Biochimica et Biophysica Acta, 591 (1980) 53—62

BBA 47835

CYTOCHROME *a*-TYPE TERMINAL OXIDASE DERIVED FROM *THIOBACILLUS NOVELLUS*

MOLECULAR AND ENZYMATIC PROPERTIES

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(Received October 9th, 1979)

Key words: Cytochrome aa_3; Cytochrome a-type terminal oxidase; Cytochrome oxidase; (Thiobacillus novellus)

Summary

Cytochrome *a*-type terminal oxidase was purified from *Thiobacillus novellus* to an electrophoretically homogeneous state and some of its properties were studied.

The enzyme shows absorption peaks at 428 and 602 nm in the oxidized form, and at 442 and 602 nm in the reduced form. The CO compound of the reduced enzyme shows peaks at 431 and 599 nm. The enzyme has 1 mol of haem *a* and 1 g-atom of copper per 55 600 g and is composed of two kinds of subunit, of 32 000 and 23 000 daltons, respectively.

The enzyme reacts rapidly with tuna, bonito and yeast cytochromes *c* as well as with *T. novellus* cytochrome *c*, while it reacts slowly with horse and cow cytochromes *c*. The reduction product of oxygen catalysed by the enzyme is water.

Introduction

Cytochrome *a*-type oxidase, i.e. cytochrome aa_3 (EC 1.9.3.1.), is widely distributed not only among eukayrotes but also among many prokaryotes [1—3]. As most studies on the cytochrome *a*-type oxidase of bacteria have been done only spectrophotometrically or spectroscopically [1—4], little is known of its molecular features, although a few of them have been purified [5—9]. It is expected that the properties of the bacterial oxidases vary from

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Abbreviations: SDS, sodium dodecyl sulphate; Mes, 2-(*N*-morpholino)ethanesulphonic acid.

organism to organism as judged from its spectral properties, e.g. a minute difference in the position of the α peak of the enzymes is observed among bacteria. Furthermore, we have already revealed that in bacteria many kinds of cytochrome *c* occur which show different reactivities with certain redox enzymes [10—12]. This fact suggests that cytochrome oxidases with different enzymatic properties may occur in bacteria, as cytochrome *c* functions as the direct electron donor for the cytochrome *a*-type oxidase.

As we have already shown, cytochrome *c* derived from *Thiobacillus novellus* resembles eukaryotic cytochrome *c* in its reactivity with certain redox enzymes while it differs from it in some of the molecular properties [13]. This suggests that cytochrome oxidase of the organism may resemble the eukaryotic oxidase with respect to its enzymatic properties but differ from the latter enzyme in its molecular features. The study of such bacterial cytochrome oxidase seems to help us to understand the structure and function relationship not only of the bacterial oxidase but also of the eukaryotic oxidase.

In the present investigation, we purified cytochrome oxidase from *T. novellus* to a homogeneous state, as judged from its gel electrophoretic behaviour, and studied some of its properties.

Materials and Methods

Growth of the bacterium. T. novellus (Starkey) was used in the present investigation, and its mass cultivation was performed as described previously [14].

Reagents. DEAE-cellulose was purchased from Serva Feinbiochemica (Heidelberg, F.R.G.), Sephadex G-150 from Pharmacia (Uppsala, Sweden) and horse cytochrome *c*, Type VI from Sigma Chemical Company. Cytochrome *c* (551, *Pseudomonas aeruginosa*) [15], cytochrome *c* (552, *Nitrosomonas europaea*) [16], cytochrome *c* (555, *Chlorobium limicola* f. *thiosulfatophilum*) [17], cytochrome *c* (550, *T. novellus*) [14], cytochrome *c* (554, *Spirulina platensis*) [18], and cytochromes *c* of wheat [19], housefly [20], tuna [21], cow [21] and man [22] were purified by methods previously established in our laboratory. Cytochrome *c* (553(550), *Chromatium vinosum*) and cytochrome *c* (550, *Paracoccus denitrificans*) were purified according to the methods of Cusanovich and Bartsch [23] and of Scholes et al. [24], respectively. Cytochrome c_2 (*Rhodospirillum rubrum*) [25], cytochromes *c* of *Saccharomyces oviformis* [26] and *Candida krusei* [27] and bonito cytochrome *c* [21] were kindly supplied by Dr. T. Horio (Institute for Protein Research, Osaka University, Japan), Sankyo Co. Ltd. (Tokyo, Japan) and Dr. M. Kakudo (Institute for Protein Research, Osaka University, Japan), respectively.

