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A CYTOCHROME *c* PEROXIDASE ISOLATED FROM *THIOBACILLUS NOVELLUS*

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SUMMARY

A cytochrome *c* peroxidase (ferrocytochrome *c*:H_2O_2 oxidoreductase, EC 1.11.1.5) was isolated and highly purified from *Thiobacillus novellus*, and its properties were studied. The enzyme has haem *c* as the prosthetic group, and shows a peak at 398 nm in the oxidized form and peaks at 415, 520 and 550 nm in the reduced form. It peroxidatically oxidizes the reduced form of cytochrome *c* (550, *Thiobacillus novellus*) and mammalian-type cytochromes *c* but does not react or reacts very poorly with bacterial *c*-type cytochromes such as cytochrome *c* (551, *Pseudomonas aeruginosa*), cytochrome *c* (552, *Nitrosomonas europaea*) and cytochrome *c* (555, *Chlorobium thiosulfatophilum*). One mole of the enzyme oxidizes peroxidatically 2450 moles of tuna ferrocytochrome *c* per min at pH 8.5 and at 18 °C. The peroxidase reaction catalysed by the enzyme is strongly inhibited by cyanide and azide.

INTRODUCTION

Cytochrome *c* peroxidase (ferrocytochrome *c*:H_2O_2 oxidoreductase, EC 1.11.1.5) oxidizes the reduced form of mammalian-type cytochromes *c* in the presence of H_2O_2. The enzyme was first isolated and partially purified from baker's yeast by Altschul *et al.*[1] and Abrams *et al.*[2], and has been studied extensively by Yonetani and his co-workers[3,4]. So far as is known the peroxidase has not been found in any organism other than yeasts. Although a cytochrome peroxidase has been isolated and purified from *Pseudomonas fluorescens*[5,6], it does not react with mammalian-type cytochromes *c* (ref. 5). In this respect, the bacterial peroxidase is distinct from the yeast enzyme. The yeast peroxidase is known to be localized at the mitochondria in the normal aerobic cells[7], while it exists even in anaerobically grown cells where mitochondria are not present[8]. This suggests that cytochrome *c* peroxidase may exist also in procaryotic cells. The physiological role of the enzyme is still uncertain. If there exists a peroxidase quite similar to the yeast enzyme in any organism other than yeasts, its study may offer evidence which may help us find out the biological significance of cytochrome *c* peroxidase.

Recently, we have succeeded in isolation and purification of a cytochrome *c* peroxidase from the facultative chemoautotroph, *Thiobacillus novellus*. Unlike the yeast enzyme, this enzyme has haem *c* as the prosthetic group, but it shows various properties very similar to those of the yeast peroxidase. In the present investigation,

we have studied its various properties. A brief description of this bacterial peroxidase has already been presented[9].

MATERIALS AND METHODS

Organism

A strain of *Thiobacillus novellus* (Starkey) was kindly supplied by Dr A. Asano (Institute for Protein Research, Osaka University, Japan). Mass cultivation of the organism was performed in 600 l of an organic medium in a stainless steel tank of 1000 l volume. The medium contained 2 g meat extract, 10 g polypeptone, 1 g yeast extract, 5 g NaCl, and 5 g $Na_2S_2O_3$ in 1000 ml. The medium inoculated with the seed culture (10 l) was incubated at 30 °C and at pH 7 for 44 h with vigorous aeration, and the cells of the organism were collected by a Sharples continuous-flow centrifuge. About 3.3 kg wet cells were obtained.

Reagents

Highly purified cow cytochrome *c* (ref. 10), tuna cytochrome *c* (ref. 11), cytochrome *c* (555, *Chlorobium thiosulfatophilum*)[12], cytochrome *c* (552, *Nitrosomonas europaea*)[13], cytochrome *c* (550, *T. novellus*)[14] and cytochrome *c* (551, *Pseudomonas aeruginosa*)[15] were prepared by the methods established in our laboratory. Yeast cytochrome *c* peroxidase[3], cytochrome *c* (550, *Saccharomyces oviformis*) and cytochrome *c* (551, *Rhodospirillum rubrum*)[16] were kindly supplied by Dr T. Yonetani (Johnson Research Foundation, University of Pennsylvania, U.S.A.), Sankyo Co. Ltd. (Tokyo, Japan) and Dr T. Horio (Institute for Protein Research, Osaka University, Japan), respectively. Horseradish peroxidase (EC 1.11.1.7) and DEAE-cellulose were purchased from Worthington Biochemical Corporation (U.S.A.) and Serva Entwicklungslabor (Germany), respectively.

