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OCCURRENCE OF BOTH *a*-TYPE AND *o*-TYPE CYTOCHROMES AS THE FUNCTIONAL TERMINAL OXIDASES IN *RHODOPSEUDOMONAS SPHEROIDES*

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SUMMARY

1. The functional terminal oxidase of the light–anaerobically grown *Rhodopseudomonas spheroides* cells was found to be the *o*-type cytochrome, whereas that of the dark–aerobically grown cells was the *a*-type cytochrome. When the dark–aerobically grown cells were further incubated under a semianaerobic condition in the dark, the content of the *o*-type cytochrome was increased in these cells, while the synthesis of the *a*-type cytochrome appeared to be repressed. In *Rhodospirillum rubrum* cells, grown either aerobically in the dark or anaerobically in the light, cytochrome *o* was the sole functional terminal oxidase.

2. Reactions with the *a*-type and *o*-type cytochromes from *Rhodopseudomonas spheroides* and also with the *o*-type cytochrome from *Rhodospirillum rubrum* were compared using reduced yeast cytochrome *c* as substrate. The reaction with the *a*-type cytochrome was far less sensitive to NaN_3 and hydroxylamine than those with the *o*-type cytochromes, whereas all the reactions were inhibited by KCN in apparently the same manner.

INTRODUCTION

Studies on the terminal oxidase of dark–aerobically grown non-sulphur purple bacteria have established that the functioning terminal oxidase in *Rhodopseudomonas spheroides* (*Rps. spheroides*) is the *a*-type cytochrome[1–3] and that in *Rhodospirillum rubrum* (*Rsp. rubrum*) is the *o*-type cytochrome[4]. Preparations containing the *a*-type or the *o*-type cytochrome catalyzed the oxidation of reduced cytochrome *c* (from horse heart and baker's yeast) and cytochrome c_2, and these reactions were inhibited by inhibitors of cytochrome oxidase. On the other hand, little is known about the terminal oxidase of the light–anaerobically grown bacteria though they perform respiration in aerobic conditions[1]. In the previous communication[3] we suggested the occurrence of the *o*-type cytochrome in light–anaerobically grown *Rps. spheroides* on the basis of the kinetic data. Further, examination of the spectral properties of the

Abbreviations: *Rps.*, *Rhodopseudomonas*; *Rsp.*, *Rhodospirillum*; DCIP, 2,6-dichlorophenolindophenol.

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particulate preparations from *Rps. spheroides* cells suggested that the *o*-type cytochrome might occur even in the dark–aerobically grown *Rps. spheroides* cells when they were harvested after the stationary phase[3].

The experiments described in this paper were designed to elucidate the nature of terminal oxidases in *Rps. spheroides* grown under different conditions.

MATERIALS AND METHODS

Preparation of bacteria

Rps. spheroides and *Rsp. rubrum*, originally obtained from Dr. Van Niel, were grown in Medium S of LASCELLES[5] containing 0.34% L-glutamate and 0.27% DL-malate as major nutrients. Stab cultures of these bacteria were used to inoculate starter culture. For the preparation of dark–aerobically grown bacteria, cells were inoculated into 140 ml of the medium in a 500-ml Sakaguchi flask, and incubated with vigorous shaking for 24 h at 28° in the dark. The culture was then transferred into a 3-l erlenmyer flask filled with 1.1 l of the medium and incubated at 28° for 16 h on a rotary shaker in the dark. Every batch was tested for cell purity by taking 0.02 ml of the cell suspension and growing the cells on an agar plate aerobically in the dark and subsequently in the light to ensure pigmentation. For the preparation of light–anaerobically grown bacteria, cells were inoculated into 500 ml of the medium in a 500-ml bottle with a glass stopper, and incubated at 28° for 3–4 days under illumination. An aliquot of this culture (40 ml) was transferred into a 5-l bottle filled to the neck with the medium and incubated at 28° for 4–5 days under illumination.

Particulate preparation

Cells were harvested by centrifugation and washed once with 0.05 M Tris–HCl buffer (pH 9.0). The cells were stored as a paste at −20°. The particulate preparation was made by resuspending 1 portion of cell paste in 5 vol. of the above buffer, and the suspension was treated with sonic oscillation with a 10-kcycles Kubota Sonicator for 10 min. After centrifugation for 10 min at 9400 × *g* to remove cell debris, the supernatant was centrifuged at 105000 × *g* for 90 min. The precipitate was resuspended in the above buffer, homogenized with a glass homogenizer and centrifuged again. The precipitate was suspended in a small volume of 0.05 M Tris–HCl buffer (pH 7.0), homogenized and centrifuged at 5000 × *g* for 10 min. The supernatant fraction was used as particulate preparation.