Physical and chemical measurements. Spectrophotometric determinations were performed in a Cary spectrophotometer, model 15 or 16, or a Hitachi spectrophotometer, model 124, using cuvettes with 1 cm light paht. Copper content was determined by atomic absorption, using a Nippon Jarrell Ash AA-1 atomic absorption spectrophotometer.

Determination of molecular weight by polyacrylamide gel electrophoresis in the presence of SDS was performed by the method of Weber and Osborn [28] as modified by Wada and Snell [29]. Amino acid composition was

analysed in a Beckman-Spinco amino acid analyser, model 120B, with an acceleration system according to the method of Spackman et al. [30].

Assay of enzymatic activity. The activity of cytochrome oxidase was determined spectrophotometrically by oxidation of ferrocytochrome *c*. The standard reaction mixture contained 10—50 mM phosphate buffer, pH 5.5, approx. 20 μM cytochrome *c* and 22—70 nM enzyme in a total volume of 1.0 ml. Oxidation of ferrocytochrome *c* was followed with time by decrease in the absorbance at the α peak of each cytochrome *c*.

Purification of cytochrome oxidase. The cells (30 g, wet wt.) which had been frozen at −20°C for a week were thawed, suspended in approx. 100 ml of distilled water and treated with a sonic oscillator (20 kHz, 500 W) for 10 min. The suspension thus treated was centrifuged at 10 000 × *g* for 30 min and the resulting supernatant was further centrifuged at 100 000 × *g* for 30 min. The debris thus obtained was suspended in 50 ml of 0.1 M phosphate buffer, pH 7.5, containing 0.5% Triton X-100 and 0.2 M KCl. The resulting suspension was allowed to stand overnight at 5°C and then centrifuged at 100 000 × *g* for 30 min. The supernatant obtained was fractionated with $(NH_4)_2SO_4$ and the precipitate formed between 25 and 50% saturation was collected. The precipitate obtained was dissolved in 3 ml of 10 mM Tris-HCl buffer, pH 8.0, containing 0.5% Triton X-100, and the resulting solution was dialysed against the same buffer as was used for the dissolution. The dialysed solution was charged on a DEAE-cellulose column which had been equilibrated with the buffer used for the dialysis. The enzyme was adsorbed on the column as a reddish-green band. When the column was washed with 10 mM Tris-HCl buffer, pH 8.0, containing 0.5% Triton X-100 and 50 mM NaCl, the enzyme adsorbed did not descend. The enzyme was then eluted by increasing the concentration of NaCl to 150 mM in the washing buffer. By this treatment, a reddish-green eluate was obtained. The eluate obtained was charged on a Sephadex G-150 column which had been washed with 50 mM Tris-HCl, pH 8.0, containing 0.5% Triton X-100 and 100 mM KCl, and the enzyme was eluted with the same buffer as was used for the washing of the Sephadex column. The enzyme was eluted almost at the void volume, while the retarded eluate contained *c*-type cytochrome. The eluate, which contained the enzyme and was green in colour, was dialysed against 10 mM Tris-HCl buffer, pH 8.0, containing 0.5% Triton X-100, and subjected to DEAE-cellulose column chromatography. The enzyme adsorbed on the column was eluted slowly with Tris-HCl buffer, pH 8.0, containing 0.5% Triton X-100 and 80 mM NaCl. The eluate thus obtained was green in colour. This eluate was used as the highly purified preparation of *T. novellus* cytochrome oxidase. The results of the purification of cytochrome oxidase from Triton X-100 extract of *T. novellus* are summarized in Table I. Although haem recovery was not shown in Table I, it was also 2—3% at the final purification stage.