Measurements of enzymatic activity

The standard reaction mixture was composed of 1.0 ml of *c*-type cytochrome dissolved in 0.1 M Tris–HCl buffer, pH 8.5, 0.01 ml of 20 mM H_2O_2 and 0.02–0.03 ml of 1 μM *T. novellus* peroxidase. The concentration of the peroxidase was determined on the basis of the millimolar extinction coefficient at 550 nm of the reduced enzyme, 16.8 (see Results). Usually, the reaction was started by addition of the enzyme and followed spectrophotometrically by the decrease in the absorbance at the α peak of each *c*-type cytochrome. The spectrophotometric determinations were performed in a Cary spectrophotometer, Model 15, using 1-cm light path cuvettes. The concentration of H_2O_2 was enzymatically determined as described by Yonetani[17].

RESULTS

Preparation of T. novellus cytochrome c peroxidase

The cells of *T. novellus* (1 kg) dispersed in 10 mM phosphate buffer, pH 7.0, were treated with a sonic oscillator (20 kcycles, 500 W; Blackstone, U.S.A.) for 10 min. The suspension thus treated was centrifuged at 13000 $\times$ *g* for 20 min. To the supernatant obtained here was added $(NH_4)_2SO_4$, and the resulting precipitate formed between 40 and 90% saturation was collected by centrifugation. The precipitate

thus obtained was dissolved in 10 mM Tris–HCl buffer, pH 8.5, and the resulting solution was dialysed for 3 days against the same buffer as used for the dissolution. The dialysed solution obtained here was centrifuged if it was turbid and charged on the DEAE-cellulose column which had been equilibrated with 10 mM Tris–HCl buffer, pH 8.5. A brownish red band appeared at the top of the column, and a broad red band was seen below it. When the column was washed with 10 mM Tris–HCl, pH 8.5, containing 0.2 M NaCl, the broad red band was eluted. This fraction contained cytochrome *c*-550. The brownish red band remained on the column after the above washing, and was eluted with 10 mM Tris–HCl buffer, pH 8.5, containing 1.0 M NaCl. The eluate thus obtained was used as *T. novellus* cytochrome *c* peroxidase preparation. About 0.15 μmole (on the basis of haem content) of the purified enzyme was obtained.

The peroxidase was also isolated from the cells of the organism cultivated in an inorganic medium[14]. The spectral properties of the peroxidase isolated from the cells cultivated in the inorganic medium were identical with those of the enzyme from the cells cultivated in the organic medium.

As the cytochrome *c* peroxidase activity was observed with the sonicate of the cells, it seemed unlikely that the peroxidase was an artificial derivative of another haemoprotein. Although, in the present investigation, the peroxidase was isolated from the cells of *T. novellus* cultivated in the organic medium in the presence of $Na_2S_2O_3$, we have observed that the peroxidase activity was also found with the sonicate prepared from the cells cultivated for 3 or 4 days in the organic medium without addition of $Na_2S_2O_3$. Therefore, the biosynthesis of the peroxidase was independent of the presence of $Na_2S_2O_3$ in the culture medium. However, it was noteworthy that the peroxidase was not obtained from the cells which were cultivated for 20 h in the organic medium in the absence of $Na_2S_2O_3$.

