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CRYSTALLINE PSEUDOMONAS CYTOCHROME OXIDASE

I. ENZYMIC PROPERTIES WITH SPECIAL REFERENCE TO THE BIOLOGICAL SPECIFICITY

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SUMMARY

The crystalline preparation of Pseudomonas cytochrome oxidase has two activities: a cytochrome oxidase and a nitrite reductase activity. One mole of the enzyme aerobically oxidizes 154 moles and 600 moles of reduced Pseudomonas cytochrome *c*-551 per min, at 16° and 27°, respectively. Anaerobically, 250 moles of reduced Pseudomonas cytochrome *c*-551 were oxidized per min per mole of enzyme, in the presence of nitrite, at 19°. The absorption spectrum of the enzyme showed that both haem a_2 and a *c*-type haem are present on the molecule. The enzyme has a strict biological specificity: it oxidizes reduced Pseudomonas cytochrome *c*-551, but scarcely acts on reduced mammalian cytochrome *c*. These properties of the enzyme were used to detect slight differences between *c*-type cytochromes. Reduced tunny-fish cytochrome *c* was oxidized by the enzyme more rapidly than reduced beef cytochrome *c*. The reduced form of *Physarum polycephalum* cytochrome *c* was not oxidized by the enzyme, but was oxidized by cytochrome *a*. Reduced wheat cytochrome *c* was oxidized faster by cytochrome *a* than by Pseudomonas cytochrome oxidase. Reduced *Porphyra tenera* cytochrome-553 was oxidized by Pseudomonas cytochrome oxidase, but not by cytochrome *a*. It is suggested that the oxidizability of the *c*-type cytochromes by Pseudomonas cytochrome oxidase offers a molecular basis for the taxonomy and evolution of organisms.

INTRODUCTION

Pseudomonas cytochrome oxidase was isolated from the cells of *Pseudomonas aeruginosa* grown anaerobically in the presence of nitrate. It was purified to an ultracentrifugally homogeneous state[1]. It possesses two haems, haem a_2 and a *c*-type haem in its molecule, but no copper. The enzyme shows the general properties of a cytochrome oxidase; aerobically it oxidizes ascorbate, hydroquinone and the reduced form of Pseudomonas cytochrome *c*-551 (see ref. 2) which is known to function in the organism just like cytochrome *c* does in animal tissues[3]. These reactions are strongly inhibited in the presence of cyanide and carbon monoxide[4]. It is noteworthy that the enzyme is extremely labile in the presence of hydrogen peroxide[5].

It was found that the enzyme acts as a nitrite reductase[6,7], it reduces nitrite

to nitric oxide with reduced Pseudomonas cytochrome *c*-551 as the electron donor, under anaerobic conditions. This reaction is strongly inhibited in the presence of cyanide but not in the presence of carbon monoxide. As the enzyme has not been isolated from cells grown aerobically in the absence of nitrate, it is very likely that the enzyme is the chemical entity of the nitrite reductase of the organism.

It was uncertain whether Pseudomonas cytochrome oxidase itself has two activities, a cytochrome oxidase and a nitrite reductase activity, or whether two distinct enzymes are present in the enzyme preparation. The enzyme has recently been obtained in a crystalline state[8], and as described briefly in the previous paper[8], was shown clearly that the crystalline enzyme has the two functions and contains the two haems described above. This fact strongly supports the idea that the Pseudomonas cytochrome oxidase acts as both a nitrite reductase and a cytochrome oxidase.

It has been reported briefly that Pseudomonas cytochrome oxidase oxidizes reduced Pseudomonas cytochrome *c*-551 but not reduced mammalian cytochrome *c* (see ref. 9). That is, the oxidation reaction of the reduced form of *c*-type cytochromes by Pseudomonas cytochrome oxidase, shows a definite biological specificity.

This paper reports on the enzymic properties of the crystalline preparation of Pseudomonas-cytochrome oxidase with special reference to its biological specificity. Its spectral properties are described in the next paper[10], and the properties of its prosthetic groups in the third paper of this series[11].

PREPARATIONS AND MATERIALS

Pseudomonas cytochrome oxidase

The extraction and the partial purification of Pseudomonas cytochrome oxidase were performed approximately according to the method of HORIO *et al.*[1], and the preparation of the crystals of the enzyme according to the previous method[8] with a slight modification.

About 500 g of acetone-dried cells of *Pseudomonas aeruginosa*, which were grown anaerobically in the presence of KNO_3, were well mixed with 10 l of 0.1 M sodium citrate (pH 7.0) in a Waring blendor. The mixture was heated at 40° for 10 min, and then allowed to stand overnight at 5° in a refrigerator. The mixture was filtered through a Büchner funnel with the aid of Celite. To the filtrate, 4% aqueous rivanol was added to a final concentration of 0.2%, and the viscous precipitates formed were removed by filtration. The filtrate was saturated with $(NH_4)_2SO_4$, and the resulting precipitate was collected. The brown precipitate was suspended in a minimum volume of water, and the suspension was dialysed against tap water for one day at 5° in a refrigerator. During the dialysis, the outside water was changed frequently. The dialysis was continued for one more day, against 0.01 M* ammonium phosphate buffer (pH 6.0). The dialysed solution was then passed through an Amberlite CG-50, type II, column (2 cm × 30 cm), which had been equilibrated with the same buffer as used in the final dialysis, and the Pseudomonas cytochrome oxidase was adsorbed at the top part of the column, forming a green zone. The adsorbed

* In this paper, the concentration of ammonium phosphate buffer is expressed with regard to the NH_4^+ concentration. For example, 0.01 M ammonium phosphate buffer contains 0.01 g ion of NH_4^+/l.

