem *a* was eluted later than the holoenzyme. The ratio of haem to the protein moiety of the purified enzyme preparations varied from 8 to 17 nmol haem *a* per mg protein.

TABLE I

PURIFICATION OF CYTOCHROME *c* OXIDASE FROM *N. EUROPAEA*

Specific activity in nmol of horse ferrocytochrome *c* oxidized per mg protein per s.

| | Total protein (mg) | Haem *a* recovery (%) | Haem *a*/ protein (nmol/mg) | Haem *c*/ haem *a* | Specific activity | |
|---|---|---|---|---|---|---|
| | | | | | without cardiolipin | with cardiolipin |
| Cell-free extract | 898 | 100 | 1.42 | 54 | 13.9 | 18.0 |
| Membrane fraction | 198 | 84 | 5.44 | 20 | 47.1 | 139 |
| Triton X-100 extract | 51.1 | 11 | 2.78 | 44 | 95.6 | 370 |
| First DEAE-cellulose | 12.1 | 8.0 | 8.43 | 0.9 | 135 | 320 |
| Second DEAE-cellulose | 10.3 | 4.5 | 5.61 | ≈ 0 | 11.8 | 64 |
| Sephacryl S-300 | 1.85 | 1.4 | 9.40 [a] | ≈ 0 | 14.1 | 258 |

[a] The best preparation so far obtained contained 17 nmol haem *a* per mg protein (see the text).

*Stability*

The enzyme preparation was stable at −20°C for several months. When the enzyme was subjected to a repeated freezing and thawing, the absorbance at 443 nm of the reduced form decreased and a concomitant increase in absorbance at 426 nm was observed, while cytochrome *c* oxidase activity did not substantially vary. When the enzyme was stored in an ice bath for two weeks, its enzymatic activity was substantially unchanged, while after one-month-storage in an ice bath, the enzyme solution became turbid.

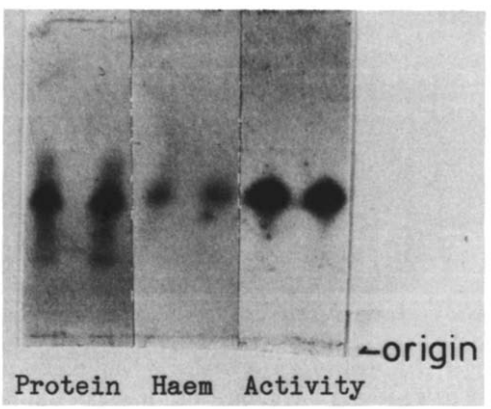


Fig. 1. Electrophoretic profiles of *N. europaea* cytochrome *c* oxidase in polyacrylamide gel. Electrophoresis was performed in polyacrylamide gel (monomer 5%) which had been equilibrated with 0.4 M Tris-HCl buffer (pH 8.9) containing 0.1% Tween 20. After the electrophoresis the slab gel was cut into three strips and these were separately stained with Coomassie brilliant blue (protein), with haem-staining reagents (haem) and by means of the Nadi reaction (activity). Two spots in each staining are duplicates.

*Absorption spectra*

*N. europaea* cytochrome *c* oxidase in the oxidized form showed absorption peaks at 278, 426 and 595 nm and a small peak at 340 nm. On addition of $Na_2S_2O_4$, peaks appeared at 443 and 597 nm (Fig. 2). Fig. 3 shows an absorption spectrum of the oxidized enzyme in the near infrared region; there was no appreciable peak or shoulder between 600 and 900 nm, whereas *Nitrobacter agilis* cytochrome *c* oxidase used as control showed an absorption peak around 840 nm and a shoulder around 655 nm [19].