Absorption spectra

As Fig. 1 shows, the peroxidase possessed absorption peaks at 252 and 398 nm in the oxidized form, and at 415, 520 and 550 nm in the reduced form. The oxidized form did not show any distinct band between 500 and 550 nm. There was a very small peak around 630 nm in the oxidized form, but its absorbance was not affected on reduction. The peak at 252 nm of the oxidized form may be attributable to the existence of a nucleic acid-like substance, but it has not been determined whether such substance is present in the peroxidase preparation. It was also noticed that the Soret band of the oxidized form was higher in absorbance than that of the reduced form, unlike other *c*-type cytochromes, although the peroxidase possessed haem *c* as presented below. The reduced form of the enzyme combined with CO (Fig. 2). The CO complex possessed a very sharp γ peak at 411 nm and small peaks at 527 and 561 nm, and the extinction coefficient of the γ peak was very large as compared with that of the original reduced form. Cyanide combined with the peroxidase in both the oxidized and reduced forms (Fig. 3). The cyanide complex of the oxidized form had the γ band at 409 nm and a broad absorption band at 530 nm. When the complex was reduced with $Na_2S_2O_4$, there appeared absorption bands at 419, 522 and 552 nm. With the cyanide complex, the γ band of the reduced form was higher than that of the oxidized form, as with other *c*-type cytochromes. As the peroxidase was easily destroyed on addition of H_2O_2 in the absence of ferrocytochrome *c* as the electron donor (see

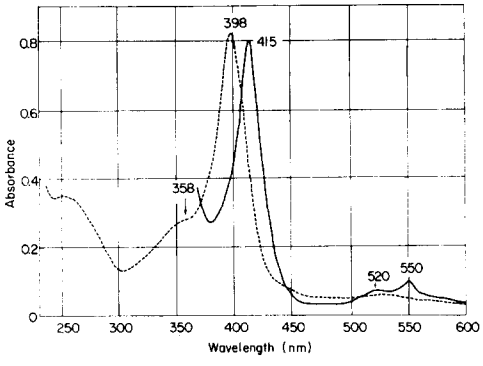
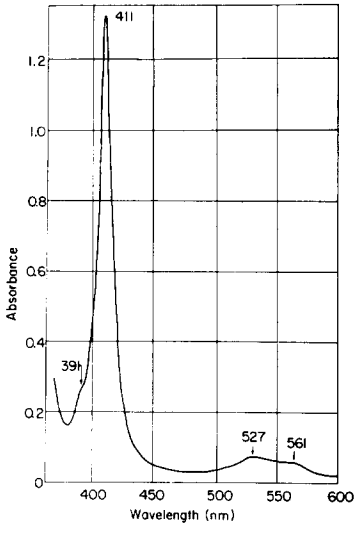


Fig. 1. Absorption spectra of *T. novellus* cytochrome *c* peroxidase. The enzyme was dissolved in 70 mM Tris–HCl buffer, pH 8.5, at a concentration of 5.7 μM. – – – –, oxidized; ———, reduced with $Na_2S_2O_4$.



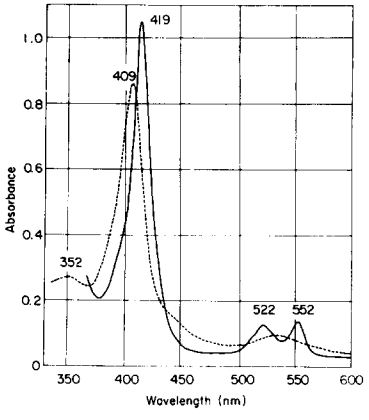


Fig. 2. Absorption spectrum of CO complex of *T. novellus* cytochrome *c* peroxidase. The reduced form of the enzyme dissolved in 70 mM Tris–HCl buffer, pH 8.5 was bubbled with CO. The concentration of the enzyme was 5.7 μM.

Fig. 3. Absorption spectra of the CN^- complex of *T. novellus* cytochrome *c* peroxidase. The enzyme was dissolved in 70 mM Tris–HCl buffer, pH 8.5, at the concentration of 5.7 μM and 10 mM KCN added to the enzyme solution. – – – –, oxidized form; ———, reduced with $Na_2S_2O_4$.

below), the absorption spectrum of the H_2O_2 complex of the enzyme could not be determined.