Pseudomonas cytochrome oxidase was eluted with 1 M ammonium phosphate buffer (pH 7.0). The cytochrome oxidase in the eluate was precipitated by the addition of $(NH_4)_2SO_4$ to 90% saturation, and collected by centrifugation at 15 000 × *g* for 10 min. The precipitate, after being dissolved in a minimum volume of water, was dialysed against 0.1 M ammonium phosphate buffer (pH 6.5) and the cytochrome was again adsorbed on an Amberlite CG-50 column which had been equilibrated with the same buffer as used for the final dialysis. The Pseudomonas cytochrome oxidase adsorbed on the column forming a broad green zone, was developed with the same buffer.

When the column volume of the buffer had been passed through the ion-exchanger column, Pseudomonas cytochrome-554 which contaminated the Pseudomonas cytochrome oxidase preparation began to be eluted. This part of the eluate was about 37 ml in volume. Next, the cytochrome oxidase was gradually eluted. The first 32 ml of this eluate which contained Pseudomonas cytochrome oxidase as well was discarded. To the next 160 ml of eluate was added $(NH_4)_2SO_4$ to 90% saturation, and the precipitate formed was collected by centrifugation at 15 000 × *g* for 10 min. The precipitate was dissolved in a minimum volume of 0.02 M phosphate buffer (pH 6.0) and the insoluble matter was removed by centrifugation at 5000 × *g* for 10 min. To the brownish green supernatant was added finely powdered $(NH_4)_2SO_4$ until the solution became slightly turbid. Then, the precipitate was discarded by centrifugation at 5000 × *g* for 10 min and the supernatant was allowed to stand at room temperature (approx. 20°) for 30–60 min. Diamond shaped crystals appeared. The crystals were collected by centrifugation at 5000 × *g* for 10 min, and dissolved in 0.02 M phosphate buffer (pH 6.0). Recrystallization was carried out in a similar way to the first crystallization. Twice recrystallized crystals, which are shown in Fig. 1, were used as the crystalline preparation of the Pseudomonas cytochrome oxidase.

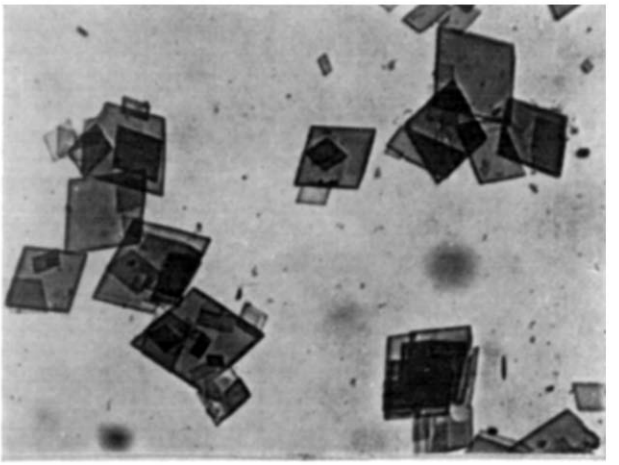
Various c-type cytochromes

Crystalline preparations of beef cytochrome *c* and tunny cytochrome *c* were prepared from corresponding heart muscles, and pigeon cytochrome *c* from pigeon-skeletal muscle according to the method of HAGIHARA *et al.*[12–14]. Crystalline yeast cytochrome *c* was prepared from baker's yeast according to the method of NOZAKI *et al.*[15]. Wheat cytochrome *c* was purified from wheat germ to 90% purity, according to the method of HAGIHARA *et al.*[16]. Pseudomonas cytochrome *c*-551 was obtained in a crystalline state from *Pseudomonas aeruginosa* cultivated anaerobically by the method of HORIO *et al.*[2]. Porphyra cytochrome-553 was obtained in a crystalline state from *Porphyra tenera* by the method of YAKUSHIJI *et al.*[17]. Physarum cytochrome *c* was purified from the plasmodium of *Physarum polycephalum* according to the method of YAMANAKA *et al.*[18].

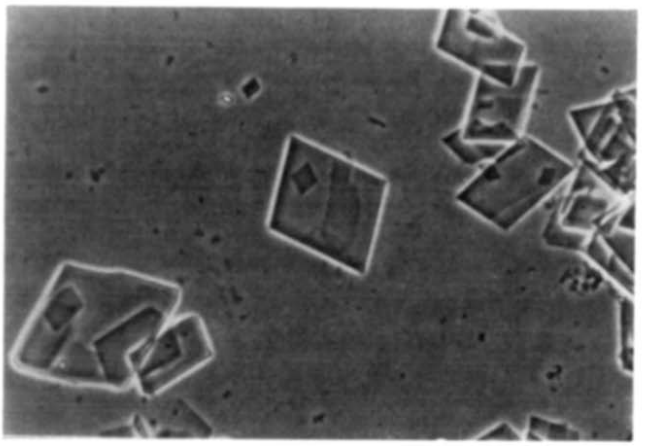
The reduced forms of these *c*-type cytochromes were prepared by the addition of a small amount of $Na_2S_2O_4$ to the solutions and subsequent dialysis overnight against 0.04 M sodium phosphate buffer (pH 6.5) containing approx. 10^{-4} M EDTA.

Cytochrome a

Cytochrome *a* was prepared from beef-heart muscle by means of cholic acid and



A



B

Fig. 1. Crystals of Pseudomonas cytochrome oxidase after two recrystallizations. A; × 90, B; × 160 (phase contrast).

fractionated with ammonium sulphate according to the method of OKUNUKI *et al.*[19].