As the pyridine ferrohaemochrome of the enzyme showed absorption peaks at 431 and 587 nm (Fig. 4), the enzyme seemed to have haem *a*. The millimolar extinction coefficient ($\epsilon_{mM}$) of the $\alpha$ peak at 597 nm of the reduced enzyme was determined to be 23 (haem $a^{-1}$) by assuming $\epsilon_{mM}$ at the $\alpha$ peak of the pyridine ferrohaemochrome *a* to be 26 [13]. In the spectrum of the pyridine ferro-

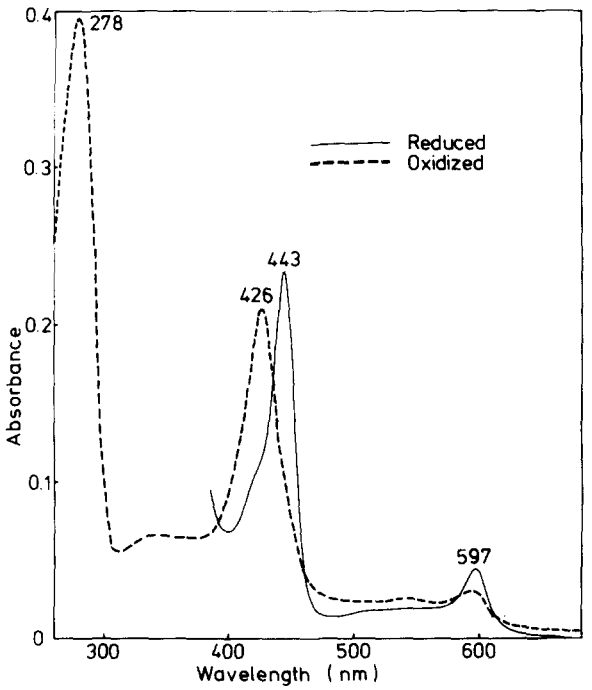


Fig. 2. Absorption spectra of *N. europaea* cytochrome *c* oxidase. The enzyme was dissolved in 10 mM Tris-HCl buffer (pH 8.0), containing 0.5% Tween 20 at a concentration of 0.96 $\mu$M. - - - - - -, Oxidized; ———, reduced with $Na_2S_2O_4$.

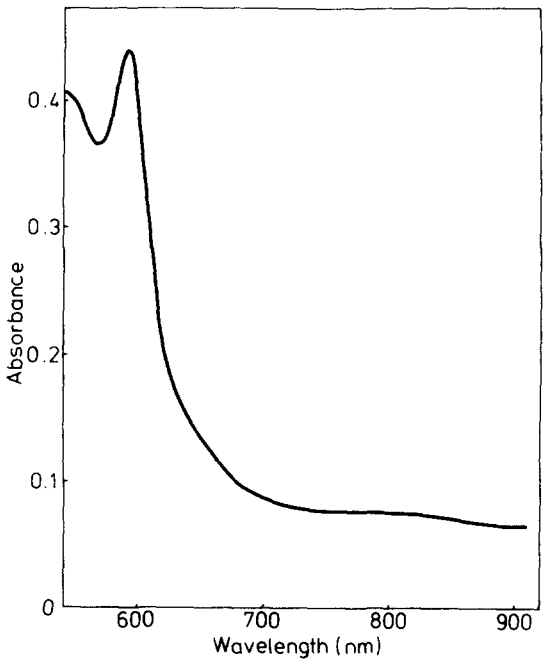


Fig. 3. Absorption spectrum in the near-infrared region of the oxidized form of *N. europaea* cytochrome *c* oxidase. The enzyme was dissolved in the buffer described in the legend for Fig. 2.

haemochrome of the enzyme, no peaks were observed which were attributed to other haems. However, when the enzyme preparation was treated with 1 mM KCN and then stored at 4°C for 2 days, small absorption peaks appeared at 416 and 550 nm and concomitant decrease in the absorbance at 443 and 597 nm was observed in the reduced form. These results seemed to show that a minute amount of cytochrome *c* occurred as a contaminant in the purified enzyme preparation, and its amount was too little to be observed by the absorption peaks in the spectra of the native enzyme or the pyridine ferrohaemochrome of the enzyme.