The haem of the peroxidase was not split off from the protein by acid–acetone, and the pyridine ferrohaemochrome of the enzyme showed absorption peaks at 412, 520 and 550 nm. Therefore, the enzyme may be concluded to have haem *c* as the prosthetic group. From the absorbance at the α peak of the pyridine ferrohaemochrome, the ε_{mM} at the α peak of the enzyme was determined to be 16.8, assuming that of the pyridine ferrohaemochrome of haem *c* is 29.1 (ref. 18).

Enzymatic properties

Oxidation of ferrocytochrome c in the presence of H_2O_2. As shown in Fig. 4, *T. novellus* cytochrome *c* peroxidase rapidly oxidized tuna ferrocytochrome *c* in the presence of H_2O_2. The reaction did not occur in the absence of H_2O_2. The activity varied with the pH of the reaction mixture; the maximal activity was observed in the pH region between 8.25 and 8.75 in 0.1 M Tris–HCl buffer (Fig. 5). The K_m for H_2O_2 was determined to be 0.43 mM in 0.1 M Tris–HCl buffer, at pH 8.5. The molecular activity was 860 per min when 18.4 μM tuna ferrocytochrome *c* reacted with 20 nM peroxidase in the presence of 200 μM H_2O_2 in 0.1 M Tris–HCl buffer, pH 8.5, at 18 °C, and was calculated to be 2450 per min from the maximal velocity as shown in Fig. 6.

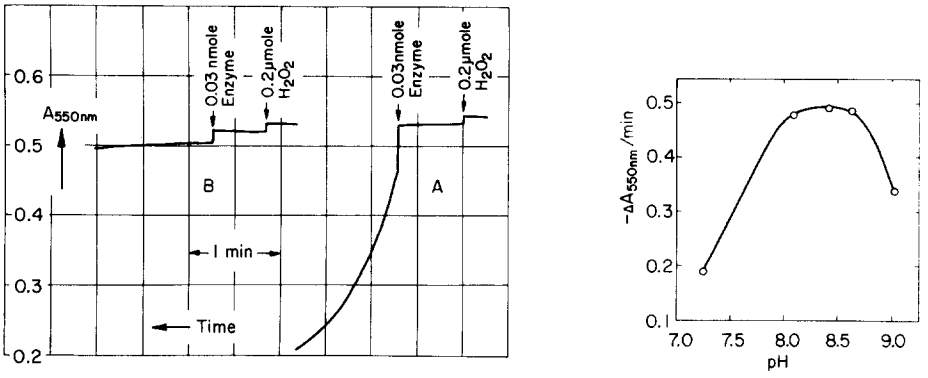
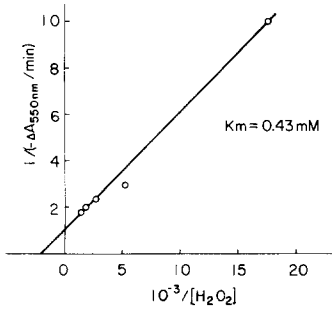


Fig. 4. Peroxidatic oxidation of tuna ferrocytochrome *c* catalysed by *T. novellus* cytochrome *c* peroxidase. (A) To 1.0 ml of 19 μM tuna ferrocytochrome *c* were added 0.01 ml of 20 mM H_2O_2 and 0.03 ml of 1.0 μM enzyme at points as indicated. (B) The composition of the reaction mixture was the same as for (A) except that 0.9 mM (final concn) KCN was added. The reactions were performed in 0.1 M Tris–HCl buffer at pH 8.5 and at 18 °C. Ferrocytochrome *c* was not oxidized by the enzyme in the absence of H_2O_2 (*cf.* Fig. 7). When tuna ferrocytochrome *c* was completely oxidized, $A_{550\ nm}$ was 0.148.

Fig. 5. Effect of pH on *T. novellus* cytochrome *c* peroxidase reaction. Reactions were performed at 18 °C in 0.1 M Tris–HCl buffer. The reaction mixture contained 18 μM tuna ferrocytochrome *c*, 600 μM H_2O_2 and 20 nM peroxidase in 1.0 ml.

When the peroxidase (3 μM) was incubated with 200 μM H_2O_2 for a few min in 0.1 M Tris–HCl buffer, pH 8.0, in the absence of ferrocytochrome *c*, it was destroyed to a great extent. The activity was about one fifth that of the intact enzyme when observed after addition of ferrocytochrome *c* to this mixture of peroxidase and H_2O_2.