The enzyme preparation thus purified was pure as judged from the results obtained by slab polyacrylamide-gel electrophoresis; a single band observed in the gel on staining with Coomassie brilliant blue, with haem staining reagents [31], or with the use of the Nadi reaction [32] (Fig. 1). In Fig. 1, the band stained with Coomassie brilliant blue was a little diffuse. Such a phenomenon is often observed with membranous protein. However, even if the diffuse band

TABLE I

PURIFICATION OF CYTOCHROME OXIDASE FROM *T. NOVELLUS*

Step	Total protein (mg)	Total activity (units *)	Specific activity (units/mg)	Purification (*n*-fold)	Recovery (%)
Triton X-100 extract	2800	1540	0.550	1	100
Ammonium sulphate fractionation (25—50% saturation)	620	1320	2.13	3.87	85.7
First DEAE-cellulose chromatography	152	638	4.21	7.65	41.0
Sephadex G-150 filtration	23.0	150	6.52	9.74	11.9
Second DEAE-cellulose chromatography	3.93	41.0	10.4	18.9	2.70

* 1 unit = oxidation of 1 μmol cytochrome *c*/min.

was caused by the contaminants, the quantity of the contaminants was calculated to be less than 5% of that of the enzyme on the basis of the blue colour intensity, so that it seems unlikely that the results obtained in the present investigation were adversely affected.

Results

Spectral properties

T. novellus cytochrome oxidase showed absorption peaks at 428 and 602 nm in the oxidized form and at 442 and 602 nm in the reduced form (Fig. 2) The absorbance at 602 nm of the peak of the oxidized form was increased when the enzyme was reduced. The peak was observed at 600 nm in the difference spectrum with a particulate fraction. Therefore, the spectrum of the enzyme appears to be unchanged after solubilization with Triton X-100. When CO was bubbled through the reduced enzyme solution, the peak at 442 nm of the reduced enzyme was shifted to 431 nm and a shoulder appeared at approx. 440 nm, while the α peak at 602 nm was shifted to 599 nm. The pyridine

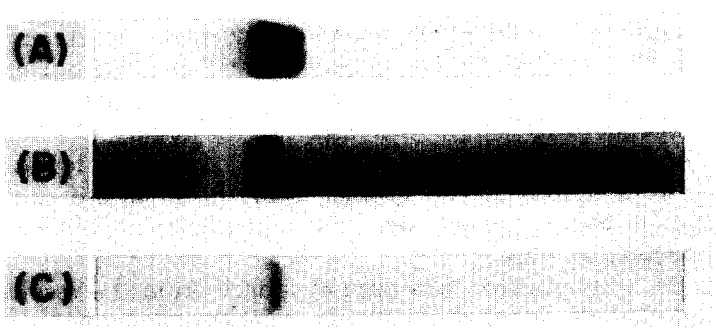

Fig. 1. Electrophoretic profiles of *T. novellus* cytochrome oxidase in polyacrylamide gel. Electrophoresis was performed in the polyacrylamide gel (monomer, 5%) which had been equilibrated with 0.1 M Tris-acetate buffer, pH 8.0, containing 0.5% Triton X-100. After the electrophoresis the slab gel was cut into three strips and these were separately stained by Coomassie brilliant blue (A), by the Nadi reaction (B) and by the haem-staining reagents (C).

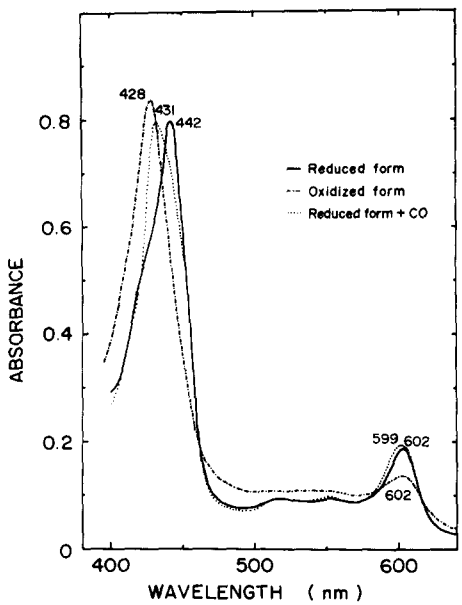

Fig. 2. Absorption spectra of *T. novellus* cytochrome oxidase. The enzyme was dissolved in 10 mM Tris-HCl buffer, pH 8.0, containing 0.5% Triton X-100, ·—·—·, Oxidized; ———, reduced with $Na_2S_2O_4$; · · · · · ·, CO was bubbled through $Na_2S_2O_4$-reduced enzyme.