When reduced cytochrome *c* was oxidized in the presence of cytochrome *a*, the addition of a non-ionic detergent "Emasol" activated the reaction. However, as it was observed that reduced Pseudomonas cytochrome *c*-551 was rapidly oxidized non-enzymically, on addition of Emasol, the detergent was not added in experiments with cytochrome *a* to avoid any influences which might appear on other *c*-type cytochromes besides mammalian cytochrome *c*.

METHODS

Spectrophotometric measurements

Spectrophotometric measurements were performed in a Cary recording spectro-

photometer, model 14, using a cuvette of 1 cm optical path. For anaerobic work, a Thunberg-type cuvette was used. Anaerobiosis was attained by evacuation of the cuvette and introduction of an atmosphere of N_2. This process was repeated at least 5 times.

RESULTS

Absorption spectra

As shown in Fig. 2, the absorption spectrum of the crystalline preparation of Pseudomonas cytochrome oxidase was very similar to that of the crude preparation of the enzyme previously reported[1]. There were peaks at 280 mμ, 412 mμ, 525 mμ and 635 mμ in the oxidized form, and at 418 mμ, 523 mμ, 549 mμ and 554 mμ in the

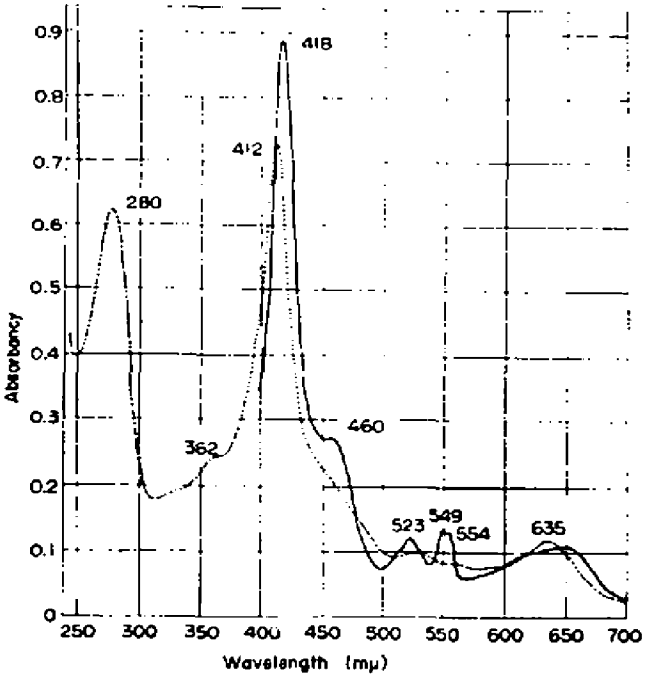


Fig. 2. The absorption spectrum of the crystalline Pseudomonas cytochrome oxidase. The crystals were dissolved in 0.2 M phosphate buffer (pH 7.0). - - - -, oxidized; ———, reduced with $Na_2S_2O_4$.

reduced form. The remarkable difference between the spectrum of the crude preparation and that of the crystalline preparation is, that in the crude preparation there was a peak at 625 mμ after reduction with $Na_2S_2O_4$, while in the crystalline preparation the peak at 635 mμ in the oxidized form was depressed by $Na_2S_2O_4$-reduction and there appeared a plateau from 620 mμ to 650 mμ accompanied by a hump around 650 mμ in the reduced form. On reoxidation with $K_3Fe(CN)_6$, the absorption peak at 635 mμ reappeared. Thus, the depression of the 635 mμ-peak on $Na_2S_2O_4$-reduction was not due to decomposition of the corresponding haem, haem a_2.

On treatment with acetone containing 0.12 N HCl, the protein moiety was

precipitated and the acetone layer became green in colour. The protein moiety precipitated showed the absorption spectrum of a *c*-type cytochrome. The haem extracted in the acetone layer was shown to be haem a_2 by formation of its pyridine haemochromogen[11]. The facts mentioned above indicate that crystalline Pseudomonas cytochrome oxidase has two haems, haem a_2 and a *c*-type haem in the molecule, or that the enzyme consists of a strong combination of a cytochrome a_2 and a *c*-type cytochrome. The fact that the enzyme was obtained in a crystalline state strongly supports the former idea.

On reduction, there appeared a bump at 460 mμ which may represent the γ-band of haem a_2. It has been mentioned that the ratio of A_γ/A_α of the cytochrome a_2 is very low[20]. This was confirmed with crystalline Pseudomonas cytochrome oxidase, as shown in Fig. 2, although cytochrome a_2 which has only haem a_2 in its molecule has not been isolated. In the crystalline preparation, $A_{412m\mu}/A_{280m\mu}$ was 1.2 which was very high and not comparable with $A_{409m\mu}/A_{278m\mu} = 0.87$ in the crude preparation. More detailed observations with respect to the spectral properties of the Pseudomonas cytochrome oxidase will be presented in the next paper of this series[10].