Inhibitors. The peroxidatic oxidation of ferrocytochrome *c* catalysed by peroxidase was strongly inhibited by KCN; 1 mM KCN completely inhibited the reaction (Fig. 4). NaN_3 also inhibited the reaction, but its mode of inhibition was a little different from that of cyanide (Fig. 7); the peroxidatic oxidation of ferrocytochrome *c* proceeded fairly rapidly at the beginning of the reaction even in the presence of about 1 mM NaN_3, was progressively inhibited and almost stopped about 15 s after the reaction had started. At higher concentrations of the compound, *e.g.* in the presence of 8 mM NaN_3, the reaction was strongly inhibited even from the start. The peroxidation of ferrocytochrome *c* was 20 % and 50 % inhibited by 1 mM and 8 mM NaF,



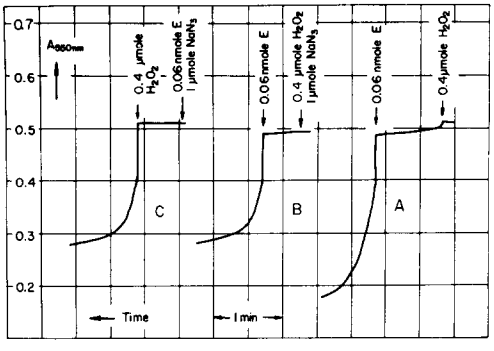


Fig. 6. Effect of H_2O_2 concentration on *T. novellus* cytochrome *c* peroxidase reaction. Reactions were performed at 18 °C in 0.1 M Tris–HCl buffer, pH 8.5. The reaction mixture contained 18 μM tuna ferrocytochrome *c* and 20 nM peroxidase in 1.0 ml besides H_2O_2. The concentration of H_2O_2 was varied from 20 to 750 μM.

Fig. 7. Effect of azide on peroxidatic oxidation of tuna ferrocytochrome *c* catalysed by *T. novellus* cytochrome *c* peroxidase. The reactions were performed at 20 °C in 0.1 M Tris–HCl buffer, pH 8.5. The reaction mixture contained besides the inhibitor 18 μM tuna ferrocytochrome *c*, 400 μM H_2O_2, and 60 nM enzyme in 1.0 ml. The concentrations of NaN_3 were 0 mM (A), and 0.91 mM (B) and (C). When tuna ferrocytochrome *c* was completely oxidized, $A_{550\ nm}$ was 0.143.

respectively. However, the reaction was also affected by salt such as NaCl; it was 32 % inhibited in the presence of 2 mM NaCl. Therefore, the inhibition by NaF may be ascribable to the salt effect.

Comparison of cytochrome c peroxidase activity with guaiacol peroxidase activity. As Table I shows, cytochrome *c* peroxidase and guaiacol peroxidase activities of

TABLE I

COMPARISON OF CYTOCHROME *c* PEROXIDASE ACTIVITY OF THE *T. novellus* PEROXIDASE WITH ITS GUAIACOL PEROXIDASE ACTIVITY

The reaction mixture for the determination of cytochrome *c* peroxidase activity was composed of 1.0 ml of 17 μM tuna ferrocytochrome *c* dissolved in 25 mM Tris–HCl, pH 7.5, 0.01 ml of 20 mM H_2O_2 and 0.02 ml of peroxidase (yeast peroxidase, 0.1 μM; *T. novellus* peroxidase, 3.1 μM; horseradish peroxidase, 8.8 μM). The reactions were performed at 23.5 °C and followed spectrophotometrically by the decrease in the absorbance at 550 nm. For the determination of guaiacol peroxidase activity, the reaction mixture consisted of 1.0 ml of 25 mM Tris–HCl buffer, pH 7.5, 0.05 ml of 20 mM guaiacol, 0.01 ml of 20 mM H_2O_2 and 0.02 ml of peroxidase (yeast peroxidase, 0.1 μM; *T. novellus* peroxidase, 3.1 μM; horseradish peroxidase, 0.88 μM). The reactions were performed at 23.5 °C and followed spectrophotometrically by the increase in the absorbance at 470 nm.