ferrohaemochrome of the enzyme showed absorption peaks at 430 and 587 nm. This seems to show that the enzyme has haem *a* as the prosthetic group. The millimolar extinction coefficient (ϵ_{mM}) of the α peak at 602 nm of the reduced enzyme was determined to be 20.7 on the basis of ϵ_{mM} at the α peak of the pyridine ferrohaemochrome of haem *a* (29.2) [33].

Molecular properties

Amino acid composition per mol of haem *a* of the enzyme is shown in Table II. The contents of glycine and alanine were large, and those of hydrophobic amino acids, such as leucine, isoleucine and valine, were also large. The polarity of the enzyme, defined as the residue percent of the polar amino acids (Lys, His, Arg, Asp, Thr, Ser, Glu) [34], was calculated to be 35.15%. This value was less than those calculated for rat liver cytochrome oxidase (38.53%) and beef heart enzyme (38.69%) [35]. Only 2 mol of cysteine residues were found per 55 600 g of the enzyme protein.

The isoelectric point of the enzyme was at pH 4.3, as determined by isoelectric focusing. This acidic property of the enzyme should provide an upper limit to amide content, namely 35 residues, as the enzyme contained 73 residues of aspartic acid and glutamic acid against 37 residues of lysine and arginine, besides ten histidine residues.

Copper content of the enzyme was determined by atomic absorption. 1 nmol of the enzyme contained 69.3 ng copper; i.e. 1 mol of the enzyme contained 1.09 g-atom of copper. In the determination of copper content, *P. aeruginosa* azurin was included as a control (Table III).

When the enzyme preparation was subjected to 7.5% polyacrylamide-

TABLE II

AMINO ACID COMPOSITION OF *T. NOVELLUS* CYTOCHROME OXIDASE

n.d., not determined.

Amino acid residue	Residues/haem *a*	Mol%
Lys	17	3.30
His	10	1.94
Arg	20	3.88
Asp	40	7.77
Thr	30	5.83
Ser	31	6.02
Glu	33	6.41
Pro	39	7.57
Gly	55	10.68
Ala	57	11.07
Cys	2	0.39
Val	44	8.54
Met	13	2.52
Ile	34	6.60
Leu	51	9.90
Tyr	17	3.30
Phe	32	6.21
Trp	n.d.	—
Total residues	525 *	101.93
Mol. wt.	55 648	
Polarity	35.15%	

* Trp is not included.

gel electrophoresis in the presence of SDS, two bands appeared in the gel stained with Coomassie brilliant blue. The two bands corresponded to proteins of 32 000 and 23 000 daltons, respectively [37]. The amounts of the two subunits seemed to be comparable as judged from the density of staining in the bands.

Enzymatic properties

The enzyme oxidized ferrocytochromes *c* of yeasts, tuna and bonito rapidly as well as ferrocytochrome *c*-550 of *T. novellus*. The reactions were strongly affected by the concentration of salt, e.g. phosphate (Fig. 3). In the cases of cytochromes *c* of *T. novellus*, tuna, bonito and other animals, the reaction rates

TABLE III

DETERMINATION OF COPPER CONTENT IN *T. NOVELLUS* CYTOCHROME OXIDASE

The concentrations were determined spectrally based on ϵ_{mM} = 20.7 at 602 nm (cytochrome oxidase) and ϵ_{mM} = 6.95 at 635 nm (azurin) [36].