*Effects of ligands*

When CO was introduced into the reduced form of *N. europaea* cytochrome *c* oxidase, the $\gamma$ band at 443 nm shifted to 433 nm and a shoulder appeared around 440 nm, while the $\alpha$ band at 597 nm shifted to 595 nm and a shoulder appeared at 588 nm (Fig. 5). The absorbance of the $\gamma$ band

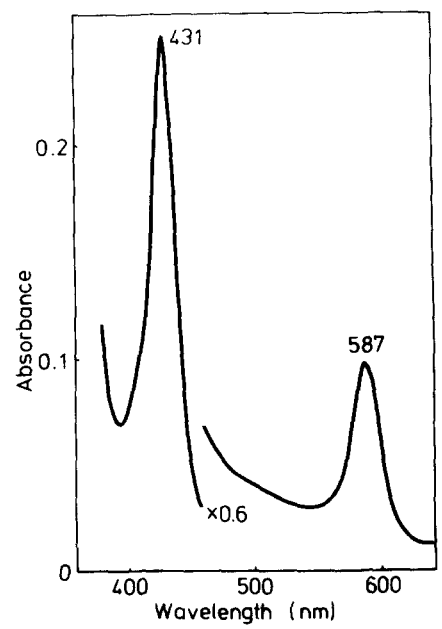


Fig. 4. Absorption spectrum of the pyridine ferrohaemochrome of *N. europaea* cytochrome *c* oxidase. To the enzyme of 1.9 $\mu$M were added 3.3 mM NaOH, 33% pyridine and a small amount of $Na_2S_2O_4$.

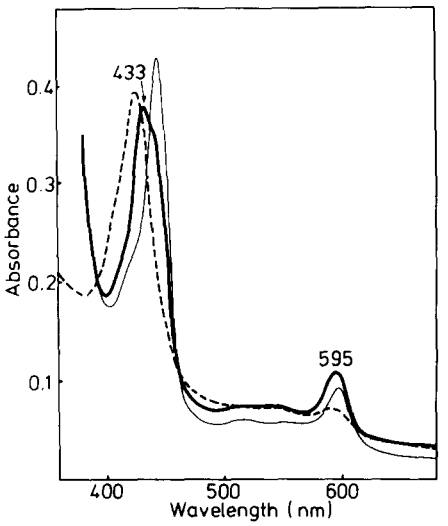


Fig. 5. Effect of carbon monoxide on the absorption spectrum of the reduced *N. europaea* cytochrome *c* oxidase. The enzyme was dissolved in the buffer described in the legend for Fig. 2 and reduced with a small amount of $Na_2S_2O_4$. The reaction with CO of the enzyme was performed in a Thunberg-type cuvette. ------, Oxidized enzyme; ———, reduced with $Na_2S_2O_4$ under Ar atmosphere; ———, reduced enzyme + CO (Ar in a Thunberg-type cuvette was replaced by 100% CO).

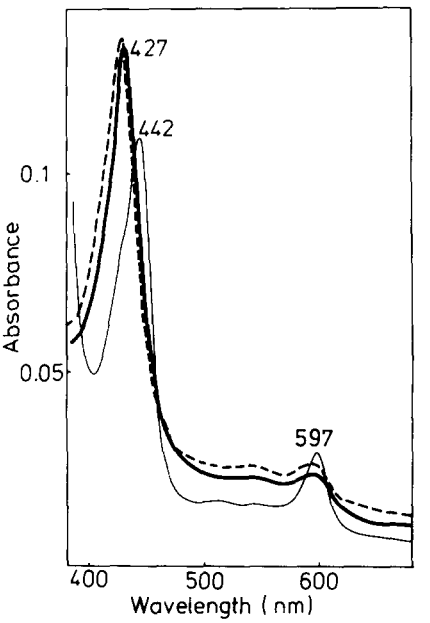


Fig. 6. Effect of KCN on the absorption spectrum of *N. europaea* cytochrome *c* oxidase. The enzyme was dissolved in the buffer described in the legend for Fig. 2. ------, Oxidized; ———, oxidized + 10 mM KCN; ———, KCN-enzyme complex + $Na_2S_2O_4$.

decreased by about 12%, while that of the $\alpha$ band was hardly changed. The difference spectrum, reduced + CO minus reduced, showed peaks at 429 and 592 nm and a trough at 449 nm (data not shown). These spectral properties of the enzyme were similar to those of mitochondrial [20,21] and other bacterial cytochromes $aa_3$ [3,19]. However, the affinity for CO of the *N. europaea* enzyme seemed unusually weak. The CO compound of the reduced enzyme which had been prepared in a Thunberg-type cuvette under 100% CO returned to the original oxidized form within several min when the atmosphere in the cuvette was replaced with air and the solution was gently shaken. The spectrum of the oxidized enzyme was not affected by CO at all.