Enzyme		$-\Delta A_{550\ nm}/min$ *per* 10^{-11} *mole enzyme* (*I*)	$+\Delta A_{470\ nm}/min$ *per* 10^{-11} *mole enzyme* (*II*)	*I/II*
Origin	*Amount (mole/cuvette)*			
T. novellus	$6 \cdot 10^{-11}$	0.364	0.093	3.9
Yeast	$2 \cdot 10^{-11}$	16.1	≈0	∞
Horseradish	$17.6 \cdot 10^{-11}$	0.026	—	0.0024
	$1.76 \cdot 10^{-11}$	—	10.9	

T. novellus peroxidase were compared with those of yeast cytochrome *c* peroxidase and horseradish peroxidase. The yeast enzyme showed a very high cytochrome *c* peroxidase activity but did not show guaiacol peroxidase activity, while the horseradish enzyme catalysed strongly the peroxidatic oxidation of guaiacol but catalysed very poorly the peroxidatic oxidation of ferrocytochrome *c*. Thus, the ratios of the cytochrome *c* peroxidase activity to the guaiacol peroxidase activity were infinity and 0.0024 for the yeast and horseradish enzymes, respectively. As seen in Table I, with *T. novellus* cytochrome *c* peroxidase, the cytochrome *c* peroxidase activity was higher than the guaiacol peroxidase activity when the two activities were presented as shown in the table. Thus, the ratio of the cytochrome *c* peroxidase activity to the guaiacol peroxidase activity was 3.9. These facts suggest that the bacterial enzyme is a cytochrome *c* peroxidase.

TABLE II

REACTIVITY WITH THE *T. novellus* PEROXIDASE OF CYTOCHROMES *c* DERIVED FROM VARIOUS ORGANISMS

The reactivity was expressed as a relative value; the molecular activity when the enzyme reacted with tuna cytochrome *c*, 860 per min, was taken as 100. Reactivity with yeast cytochrome *c* peroxidase was also presented (cited from ref. 19).

Organism	*α peak (nm)*	*Cytochrome c concn (μM)*	*T. novellus cytochrome c peroxidase*	*Yeast cytochrome c peroxidase*
P. aeruginosa	551	20	0	0
N. europaea	552	10	5.9	≈0
T. novellus	550	16	97	107
C. thiosulfatophilum	555	21	5.8	≈0
R. rubrum	551	21	24	12
S. oviformis	550	19	45	106
Tuna	550	16	100	100
Cow	550	17	88	64

Specificity for cytochrome c as the electron donor. As presented in the preceding section, the *T. novellus* peroxidase oxidized peroxidatically the reduced form of tuna cytochrome *c*. As Table II shows, the peroxidase also reacted rapidly with other mammalian-type cytochromes *c* such as cytochrome *c* (550, *S. oviformis*) and cow cytochrome *c*. On the other hand, the enzyme did not react with cytochrome *c* (551, *P. aeruginosa*). Other bacterial cytochromes *c*, cytochrome *c* (555, *C. thiosulfatophilum*) and cytochrome *c* (552, *N. europaea*) reacted very poorly with the peroxidase. Cytochrome *c* (551, *R. rubrum*) reacted fairly rapidly with the enzyme. Cytochrome *c* (550, *T. novellus*) reacted with the enzyme as rapidly as tuna cytochrome *c*, suggesting that the bacterial cytochrome *c* functions in the cells as the electron donor to the enzyme. As cytochrome *c* (550, *T. novellus*) also reacts very rapidly with yeast cytochrome *c* peroxidase, it seems to have a conformation similar to that of mammalian-type cytochrome *c* (ref. 19). From these facts, the specificity of the bacterial peroxidase for cytochrome *c* as the electron donor was seen to be very similar to that of yeast cytochrome *c* peroxidase.