Enzyme or protein	Concentration (nmol/ml)	Cu	
		ng/ml	ng-atom/ml
T. novellus cytochrome oxidase	4.76	330	5.19
P. aeruginosa azurin	3.93	270	4.25
P. aeruginosa azurin	7.87	530	8.34

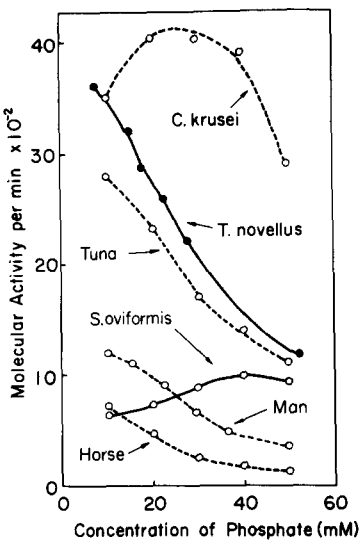

Fig. 3. Effect of phosphate concentration on the oxidation rate of cytochromes *c* catalysed by *T. novellus* cytochrome oxidase. The concentrations of cytochromes *c* were 12—16 μM, and that of the enzyme 22 nM.

decreased rapidly with the increase in concentration of the phosphate buffer. On the other hand, the reaction rates of yeast cytochromes *c* with the enzyme increased with the increase in the phosphate concentration when the salt concentration was lower than 30—40 mM, but when the salt concentration was further increased the reaction rates decreased. The effect of salt concentration on the reaction rate of the enzyme was also observed with other salts, e.g. NaCl. The effects of NaCl and phosphate were similar at the same concentrations. The observation that the reaction rates were affected by the concentration of the salts rather than by the ionic strength seems to be attributable to the pH at which the reactions were performed. Phosphate exists practically as a monovalent anion at pH 5.5.

Phosphate was found to compete with cytochrome *c* for the enzyme when the dependency of the reaction rates on cytochrome *c* concentrations in the presence of different concentrations of the salt was analysed by Lineweaver-Burk plots. The K_m value of the enzyme for cytochrome *c* did not differ much between cytochromes *c* which react rapidly with the enzyme and those which react poorly (about 11 μM), when phosphate concentration was lower than approx. 30 mM, while at higher concentrations of the salt, e.g. 100 mM, the value for *S. oviformis* cytochrome *c* (37 μM) was considerably lower than that for tuna cytochrome *c* (200 μM).

The optimum pH of the enzyme was approx. 5.5, irrespective of the kind of cytochrome *c* tested, when the reaction was performed in 50 mM phosphate buffer. When the reaction was carried out in 0.1 M Mes buffer, the optimum pH was approx. 5.

The oxidation of ferrocytochrome *c* catalysed by the enzyme was strongly inhibited by cyanide. The reaction performed in 10 mM phosphate buffer,

with tuna cytochrome *c* as an electron donor, was 50% inhibited in the presence of 4 μM KCN when the enzyme concentration was approx. 15 nM, while 14 μM NaN_3 was necessary to cause the same inhibition. The enzyme activity was not affected by 2% Triton X-100, while the detergent at concentrations over 2% was a little inhibitory; the activity was 15% inhibited by 3% Triton X-100.

1 mol of the enzyme oxidized about 1200 mol of *T. novellus* ferrocytochrome *c* per min in 12.5 mM phosphate buffer at pH 5.5 and 20°C when the concentration of the cytochrome was approx. 12 μM. The molecular activity per min was about 1300 when it was determined from *V.* The molecular activity per min with *C. krusei* cytochrome *c* was 2750 in 30 mM phosphate buffer at pH 5.5 and 18°C when the concentration of the cytochrome was 14 μM.

In the oxidation of ferrocytochrome *c* catalysed by the enzyme, the ratio in mol of the cytochrome oxidized to oxygen consumed was approc. 4 and addition of catalase did not affect the reaction. Therefore, we can conclude that the reduction product of oxygen catalysed by the enzyme is water.

The enzyme showed specificity for cytochrome *c* as an electron donor (Table IV). The reactivity with the enzyme of cytochromes *c* derived from various organisms was determined in 50 mM phosphate buffer at pH 5.5 and 18°C. Although the difference in the reaction rates was clearer in 10 mM phosphate buffer than in 50 mM phosphate buffer, as seen from the results shown in Fig. 3, the reactivities in 50 mM of the buffer were compared because even minute variations in the concentration of the buffer might affect the reactivity more in 10 mM buffer than in 50 mM buffer. The enzyme reacted very poorly with cytochrome *c* (551, *P. aeruginosa*), cytochrome *c* (552,

TABLE IV

REACTIVITY OF VARIOUS CYTOCHROMES *c* WITH *T. NOVELLUS* CYTOCHROME OXIDASE

Reactivity of cytochromes *c* is expressed as relative value; the molecular activity is taken as 100%, which was observed when the enzyme reacted with *T. novellus* cytochrome *c*.