Addition of 10 mM KCN to the oxidized enzyme caused a shift of the $\gamma$ band at 426 nm to 427 nm and a slight decrease in its absorbance. On subsequent addition of $Na_2S_2O_4$ to the cyanide-enzyme complex, absorption peaks appeared at 442 and 597 nm (Fig. 6). The difference spectrum, oxidized + KCN minus oxidized, showed peaks at 440 and 600 nm, and a trough at 413 nm (data not shown). When 3 mM KCN was added to the reduced enzyme in a Thunberg-type cuvette under anaerobic conditions, absorption peaks appeared at 440 and 594 nm and the latter peak had a shoulder around 585 nm. The absorbance of the $\gamma$ band decreased slightly, while that of the $\alpha$ band was hardly changed (data not shown).

*Molecular properties*

When the enzyme preparation was subjected to polyacrylamide gel (monomer 12.5%) electrophoresis in the presence of sodium dodecyl sulphate (SDS), two bands appeared in the gel stained with Coomassie brilliant blue (Fig. 7). The two bands corresponded to two subunits of 50 and 33 kDa, respectively. In Fig. 7, minor bands are observed which correspond to the proteins of 27 and 88 kDa, respectively. The latter band seemed to be attributable to undissociated enzyme. The presence of $\beta$-mercaptoethanol during pretreatment of the enzyme did not affect the splitting of the enzyme molecule.

The copper content of the enzyme was de-

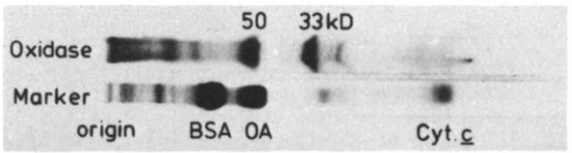


Fig. 7. Polyacrylamide gel electrophoresis of *N. europaea* cytochrome *c* oxidase in the presence of SDS. The electrophoresis was performed in 0.4 M Tris-HCl (pH 8.8) and in the presence of 0.1% SDS. The concentration of acrylamide was 12.5%. BSA, bovine serum albumin; OA, ovalbumin; Cyt *c*, horse cytochrome *c*.

TABLE II

COPPER CONTENT OF *N. EUROPAEA* CYTOCHROME *c* OXIDASE

The concentration is determined spectrophotometrically based on $\epsilon_{mM} = 23$ (haem $a^{-1}$) at 597 nm (oxidase) and $\epsilon_{mM} = 6.95$ at 635 nm (azurin).

| Enzyme or protein | Concentration (nmol/ml) | Copper | |
|---|---|---|---|
| | | (ng/ml) | (ng atom/ml) |
| *N. europaea* oxidase | 3.37 | 150 | 2.36 |
| *P. aeruginosa* azurin | 11.3 | 860 | 13.5 |

termined by atomic absorption. The enzyme preparation was dialyzed overnight against 10 mM Tris-HCl buffer (pH 8.0) containing 0.5% Tween 20 and 10 mM EDTA-2Na, before determination of the metal content. The outer solution after the dialysis did not contain detectable amount of copper. As shown in Table II, the enzyme contained 0.7 g atom of copper per mol of haem *a*. In the determination of copper *Pseudomonas aeruginosa* azurin was included as a standard of the measurement.

The amino acid composition of *N. europaea* cytochrome *c* oxidase is shown in Table III. The composition closely resembled that of *Nitrobacter agilis* cytochrome *c* oxidase as shown in Fig. 8a, while it was less similar to a summation of the compositions of the subunits I and II derived from the bovine heart mitochondrial oxidase (Fig. 8b).