Purification of cytochrome oxidase

The solubilized oxidase preparation from dark–aerobically grown *Rsp. rubrum* was prepared and purified by the method described previously[2]. For the purification of the cytochrome oxidase from dark–aerobically grown *Rps. spheroides*, the previous method[2] was improved. The following is a typical protocol. Unless otherwise stated, all buffer solutions were 0.05 M Tris–HCl (pH 9.0), and all operations were carried out at 0–4°. Cells (200 g wet weight) were suspended in a total of 400 ml of buffer and sonicated in 30-ml batches for 10 min. Triton X-100 (8 ml) was added to the solution which was then stirred overnight. The solution was centrifuged at 77000 × *g* for 30 min, and the supernatant was collected. The precipitate was resuspended in a total of 200 ml of buffer containing 4 ml of Triton X-100, and was treated as described

above. The supernatant solution was collected and added to the previous one. 80 ml of $(NH_4)_2SO_4$, saturated at 4° and adjusted to pH 9.0 with ammonia, were added to 535 ml of the above solution, and the mixture was stirred for 1 h. The solution was centrifuged at 9000 × *g* for 10 min, and the precipitate was discarded. To the supernatant solution, 182 ml of saturated $(NH_4)_2SO_4$ were added. After standing for 1 h, the solution was centrifuged at 9000 × *g* for 10 min. A brown layer which appeared on the top was collected and dissolved in 80 ml of buffer and dialyzed overnight against 3 l of buffer. After the protein concentration of the solution was adjusted to 3 mg/ml by buffer, the solution was brought to 1% (w/v) with sodium cholate. Saturated $(NH_4)_2SO_4$ (320 ml) was added to 720 ml of the solution and the solution was stirred for 20 min. After centrifugation at 8000 × *g* for 10 min, the precipitate was discarded. Saturated $(NH_4)_2SO_4$ (50 ml) was added to the supernatant solution which was then stirred for 10 min and centrifuged as above. The precipitate was discarded. To the resulting supernatant, another 50 ml of saturated $(NH_4)_2SO_4$ solution were added and the mixture was processed in the same way. This procedure was repeated once. To the supernatant solution obtained after the third addition of 50 ml of $(NH_4)_2SO_4$ solution, 60 ml of saturated $(NH_4)_2SO_4$ were further added, and the solution was centrifuged at 8000 × *g* for 10 min. A brownish-green layer which appeared on the top was collected and dissolved in a small amount of buffer and dialyzed overnight against buffer. To the solution (45 ml), 0.45 g of sodium cholate and 24 ml of saturated $(NH_4)_2SO_4$ were added and the mixture was stirred for 20 min. The solution was centrifuged at 8000 × *g* for 10 min, and the precipitate was dissolved in a small volume of buffer and dialyzed against buffer for 12 h. The final product was clear and dark green.

Determination of cytochrome c oxidase activity

Yeast cytochrome *c*, kindly supplied by Sankyo and Co., Tokyo, was reduced by the addition of sodium dithionite and passed through a column of Sephadex G-25 to eliminate dithionite. The activity of cytochrome *c* oxidase was measured by the decrease of absorbance at 550 mμ at 20° traced by a Hitachi recording spectrophotometer, Type EPS-2. When inhibitors were used, the oxidase preparation was incubated for 1 min with the inhibitor, and reaction was started by the addition of reduced cytochrome *c*.

Protein determination

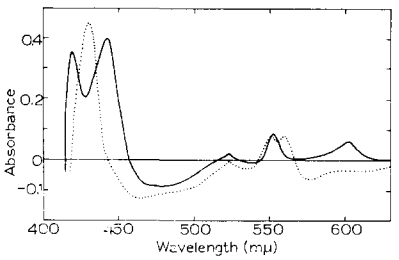
Protein was determined by the method of LOWRY *et al.*[6] using bovine serum albumin as standard.

RESULTS

Properties of purified oxidase preparations from dark–aerobically grown cells

Difference spectra (dithionite-reduced *minus* oxidized) of purified oxidase preparations from dark–aerobically grown *Rps. spheroides* and *Rsp. rubrum* cells are shown in Fig. 1. As can be seen from the figure, the preparation obtained from *Rps. spheroides* contained an *a*-type cytochrome with an α-peak at 603 mμ and a γ-peak at 443 mμ. The preparation still contained a *c*-type cytochrome whose properties were described previously[2], but there was no absorption corresponding to a *b*-type cytochrome. On

the contrary, the preparation obtained from *Rsp. rubrum* contained no *a*-type cytochrome but *b*- and *c*-type cytochromes. An appreciable amount of the *b*-type cytochrome in the preparation was reducible with ascorbate–2,6-dichlorophenolindophenol (ascorbate–DCIP) system and this cytochrome was found to react with CO.