Cytochrome *c*		Relative reactivity
Organism	α Peak (nm)	
P. aeruginosa	551	0.78
N. europaea	552	2.5
C. limicola f. *thiosulfatophilum*	555	18
C. vinosum	553 (550)	0
R. rubrum	550	1.5
T. novellus	550	100
S. platensis	554	3.8
S. oviformis (iso-1-)	550	77
C. krusei	549	290
Wheat	550	26
Tuna	550	93
Bonito	550	91
Housefly (larva)	550	12
Cow	550	4.7
Horse	550	4.7
Human	550	14

N. europaea), cytochrome c_2 (550, *R. rubrum*), cytochrome *c* (553(550), *C. vinosum*) and cytochrome *c* (554, *S. platensis*). The enzyme reacted rapidly with cytochromes *c* derived from *S. oviformis*, *C. krusei*, tuna and bonito, while it reacted slowly with cow and horse cytochromes *c*. It reacted slowly with wheat, housefly and human cytochromes *c*, but its reactivity with these cytochromes was still larger than that with cow and horse cytochromes *c*.

Discussion

Although cytochrome *a*-type terminal oxidase has been known to function in many bacteria, little is known of its molecular features [1—4]. In the present investigation, we have purified a cytochrome *a*-type oxidase from *T. novellus* to a homogeneous state as judged from its electrophoretic behaviour.

The enzyme has haem *a* and copper as the prosthetic groups, shows spectral properties very similar to those of the eukaryotic enzymes, and reacts rapidly with some of eukaryotic cytochromes *c* as well as with the native cytochrome *c*, cytochrome *c* (550, *T. novellus*).

The minimum molecular weight of the bacterial enzyme has been determined to be 55 600 on the basis of the haem *a* content and amino acid composition. This value is smaller than that of the eukaryotic enzyme. The eukaryotic enzyme is known to be composed of 6—7 kinds of subunit [38—48], while the bacterial enzyme is composed of only two kinds of subunit, of 32 000 and 23 000 daltons, respectively. These subunits resemble, respectively, the subunits I and II of the eukaryotic enzyme with respect to molecular weight, although, as their molecular weights were determined with 7.5% gel, the true values may differ a little from those shown by the present work. In any case, the enzyme molecule of *T. novellus* cytochrome oxidase seems to be composed of two kinds of subunit which are similar to those of the eukaryotic enzyme biosynthesized in the mitochondrion [49,50]. At present, we do not know whether or not all cytochrome *a*-type oxidases of bacteria are composed of the two kinds of subunit observed in the *T. novellus* enzyme. However, the oxidase purified from *Nitrobacter agilis* has also been found to be composed of two kinds of subunit which are similar to the mitochondrial subunits of the eukaryotic enzyme [37] with respect to molecular weight.

The specificity for cytochrome *c* of the *T. novellus* enzyme differs from that of the eukaryotic enzyme [10,11,51—53]. The bacterial enzyme reacts rapidly with several eukaryotic cytochromes *c*, while it reacts poorly with horse and cow cytochromes *c*. Namely, it distinguishes between some of the eukaryotic cytochromes *c*.

Acknowledgements

The authors wish to thank Professor H. Matsubara for his valuable discussions during the course of this work, Mr. Y. Fukumori for mass cultivation of *T. novellus*, and Dr. T. Hase for his technical assistance in amino acid analysis. They are also grateful to Drs. M. Kakudo and T. Horio (Institute for Protein Research, Osaka University, Japan) and Sankyo Co. Ltd. (Tokyo, Japan) for their generosity in supplying respective cytochromes as described in the text.

This work has been supported in part by grants-in-aid for Special Project Research Nos. 312107 and 421319 from the Ministry of Education, Science and Culture of Japan.

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