Cytochrome oxidase activity

As shown in Fig. 3, reduced Pseudomonas cytochrome *c*-551 was rapidly oxidized by Pseudomonas cytochrome oxidase under aerobic conditions. This reaction was 96% inhibited in the presence of $3 \cdot 10^{-4}$ M KCN, and was almost completely

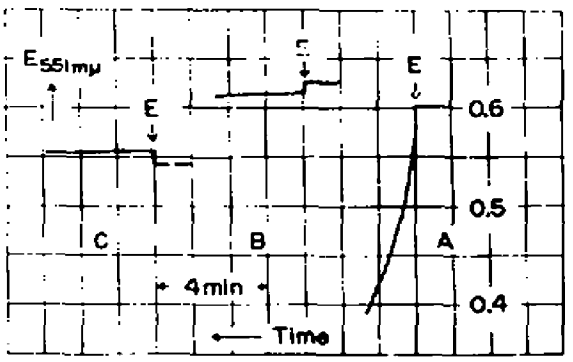


Fig. 3. The aerobic oxidation of reduced Pseudomonas cytochrome *c*-551 by Pseudomonas cytochrome oxidase. The standard reaction mixture consisted of 1.5 ml of 0.03 M phosphate buffer, 0.3 ml of $1.4 \cdot 10^{-4}$ M reduced Pseudomonas cytochrome *c*-551 and 0.03 ml of $8.7 \cdot 10^{-4}$ M Pseudomonas cytochrome oxidase. A, control; B, 0.03 ml of $2 \cdot 10^{-3}$ M KCN added (final concentration, $3.2 \cdot 10^{-4}$ M); C, under the gas mixture, CO-N_2-O_2 (50 : 40 : 10). For C, a Thunberg-type cuvette was used.

inhibited under CO-N_2-O_2 (50 : 40 : 10) in a Thunberg-type cuvette. The turnover number (consumption of reduced *c*-type cytochrome in moles/mole of Pseudomonas cytochrome oxidase) per min, at 16° was 154 with reduced Pseudomonas cytochrome *c*-551 as the electron donor. The concentration of Pseudomonas cytochrome oxidase was determined from $A_{549m\mu} = 30.2 \cdot 10^3$ M^{-1}/cm in the crude preparation[1] assuming that the absorbancy around 550 mμ was not affected by the impurities which might be present in the crude preparation. The turnover number was considerably increased

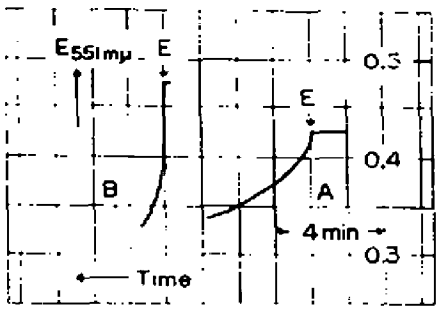


Fig. 4. Temperature dependence of the aerobic oxidation of reduced Pseudomonas cytochrome *c*-551 by Pseudomonas cytochrome oxidase. The reaction mixture was the same as for Fig. 3, except that the concentration of the Pseudomonas cytochrome oxidase was $8.7 \cdot 10^{-7}$ M. A, at 16°; B, at 27°.

at 27°; it was 600 per min. The activation energy for the reaction was calculated as 18.3 kcal. However, the temperature in the reaction mixture was not very precisely controlled and the figure for the activation energy obtained represents only a rough value.

Nitrite reductase activity

It has been established that the crude preparation of the Pseudomonas cytochrome oxidase reduces NO_2^- to NO with reduced Pseudomonas cytochrome *c*-551 as the electron donor. However, there was some doubt as to whether the Pseudomonas cytochrome oxidase itself functions as a nitrite reductase or whether the nitrite-reducing activity was due to another enzyme, nitrite reductase, contaminating the Pseudomonas cytochrome oxidase preparation. As shown in Fig. 5, crystalline Pseudomonas cytochrome oxidase also functioned as a nitrite reductase. This sup-

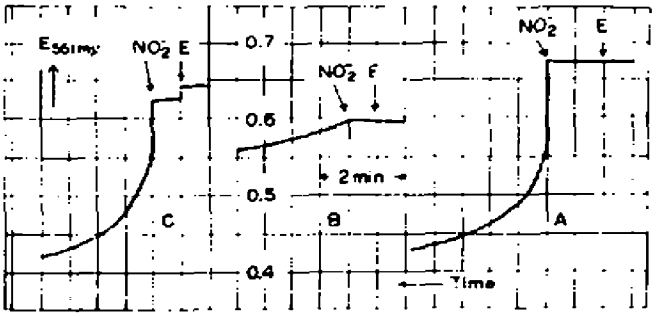


Fig. 5. The anaerobic oxidation of reduced Pseudomonas cytochrome *c*-551 by Pseudomonas cytochrome oxidase in the presence of NO_2^-. The standard reaction mixture consisted of 2.0 ml of 0.04 M phosphate buffer, (pH 6.5), 0.1 ml of $1.4 \cdot 10^{-4}$ M reduced Pseudomonas cytochrome *c*-551, 0.03 ml of 0.2 M KNO_2, and 0.2 ml of $8.7 \cdot 10^{-7}$ M Pseudomonas cytochrome oxidase. Reactions were carried out in Thunberg-type cuvettes which had two side chambers. At the point E, Pseudomonas-cytochrome oxidase was added and the fact that the absorbancy at 551 mμ did not decrease with time showed that the anaerobiosis was complete. On addition of NO_2^-, reduced Pseudomonas cytochrome *c*-551 was rapidly oxidized in A and C. The time required for mixing of NO_2^- with the reaction mixture in the main chamber was approx. 15 sec. Reactions were carried out at 19°, and pH 6.5. A, control; B, 0.1 ml of $2 \cdot 10^{-2}$ M KCN was added (final concentration, $8 \cdot 10^{-4}$ M); C, under 100% CO.

ports the idea that Pseudomonas cytochrome oxidase is the chemical entity of the nitrite reductase of *Pseudomonas aeruginosa*. The nitrite-reducing activity was 94% inhibited by $8 \cdot 10^{-4}$ M KCN. As shown in Fig. 5, the anaerobic oxidation of reduced Pseudomonas cytochrome *c*-551 by the Pseudomonas-cytochrome oxidase with NO_2^- as the electron acceptor proceeded rapidly in the early phase of the reaction but the reaction velocity soon became greatly depressed. In the initial stage of the reaction, the turnover number was 250 per min at 19°. When a Thunberg-type cuvette was used, the temperature was not controlled appreciably. Therefore, the reaction was carried out only at room temperature, and the effect of temperature on the reaction velocity was not examined. Nitrite reduction by the crystalline preparation of Pseudomonas cytochrome oxidase was not inhibited under an atmosphere of 100% CO. This was in good accordance with results obtained with the crude preparation[7].