The molecular weight per haem *a* of the *N. europaea* enzyme was estimated to be 59000 on the basis of 17 nmol haem *a* per mg protein. This value was the highest haem *a* content in the en-

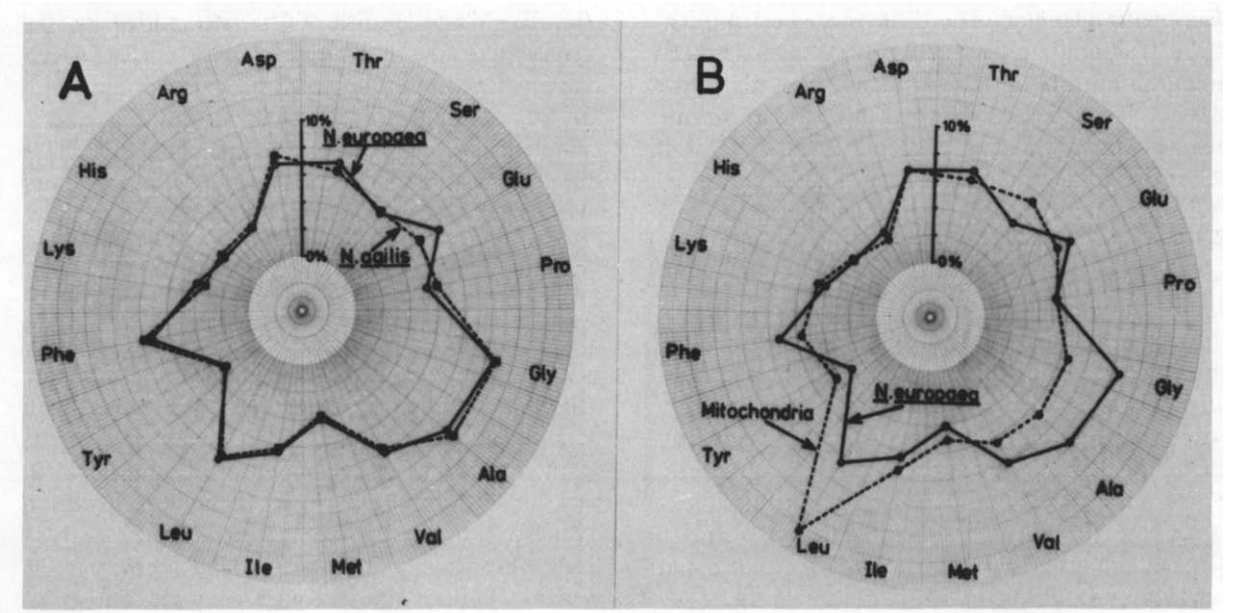


Fig. 8. Comparison of the amino acid compositions of *N. europaea* cytochrome *c* oxidase with those of *Nitrobacter agilis* cytochrome *c* oxidase (A) and those of subunits I and II of the bovine heart mitochondrial cytochrome *c* oxidase (B). The compositions were expressed in mol%. The composition of *N. agilis* and bovine heart mitochondrial enzymes were calculated from the data reported by Yamanaka et al. [19] and Yasunobu et al. [22], respectively.

TABLE III

AMINO-ACID COMPOSITION OF *N. EUROPAEA* CYTOCHROME *c* OXIDASE

Contents of cysteine and tryptophan residues were not determined.

| Amino-acid residue | Mol % |
|---|---|
| Asp | 7.0 |
| Thr | 7.1 |
| Ser | 5.2 |
| Glu | 7.7 |
| Pro | 5.4 |
| Gly | 10.6 |
| Ala | 9.8 |
| Val | 8.1 |
| Met | 4.1 |
| Ile | 6.4 |
| Leu | 8.4 |
| Tyr | 2.9 |
| Phe | 7.3 |
| Lys | 4.1 |
| His | 2.9 |
| Arg | 3.1 |

zyme obtained because haem *a* of the enzyme was easily released.