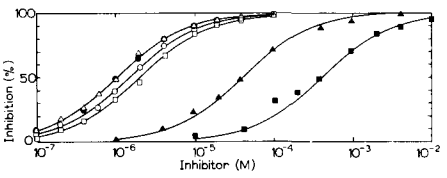


Fig. 1. Reduced-*minus*-oxidized difference spectra of the purified oxidase preparations from dark–aerobically grown *Rps. spheroides* and *Rsp. rubrum* cells. Samples in measuring cuvettes were reduced with sodium dithionite. ———, *Rps. spheroides*; ·····, *Rsp. rubrum*.

Fig. 2. Relationships between the concentrations of inhibitors and the inhibition rates of cytochrome *c* oxidation by the purified oxidase preparations. The reaction mixture contained: 200 μmoles phosphate buffer (pH 7.0), 75 mμmoles yeast cytochrome *c*, inhibitor and enzyme preparation (*Rps. spheroides*, 0.37 mg as protein and *Rsp. rubrum*, 5.3 mg as protein) in a total volume of 3 ml. Activities of 100% were 37 mμmoles cytochrome *c*/min ($\Delta A_{550\ m\mu} = 0.34$/min) with *Rps. spheroides* and 12 mμmoles cytochrome *c*/min ($\Delta A_{550\ m\mu} = 0.112$/min) with *Rsp. rubrum*. Open symbols, *Rsp. rubrum*; closed symbols, *Rps. spheroides*; ○—○, KCN; △—△, NaN_3; □—□, hydroxylamine.

These preparations catalyzed the oxidation of reduced yeast cytochrome *c*, and the reaction was inhibited by inhibitors of cytochrome oxidase such as KCN, NaN_3 and hydroxylamine. The effects of inhibitors upon oxidation of yeast cytochrome *c* by these preparations are depicted in Fig. 2. The relationships between the per cent inhibition of the reaction and the concentrations of inhibitors were always found to follow Hill's equation where $n = 1$. Of particular interest is the effect of NaN_3 and hydroxylamine. These two substances were more effective on the oxidase preparation from *Rsp. rubrum* than on that from *Rps. spheroides*, whereas KCN affected both the preparations to the same degree. K_i values (concentrations of inhibitors for 50% inhibition) calculated from the several experiments were as follows. (1) For the oxidase preparation from *Rps. spheroides*: KCN, 1 μM; NaN_3, 50 μM; and hydroxylamine, 400 μM. (2) For the oxidase preparation from *Rsp. rubrum*: KCN, 1.5 μM; NaN_3, 1 μM and hydroxylamine, 2.5 μM. The effect of pH on the activity of yeast cytochrome *c* oxidation was also different in these two preparations, the optimal pH's being 6.8 for *Rps. spheroides* and 5.6 for *Rsp. rubrum*. The observed differences in sensitivities to the inhibitors and H^+ concentration between *Rps. spheroides* and *Rsp. rubrum* are due to the difference in nature of their terminal oxidases. Hence it may be concluded that the *a*-type cytochrome in *Rps. spheroides* is less sensitive to NaN_3 and hydroxylamine than the *o*-type cytochrome in *Rsp. rubrum*. The terminal oxidase of dark–aerobically grown *Rsp. rubrum* has been reported to be cytochrome *o* by TANIGUCHI AND KAMEN[4]. The same K_i values for these inhibitors as obtained with the purified preparation from *Rsp. rubrum* in the oxidation of yeast cytochrome *c* were also obtained with crude particulate preparations from dark–aerobically grown

Rsp. rubrum cells, indicating that *Rsp. rubrum* may have only one species of terminal oxidase, cytochrome *o*.