Biological specificity of the reaction catalysed by Pseudomonas cytochrome oxidase

It has been briefly reported that Pseudomonas cytochrome oxidase oxidized reduced Pseudomonas cytochrome *c*-551 but did not reduce mammalian cytochrome *c* (see ref. 9). As shown in Fig. 6, Pseudomonas cytochrome oxidase oxidized reduced

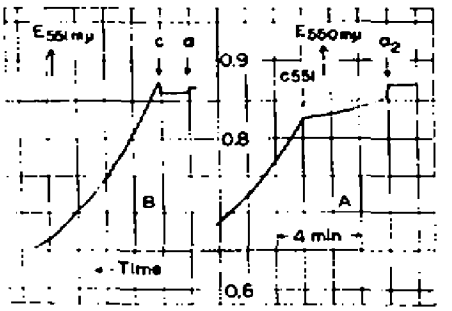


Fig. 6. Influence of a small amount of Pseudomonas cytochrome *c*-551 and yeast cytochrome *c* on the aerobic oxidation of reduced yeast cytochrome *c* by Pseudomonas cytochrome oxidase (A), and that of reduced Pseudomonas cytochrome *c*-551 by cytochrome *a* (B), respectively. In A, the reaction mixture consisted of 3.0 ml of 0.04 M phosphate buffer (pH 6.5), 0.1 ml of $9.7 \cdot 10^{-4}$ M reduced yeast cytochrome *c*, 0.03 ml of $8.7 \cdot 10^{-6}$ M Pseudomonas cytochrome oxidase and 0.03 ml of $1.4 \cdot 10^{-3}$ M oxidized Pseudomonas cytochrome *c*-551. At the points indicated by a_2 and *c*-551 in the figure, were added Pseudomonas cytochrome oxidase and Pseudomonas cytochrome *c*-551, respectively. The reaction was carried out at 15°, and pH 6.5. In B, the reaction mixture consisted of 1.3 ml of 0.04 M phosphate buffer, 0.3 ml of $1.4 \cdot 10^{-4}$ M reduced Pseudomonas cytochrome *c*-551, 0.03 ml of $5.2 \cdot 10^{-6}$ M cytochrome *a* and 0.03 ml of $9.7 \cdot 10^{-4}$ M oxidized yeast cytochrome *c*. At the points indicated by *a* and *c* in the figure, were added cytochrome *a* and yeast cytochrome *c*, respectively. The reaction was carried out at 15°, and pH 6.5.

yeast cytochrome *c* very slowly, while it oxidized reduced Pseudomonas cytochrome *c*-551 very rapidly. The oxidation of reduced yeast cytochrome *c* by Pseudomonas cytochrome oxidase was highly accelerated by the addition of a small amount of Pseudomonas cytochrome *c*-551 (Fig. 6A). As Fig. 6B shows, cytochrome *a* did not oxidize reduced Pseudomonas cytochrome *c*-551 at all. But on the addition of a small amount of yeast cytochrome *c*, reduced Pseudomonas cytochrome *c*-551 was rapidly oxidized by cytochrome *a*. Reduced yeast cytochrome *c* was not oxidized by Pseudomonas cytochrome oxidase in the presence of NO_2^- under anaerobic conditions.

Enzymic oxidation of reduced cytochrome c's obtained from various sources by Pseudomonas cytochrome oxidase

As already mentioned, Pseudomonas cytochrome oxidase rapidly oxidized reduced Pseudomonas cytochrome c-551 but oxidized reduced yeast cytochrome *c* very slowly. Other c-type cytochromes were tested to see if they were oxidized by Pseudomonas cytochrome oxidase. The results are shown in Figs. 7–12. Reduced beef and pigeon cytochrome *c*'s were oxidized less rapidly than reduced yeast cytochrome *c*.

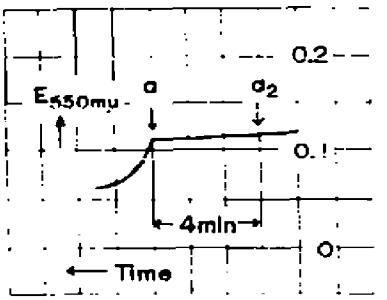


Fig. 7. Oxidation of the reduced form of *Physarum polycephalum* cytochrome *c* by Pseudomonas cytochrome oxidase and cytochrome *a*. The reaction mixture consisted of 1.5 ml of reduced *Physarum polycephalum* cytochrome *c* dissolved in 0.2 M phosphate buffer (pH 7.0), 0.03 ml of $8.7 \cdot 10^{-6}$ M Pseudomonas cytochrome oxidase and 0.03 ml of $5.2 \cdot 10^{-5}$ M cytochrome *a*. At the points indicated by a_2 and *a* in the figure, were added Pseudomonas cytochrome oxidase and cytochrome *a*, respectively. The reaction was carried out at 20° and pH 7.0