DISCUSSION

Cytochrome *c* peroxidase has been thought to exist only in yeasts. This peroxidase peroxidatically oxidizes the reduced form of mammalian-type cytochromes *c* but does not react with bacterial cytochromes *c* such as cytochrome *c* (551, *P. aeruginosa*) and cytochrome *c* (554, *Micrococcus* species)[19]. Namely, the specificity for cytochrome *c* as the electron donor of the peroxidase is quite similar to that of cow cytochrome oxidase[19,20].

In the present investigation, we have succeeded in purification of a cytochrome *c* peroxidase from the facultative chemoautotroph, *T. novellus*. This peroxidase oxidizes the reduced form of mammalian-type cytochromes *c* in the presence of H_2O_2. Although the molecular activity of the bacterial peroxidase is about one tenth as high as that of the yeast peroxidase, its specificity for cytochrome *c* is very similar to that of the yeast enzyme. Further, the cytochrome *c* peroxidase activity of the bacterial enzyme is much higher than its guaiacol peroxidase activity. This is one of the characteristics of cytochrome *c* peroxidase. The fact that distinguishes the bacterial peroxidase from the yeast enzyme is that the former has haem *c* as the prosthetic group while the latter possesses protohaem[2,3]. Therefore, strictly it may still be true that a cytochrome *c* peroxidase just like the yeast enzyme has not been found in any organisms other than yeasts. However, it may be said that in the functional sense cytochrome *c* peroxidase exists in *T. novellus* as well as in yeasts.

Although the bacterial peroxidase possesses haem *c* as the prosthetic group, its spectral properties seem to be similar to that of the yeast enzyme which has protohaem; the extinction coefficient of the Soret peak in the oxidized form changes little on reduction, unlike other *c*-type cytochromes. This is observed with many other peroxidases including the yeast peroxidase, although the extinction coefficient of the Soret peak of the bacterial enzyme becomes a little smaller on reduction, unlike the other peroxidases. On addition of cyanide the spectral properties of the bacterial peroxidase are changed into the "normal cytochrome type"; there appears a broad band around 530 nm in the oxidized form and the Soret peak of the reduced form is larger than that of the oxidized form in the extinction coefficient. The spectral similarity mentioned above between the bacterial peroxidase and the yeast enzyme may be attributable to the conformation which is necessary for the peroxidase activity regardless of the kind of the haem, haem *c* or protohaem.

The physiological role of yeast cytochrome *c* peroxidase has not yet been clarified. It may give us a clue to elucidation of the role of this enzyme to know that a cytochrome *c* peroxidase quite similar to the yeast enzyme in enzymatic properties exists in *T. novellus*. We have first wondered if the bacterial enzyme participates in the oxidation of $Na_2S_2O_3$ in the organism, as the enzyme has been isolated from cells cultivated in the presence of this salt. However, as it has been found that the enzyme exists even in cells cultivated in the absence of the salt, the possibility that the enzyme may play a role in the oxidation of $Na_2S_2O_3$ seems to have been ruled out.

We have established in previous studies[19,20] that there is a distinct biological specificity in the reaction of cytochrome *c* with cytochrome oxidases. Generally, cytochromes *c* derived from the primitive organisms react rapidly with *P. aeruginosa* nitrite reductase (= *Pseudomonas* cytochrome oxidase) but do not react or react very poorly with cow cytochrome oxidase, while those from the higher organisms react

very poorly with nitrite reductase but react very rapidly with the animal oxidase. Cytochrome *c* (550, *T. novellus*) reacts with the cow oxidase fairly rapidly while it reacts poorly with the nitrite reductase in spite of its bacterial origin[14]. Further, it has been shown that sulphite cytochrome *c*: oxidoreductase, another enzyme isolated from *T. novellus*, reduces very rapidly mammalian-type ferricytochrome *c* in the presence of sulphite but does not react with bacterial cytochromes *c* such as cytochrome *c* (551, *P. aeruginosa*), cytochrome *c* (552, *P. stutzeri*) and cytochrome *c* (555, *C. thiosulfatophilum*)[14]. These facts suggest that *T. novellus* may be very closely related to eucaryotes such as yeast in biochemical respects. The specificity for cytochrome *c* of the bacterial peroxidase studied in the present investigation seems also to support this idea.

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