Properties of particulate preparations obtained from light–anaerobically grown cells

Particulate preparations obtained from light–anaerobically grown cells of either *Rps. spheroides* or *Rsp. rubrum* catalyzed the oxidation of yeast cytochrome *c*. To determine the species of cytochrome responsible for the oxidase activity in these light–anaerobically grown cells, inhibition studies were performed. With the particulate preparations from either *Rps. spheroides* or *Rsp. rubrum* cells, the relationships between the concentrations of inhibitors and the inhibition rates were again found to follow Hill's equation where $n = 1$, but with these preparations practically no difference could be observed in sensitivities toward NaN_3 and hydroxylamine between *Rps. spheroides* and *Rsp. rubrum*. The K_i values for all inhibitors were found to be in the range of 1 to 2 μM, and these values are in good agreement with the values obtained with the purified oxidase preparation from dark–aerobically grown *Rsp. rubrum*. These results strongly suggest that the terminal oxidase in each of the light–anaerobically grown *Rps. spheroides* and *Rsp. rubrum* cells is the *o*-type cytochrome.

Replacement of the a-type cytochrome by the o-type cytochrome in dark–aerobically grown Rps. spheroides cells resulting from a semi-anaerobic incubation

When cytochrome *c* oxidase activity was examined in particulate preparations obtained from several different batches of dark–aerobically grown *Rps. spheroides*, it was noticed that the curves indicating the relationships between the inhibition rates and the concentrations of NaN_3 and hydroxylamine did not always follow Hill's equation where $n = 1$, and the K_i values for these inhibitors sometimes appeared to be reduced. The aspects of the curves suggested the possibility of the existence of two kinds of oxidase in dark–aerobically grown *Rps. spheroides*. As described previously[3], the existence of the *o*-type cytochrome in addition to the *a*-type cytochrome in lightly pigmented cells was suggested by the spectral properties of the particulate preparation obtained from those cells. Since the terminal oxidase of dark–aerobically grown *Rps. spheroides* was shown to be the *a*-type cytochrome whereas that of the light–anaerobically grown cells appeared to be the *o*-type cytochrome, it seems possible that the relative contents of the *a*-type and *o*-type cytochromes in the cells are dependent on the aerobiosis of the growth condition.

To confirm this idea, the dark–aerobically grown *Rps. spheroides* cells were further incubated under a relatively low O_2 tension, that is, a relatively heavy suspension of the cells was further incubated for 3 h in air in the dark with gentle shaking as described by HIGUCHI *et al.*[7]. A particulate preparation from these cells was made as described in MATERIALS AND METHODS, and the spectral properties as well as the sensitivities to KCN, NaN_3 and hydroxylamine of the oxidase activity in the particulate preparation were examined.

To record difference spectra, the particulate preparation was incubated with 3% Triton X-100 at 30° for 10 min to obtain well-dispersed material, and the sample was then reduced with either dithionite or the ascorbate–DCIP system. In Fig. 3 are shown the difference spectra (with and without the reducing system) of particulate preparations obtained from the cells before and after the incubation. The amount of the *a*-type cytochrome, as judged from the $A_{603\ m\mu}$, was lessened after incubation,

while the amount of a *b*-type cytochrome reducible with the ascorbate–DCIP system was increased. The CO difference spectrum of the particulate preparation from the incubated cells is depicted in Fig. 4. There are absorption maxima at about 415, 540 and 570 mμ and troughs at about 430 and 560 mμ. These spectral properties are in

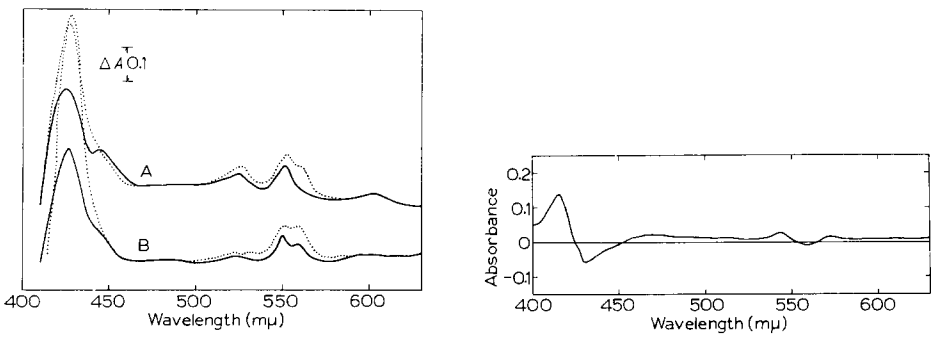


Fig. 3. Difference spectra (reduced *minus* oxidized) of the respective particulate preparations obtained from dark–aerobically grown *Rps. spheroides* cells before and after the further incubation under a dark–semianaerobic condition. A, before the semianaerobic incubation; B, after the semianaerobic incubation. ———, reduced with ascorbate–DCIP (ascorbate 30 μmoles and DCIP 3 mμmoles in 3 ml); ·····, reduced with sodium dithionite.