It is noteworthy, however, that reduced tunny cytochrome *c* was oxidized more rapidly than reduced beef and pigeon cytochrome *c*'s. Reduced wheat cytochrome *c* was very slowly oxidized by Pseudomonas cytochrome oxidase. The reduced form of *Physarum polycephalum* cytochrome *c* was oxidized fairly rapidly by cytochrome *a*, but scarcely oxidized by Pseudomonas cytochrome oxidase. Previously YAKUSHIJI *et al.*[14] showed qualitatively that reduced *Porphyra tenera* cytochrome-553 was oxidized by Pseudomonas cytochrome oxidase, but not by cytochrome *a*. In Fig. 8,

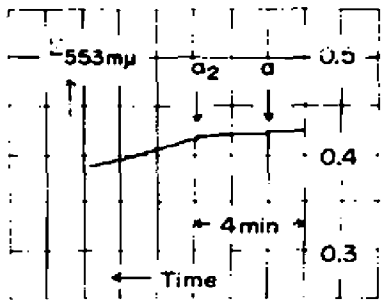


Fig. 8. Oxidation of the reduced form of *Porphyra tenera* cytochrome-553 by cytochrome *a* and Pseudomonas cytochrome oxidase. The reaction mixture consisted of 2.0 ml of 0.04 M phosphate buffer (pH 6.5), 0.4 ml of reduced *Porphyra tenera* cytochrome-553, 0.03 ml of $8.7 \cdot 10^{-6}$ M Pseudomonas cytochrome oxidase and 0.03 ml of $5.2 \cdot 10^{-5}$ M cytochrome *a*. At the points indicated by *a* and a_2, were added cytochrome *a* and Pseudomonas cytochrome oxidase, respectively. The reaction was carried out at 18° and pH 6.5.

their findings are shown quantitatively. In Figs. 7–12, the oxidation of these reduced *c* type cytochromes by cytochrome *a* are also shown. It is apparent that reduced beef cytochrome *c* was oxidized less rapidly than reduced pigeon, tunny and yeast cytochrome *c*'s.

It was shown, in the case of tunny cytochrome *c*, that the oxidation velocity of

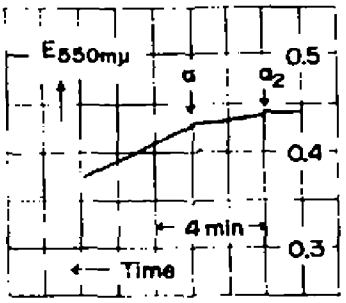


Fig. 9. Oxidation of reduced wheat cytochrome *c* by Pseudomonas cytochrome oxidase and cytochrome *a*. The reaction mixture consisted of 2.0 ml of 0.04 M phosphate buffer (pH 6.5), 0.6 ml of reduced wheat cytochrome *c*, 0.03 ml of $8.7 \cdot 10^{-6}$ M Pseudomonas cytochrome oxidase, 0.03 ml of $5.2 \cdot 10^{-6}$ M cytochrome *a*. At the points indicated in the figure by a_2 and *a*, were added Pseudomonas cytochrome oxidase and cytochrome *a*, respectively. The reaction was carried out at 18°.

reduced cytochrome *c* in the presence of both Pseudomonas cytochrome oxidase and cytochrome *a* was not the sum of the activities of the two enzymes, but was approximately the same as that of cytochrome *a* only. Therefore, it may be that the rate of oxidation of the reduced form of beef, pigeon and *Physarum polycephalum* cytochrome *c*'s by cytochrome *a* was scarcely affected by the co-existence of Pseudomonas cytochrome oxidase. The turnover numbers of some *c*-type cytochromes in the oxidation reaction are presented in Table I. The molar extinction coefficient of beef and yeast cytochrome *c*'s at 550 mμ is known to be $27.8 \cdot 10^3$ M^{-1}/cm (see ref. 23), and

TABLE I

THE OXIDATION OF VARIOUS *c* TYPE CYTOCHROMES BY PSEUDOMONAS CYTOCHROME OXIDASE AND CYTOCHROME *a*

The numbers in the parentheses represent the temperature under which the experiments were carried out.

Source	Turnover number (moles/mole of enzyme/min)	
	Pseudomonas cytochrome oxidase	Cytochrome *a*
Pseudomonas aeruginosa	154 (16°)	0 (15°)
Porphyra tenera	4.1 (18°)	Trace (18°)
Physarum polycephalum	0.66 (20°)	3.8 (20°)
Yeast (baker's)	6.8 (15°)	50.3 (16°)
Wheat	1.2 (18°)	13.2 (18°)
Tunny	4.8 (18°)	21.7 (18°)
Pigeon	0.68 (18°)	35.3 (18°)
Beef	0.81 (18°)	20.0 (18°)

that of Pseudomonas cytochrome *c*-551 at 551 mμ is $28.3 \cdot 10^3$ M^{-1}/cm. However, as the molar extinction coefficients for *Physarum polycephalum*, wheat, and tunny cytochrome *c*'s were not determined, the turnover numbers in Table I are calculated assuming that the molar extinction coefficients of the α-bands of all *c*-type cytochromes are all $28 \cdot 10^3$ M^{-1}/cm. This assumption is perhaps reasonable. In the case of *Physarum polycephalum* cytochrome *c*, the concentration of cytochrome *a* was 10-times that used in the case of other cytochrome *c*'s. However, it was found that in the oxidation of reduced Pseudomonas cytochrome *c*-551 by the Pseudomonas