*Enzymatic activities*

*N. europaea* cytochrome *c* oxidase oxidized horse and yeast (*Candida krusei*) ferrocytochromes *c* and *N. europaea* ferrocytochrome *c*-552. Cardiolipin stimulated the reactivity of the enzyme with eukaryotic cytochromes *c*, while the phospholipid did not affect its reactivity with *N. europaea* cytochrome *c*-552. Table IV shows some enzymatic properties of the *N. europaea* enzyme with different cytochromes *c* as the electron donor and the effect of cardiolipin on its activity. When cardiolipin was added to the reaction mixtures, the activities of the enzyme were enhanced by 60-fold and 9-fold with horse and *C. krusei* ferrocytochromes *c*, respectively, and $K_m$ values of the enzyme for these cytochromes *c* decreased from 8.1 to 3.8 and from 16 to 3.7 $\mu$M, respectively. The concentrations of KCN and $NaN_3$ which inhibited the oxidase activity by 50% are also shown in Table IV.

The oxygen consumption during the oxidation of ferrocytochrome *c* catalyzed by the enzyme was determined with a Clark-type oxygen electrode with limited amounts of ferrocytochrome *c*; the oxygen consumed ($\mu$M) was measured when 40–300 $\mu$M horse ferrocytochrome *c* was completely oxidized in 20 mM sodium phosphate buffer, pH 6.0. The molar ratio of the ferrocytochrome *c* oxidized to oxygen consumed was 4.5. This result indicated that the reduction product of oxygen in the reaction catalyzed by the enzyme was water.

TABLE IV

SOME ENZYMATIC PROPERTIES OF *N. EUROPAEA* CYTOCHROME *c* OXIDASE

| Cytochrome *c* | pH optimum | $K_m$ ($\mu$M) | Molecular activity (mol ferrocytochrome *c* oxidized per mol haem *a* per s) | Concn. of KCN required for 50% inhibition ($\mu$M) | Concn. of $NaN_3$ required for 50% inhibition (mM) |
|---|---|---|---|---|---|
| Horse | | | | | |
| without cardiolipin | 6.1 | 8.1 | 0.57 | 130 | 1.0 |
| with cardiolipin | | 3.8 | 32 | 80 | 0.85 |
| *C. krusei* | | | | | |
| without cardiolipin | 6.1 | 16 | 1.6 | 60 | 6.5 |
| with cardiolipin | | 3.7 | 14 | 80 | 7.0 |
| *N. europaea* (*c*-552) | | | | | |
| without cardiolipin | 5.6 | 31 | 1.8 | 50 | 1.5 |
| with cardiolipin | | | $\approx 0$ | | |

## Discussion

Cytochrome *c* oxidase purified from *N. europaea* possesses many properties similar to those of other bacterial $aa_3$-type terminal oxidases [3,19] and mitochondrial oxidases [20,21] as shown by the results obtained in the present studies, although the enzyme is classified as cytochrome $a_1$-type as its $\alpha$ peak is located at 597 nm.

The position of the $\alpha$ band in the reduced form of the enzyme has not changed during the purification procedure in the present investigation, although Erickson et al. [8] have reported that it shifts from 600 nm to 595 nm upon solubilization of the enzyme with Triton X-100. Although they have reported that the $\alpha$ peak of the enzyme in the cell-free extracts is detected around 600 nm, its position seems to be erroneously observed because it is located as a very small peak at the large trough due to *b*- and *c*-type cytochromes in the reduced-minus-oxidized difference spectrum. So the spectral properties of the enzyme in the membrane bound state seem to be the same as those in the solubilized enzyme.

The ability to oxidize horse ferrocytochrome *c* of the enzyme gradually decreased as its purification proceeded (Table I). The results may be attributable to removal of phospholipids from the enzyme molecule. Thus, the reactivity of the enzyme with eukaryotic cytochrome *c* is enhanced by cardiolipin as is the activity of *Nitrobacter agilis* cytochrome *c* oxidase [23].