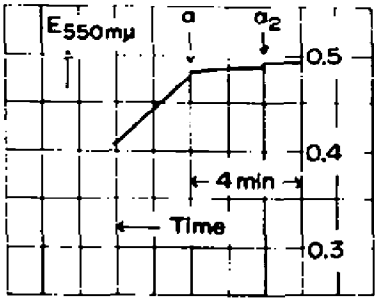


Fig. 10. Oxidation of reduced beef cytochrome *c* by Pseudomonas-cytochrome oxidase and cytochrome *a*. The reaction mixture consisted of 2.0 ml of 0.04 M phosphate buffer (pH 6.5), 0.2 ml of reduced beef cytochrome *c*, 0.03 ml of $8.7 \cdot 10^{-5}$ M Pseudomonas cytochrome oxidase, and 0.03 ml of $5.2 \cdot 10^{-5}$ M cytochrome *a*. At the points indicated in the figure by a_2 and *a*, Pseudomonas cytochrome oxidase and cytochrome *a* were added, respectively. The reaction was carried out at 18°.

cytochrome oxidase, 10-times the concentration of enzyme only accelerated the oxidation of the reduced Pseudomonas cytochrome *c*-551, 5-times at most, when the concentrations of the Pseudomonas cytochrome oxidase and Pseudomonas cytochrome *c*-551 were approx. 10^{-6} M. Thus, the turnover number in the oxidation of the reduced form of *Physarum polycephalum* cytochrome *c* is probably twice that calculated from the results shown in Fig. 7. In Table I, the value of the turnover number corrected is shown as for *Physarum polycephalum* cytochrome *c*.

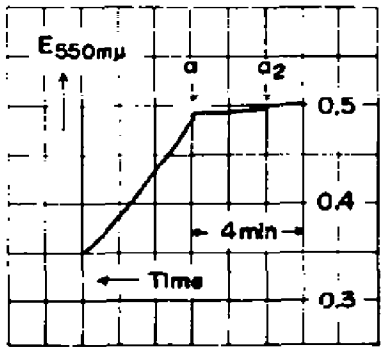


Fig. 11. Oxidation of reduced pigeon cytochrome *c* by Pseudomonas cytochrome oxidase and cytochrome *a*. The reaction mixture consisted of 2.0 ml of 0.04 M phosphate buffer, 0.6 ml of reduced pigeon cytochrome *c*, 0.03 ml of $8.7 \cdot 10^{-5}$ M Pseudomonas cytochrome oxidase, and $5.2 \cdot 10^{-5}$ M cytochrome *a*. At the points indicated in the figure by a_2 and *a*, were added Pseudomonas cytochrome oxidase and cytochrome *a*, respectively. The reaction was carried out at 18°.

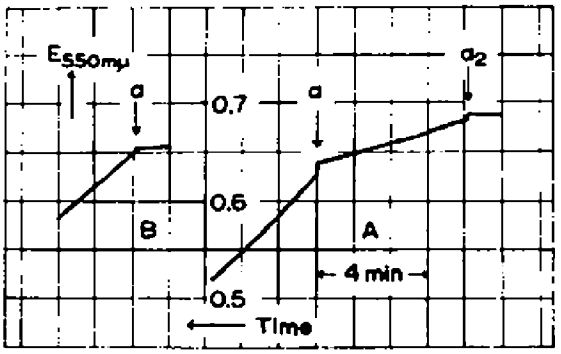


Fig. 12. Oxidation of reduced tunny cytochrome *c* by Pseudomonas cytochrome oxidase. In A, the reaction mixture consisted of 2.0 ml of 0.04 M phosphate buffer (pH 6.5), 0.6 ml of reduced pigeon cytochrome *c*, 0.03 ml of $8.7 \cdot 10^{-6}$ M Pseudomonas-cytochrome oxidase and 0.03 ml of $5.2 \cdot 10^{-6}$ M cytochrome *a*. In B, the reaction mixture was the same as for A, except that Pseudomonas cytochrome oxidase was omitted. At the points indicated in the figures by a_2 and *a*, were added Pseudomonas cytochrome oxidase and cytochrome *a*, respectively. The reactions were carried out at 18°.

DISCUSSION

It has been established with a crude preparation of Pseudomonas cytochrome oxidase that it has two haems, haem a_2 and a *c*-type haem, in the molecule, and that it has two activities: cytochrome oxidase and nitrite reductase activity. These properties have been confirmed using a crystalline preparation of the enzyme. However, it was doubtful whether the Pseudomonas cytochrome oxidase molecule had two haems in the one molecule or whether it consisted of a strong combination of two proteins; a haem a_2-containing protein and a haem *c*-containing one. The former seemed the more probable because Pseudomonas cytochrome oxidase was obtained in a crystalline state with both haems. There are the same two possibilities with regard to the two functions of the enzyme. Two enzymes, a cytochrome oxidase and a nitrite reductase, might represent the strong combination of two protein molecules. This possibility is apparently supported by the fact that the cytochrome oxidase activity of the enzyme was inhibited in the presence of cyanide and carbon monoxide, whereas the nitrite reductase activity was inhibited in the presence of cyanide but not of carbon monoxide. However, spectral studies showed that nitrite has a stronger affinity for the enzyme than carbon monoxide[10]. The fact that Pseudomonas cytochrome oxidase was obtained in crystalline state supports the idea that the Pseudomonas cytochrome oxidase itself functions both as a nitrite reductase and as a cytochrome oxidase.