The position of the $\gamma$ band of the oxidized form at 426 nm is different from that at 415 nm as reported by Erickson et al. [8]. The spectrum of the reoxidized enzyme preparation which had been reduced with $Na_2S_2O_4$ sometimes showed the $\gamma$ band at 415–420 nm also with the present preparation. However, the band returned to 426 nm when the $Na_2S_2O_4$-reduced enzyme was dialysed. On addition of the oxidation products of $Na_2S_2O_4$, obtained by vigorous shaking of an $Na_2S_2O_4$ solution, the $\gamma$ band of the oxidized enzyme appeared at 415 nm. Therefore, the spectrum of the enzyme preparation obtained by Erickson et al. [8] seems to have suffered a change to some extent by the oxidation products of $Na_2S_2O_4$.

The absorption spectrum of *N. europaea* cytochrome *c* oxidase in the oxidized form shows no peak around 830 and 655 nm. In the case of mitochondrial cytochrome *c* oxidase at the oxidized state, the absorbance at 830 and 655 nm concomitantly decrease with the enzymatic activity by removal of EPR-detectable copper ($Cu_A$) [24]. If the absence of the absorption peaks around 830 and 655 nm in the *N. europaea* oxidase means lack of $Cu_A$, the copper seems unnecessary for the enzymatic activity of the bacterial oxidase. Indeed, from a bacterium, $aa_3$-type cytochrome *c* oxidase has been recently obtained which contains one atom of copper in the molecule and shows a high cytochrome *c* oxidase activity (unpublished results). These results suggest that the *N. europaea* enzyme may contain 2 mol of haem *a* and one g atom of copper in one mol when it is intact. The ratio of haem *a* to copper (1 : 0.7) in the present enzyme preparation will be explained by the results that haem *a* of the enzyme is easily dissociable during the purification; i.e., it can be that the content of haem *a* in the present enzyme preparation is lower than that in the intact enzyme, while that of copper in the preparation is not modified from the value in the intact enzyme. If we assume that the enzyme contains one atom of copper and two molecules of haem *a* in the molecule, the molecular weight of the enzyme per copper is 83000 (or 41000 per haem *a*). This value per copper is in good agreement with the summation of molecular weights of two subunits.

The affinity for CO of the *N. europaea* enzyme is considerably weak as compared with that of the mitochondrial and other bacterial enzymes as it has been reported by Erickson et al. [8]; the CO compound of the reduced *N. europaea* enzyme is obtained only under 100% CO atmosphere and the CO compound is easily dissociable to the enzyme and CO in air. The activity of the *N. europaea* enzyme is considerably resistant to KCN and $NaN_3$; concentrations of KCN and $NaN_3$ required for 50% inhibition of the enzymatic activity are about 100 $\mu$M and 1000 $\mu$M, respectively, while the values for other bacterial and mitochondrial enzymes are about 4 and 4–14 $\mu$M, respectively [4,19,25]. The low affinity for CO and high resistance to KCN and $NaN_3$ of the enzyme may be correlated with lack of the 830 nm band.

The maximum $O_2$ consumption rate by the enzyme in the cell-free extracts was 38 $\mu$mol $O_2$/min

per g protein with *N. europaea* cytochrome *c*-552 as electron donor and 680 μmol $O_2$/min per g protein with horse cytochrome *c*. As washed whole cells of *N. europaea* consume 880 μmol of $O_2$/min per g protein [8], the $O_2$ consumption rate by the enzyme with *N. europaea* cytochrome *c*-552 cannot explain all of the the $O_2$ uptake by whole cells. The discrepancy may be attributable to the following reasons. (1) Some other oxygenases and/or oxidases such as ammonia oxygenase and hydroxylamine oxidoreductase besides cytochrome *c* oxidase utilize oxygen in whole cells. (2) An unidentified activator for the electron transfer between cytochrome *c* oxidase and cytochrome *c*-552 may function in whole cells. (3) Another cytochrome *c* may be present which is the real electron donor for cytochrome *c* oxidase. Further studies to solve these problems are in progress in our laboratory.

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