WALKER AND NICHOLAS[22] obtained a nitrite reductase from *Pseudomonas aeruginosa*. Although their enzyme was still very crude, its absorption spectrum was very similar to that of Pseudomonas cytochrome oxidase. The chemical entity of their enzyme preparation perhaps corresponds to Pseudomonas-cytochrome oxidase. As already indicated[5], Pseudomonas cytochrome oxidase has not been obtained from cells of the organism grown aerobically in the absence of nitrate. Recently we have shown that Pseudomonas cytochrome oxidase is an adaptive enzyme and that nitrate is an essential factor for its biosynthesis[23]. The facts mentioned above strongly

support the idea that Pseudomonas cytochrome oxidase is the chemical entity of the nitrite reductase of *Pseudomonas aeruginosa*, and also acts as a cytochrome oxidase. Thus, it will be concluded that the nitrite reductase of *Pseudomonas aeruginosa*, and if two types exist, especially the enzyme of the respiratory type, is a cytochrome and it is this which was obtained in a crystalline state.

The turnover numbers in the aerobic oxidation of reduced Pseudomonas cytochrome *c*-551 by the Pseudomonas cytochrome oxidase were 154 and 600 per min, at 16° and 27° respectively. From these data, the turnover number at 37° is calculated to be 2400. This corresponds to 600 moles of oxygen consumption, and consequently to 13 440 l oxygen consumption/mole of Pseudomonas cytochrome oxidase/min, at 37°. In nitrite reduction, 250 moles of reduced Pseudomonas cytochrome *c*-551 were oxidized anaerobically per mole of Pseudomonas cytochrome oxidase in the presence of nitrite, at 19°. As reported previously[7], one mole of nitrite is reduced with consequent oxidation of one mole of reduced Pseudomonas cytochrome *c*-551. If the temperature dependence of the reaction velocity is the same as that in the aerobic oxidation of reduced Pseudomonas cytochrome *c*-551, about 4000 moles of nitrite are reduced/mole of the Pseudomonas-cytochrome oxidase/min, at 37°, at normal growth temperature.

It has been reported briefly by HORIO[9] that the Pseudomonas cytochrome oxidase preparation oxidizes reduced Pseudomonas cytochrome *c*-551 but not reduced mammalian cytochrome *c*, and that cytochrome *a* oxidizes reduced mammalian cytochrome *c* but not reduced Pseudomonas cytochrome *c*-551. Similar results have been presented by KAMEN AND VERNON[24] for crude preparations of bacterial and mammalian cytochrome oxidases. The present experiments extend the results of HORIO. It was clearly shown in this paper, that, in order to prove the biological specificity of the oxidation of reduced *c*-type cytochromes by cytochrome oxidase, the cytochrome oxidase preparation should be free of *c*-type cytochrome present in the organism from which the cytochrome oxidase was isolated. Thus, it is very important to purify each component, in order to demonstrate biological specificity in the oxidation of *c*-type cytochromes by the cytochrome oxidase. Fig. 6 also shows that, if there are other kinds of *c*-type cytochromes in the cytochrome *c* preparation isolated from one organism, the oxidation velocity of the cytochrome *c*'s by cytochrome oxidase is the same as that of the cytochrome *c* which reacts most rapidly with the cytochrome oxidase.

A clear difference was found between the rate of oxidation of reduced tunny and beef cytochrome *c*'s, by the Pseudomonas cytochrome oxidase. This suggests that there is a great difference between the enzymic properties of tunny and beef cytochrome *c*'s, although there was no difference in their absorption spectra, adsorbability on a cation-exchange resin, and both appeared in the same ammonium sulphate fraction. The difference in the rate of oxidation of these *c*-type cytochromes by Pseudomonas cytochrome oxidase, therefore, detected more delicate differences in their molecular structures than the techniques mentioned above.

Reduced pigeon cytochrome *c* was oxidized by Pseudomonas-cytochrome oxidase at almost the same velocity as reduced beef cytochrome *c*. Reduced yeast cytochrome *c* was oxidized by Pseudomonas-cytochrome oxidase considerably faster than reduced beef cytochrome *c*. This seems natural, since yeast is closer to bacteria phylogenetically than to animals. It is very curious, however, that the

reduced forms of yeast, pigeon and tunny cytochrome *c*'s were oxidized faster than reduced beef cytochrome *c* by cytochrome *a*, although the cytochrome *a* was isolated from beef heart. From the viewpoint of comparative biochemistry, the fact that reduced tunny cytochrome *c* was more rapidly oxidized by the Pseudomonas cytochrome oxidase than reduced pigeon and beef cytochrome *c*'s is in good accordance with the fact that fish is more primitive than birds and animals.

It is very noteworthy that the reduced form of *Physarum polycephalum* cytochrome *c* was rapidly oxidized by cytochrome *a*, whereas it was oxidized very slowly by Pseudomonas cytochrome oxidase. In present taxonomies, slime mould is classified as a plant. The oxidation of the reduced form of *Physarum polycephalum* cytochrome *c* by cytochrome *a*, however, strongly suggests that the slime mould is more closely related to animals than to plants in its biochemical properties, at least in the plasmodial form. This is supported in part by the fact found by NAKAJIMA[26] that actomyocin isolated from the slime mould behaved in a similar manner to the enzyme isolated from the muscle of vertebrates.

It seems natural that the reduced form of *Porphyra tenera* cytochrome-553 was oxidized by Pseudomonas cytochrome oxidase whereas it was scarcely oxidized by cytochrome *a*, since algae are more closely related to bacteria than to animals.

We are purifying various *c*-type cytochromes other than those cited in the present paper, and are studying the reactivity of these cytochrome *c*'s with Pseudomonas cytochrome oxidase and cytochrome *a*, from the viewpoint of comparative biochemistry. It may give a molecular basis to the taxonomy and the evolution of these organisms to study the enzymic oxidizability of *c*-type cytochromes from various organisms catalysed by the Pseudomonas cytochrome oxidase and cytochrome *a*.

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