Fig. 4. CO difference spectrum of the particulate preparation obtained from *Rps. spheroides* cells grown aerobically in the dark and further incubated semianaerobically in the dark.

accord with the CO difference spectra obtained from the membrane fragment of dark–aerobically grown *Rsp. rubrum*[4]. The amount of the *o*-type cytochrome in the dark–aerobically grown *Rps. spheroides* cells increased during the semianaerobic incubation.

The effects of inhibitors on the oxidation of yeast cytochrome *c* catalyzed by the particulate preparations are compared in Fig. 5. Although the behaviour of KCN inhibition did not change after the semianaerobic incubation of the cells, the curves indicating the relationships between the inhibition rates and the concentrations of NaN_3 and hydroxylamine became more biphasic after the cell incubation, and the K_i values were reduced as shown in Fig. 5. These findings would establish the above

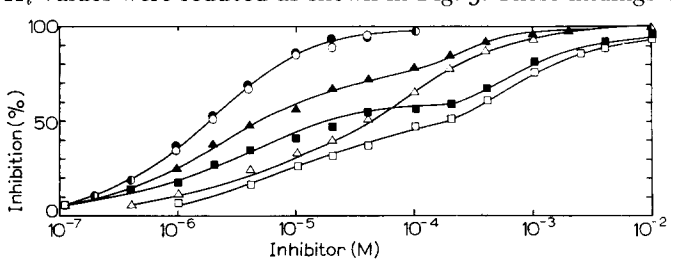


Fig. 5. Relationships between the concentrations of inhibitors and the inhibition rates of cytochrome *c* oxidation by the respective particulate preparations from dark–aerobically grown *Rps. spheroides* cells before and after the further incubation in a dark–semianaerobic condition. The composition of the reaction mixture was the same as for Fig. 2 except for the amount of enzyme preparation (enzyme preparation from cells before the semianaerobic incubation, 0.56 mg as protein and that after the semianaerobic incubation, 0.3 mg as protein). 100% activities were 49 mμmoles/min ($\Delta A_{550\,m\mu}$ = 0.45/min) with the enzyme preparation before the incubation, and 54 mμmoles/min ($\Delta A_{550\,m\mu}$ = 0.51/min) with that after the incubation. Open symbols, before the semianaerobic incubation; closed symbols, after the semianaerobic incubation. ○—○, KCN; △—△, NaN_3; □—□, hydroxylamine.

TABLE I

YEAST CYTOCHROME *c* OXIDATION BY PARTICULATE PREPARATIONS

Each reaction mixture contained 70 mM phosphate buffer (pH 7.0), appropriate amounts of reduced yeast cytochrome *c* and particulate preparation.

Species	*Growing condition*	*Specific activity (mμmoles/min per mg protein)*	*K_m for cytochrome c (μM)*
Rps. spheroides	Light–anaerobic	49	13
	Dark–aerobic	162	6.6
	Dark–semianaerobic*	238	6.6–10.0
Rsp. rubrum	Light–anaerobic	5	40
	Dark–aerobic	16	40

* Dark–aerobically grown cells were transferred to a semianaerobic condition and incubated further for 3 h in the dark.

notion. Michaelis constants in the oxidation of yeast cytochrome *c* with the particulate preparations obtained from cells grown under different conditions are shown in Table I. When the dark grown *Rps. spheroides* cells were further incubated semianaerobically, the K_m value obtained with the particulate preparation from the dark grown cells became close to that found for the light–anaerobically grown *Rps. spheroides* cells. With respect to *Rsp. rubrum*, however, apparently the same K_m value was obtained with either dark–aerobically grown or light–anaerobically grown cells. These findings also appear to support the above idea.

DISCUSSION

The data presented here confirm the existence of the *a*-type cytochrome in *Rps. spheroides* grown aerobically in the dark and the *o*-type cytochrome in *Rsp. rubrum* grown under the same condition. Both kinds of cytochrome were found to catalyze the oxidation of reduced yeast cytochrome *c* though the *o*-type was less active than the *a*-type. The cytochrome *c* oxidation by either type of cytochrome was inhibited by inhibitors for cytochrome oxidase. SEKUZU *et al.*[8] reported that NaN_3 and hydroxylamine were less effective than KCN in inhibiting cytochrome *c* oxidation by cytochrome *a* obtained from *Saccharomyces oviformis*. This was also so for the *a*-type cytochrome from *Rps. spheroides*. On the contrary, NaN_3 and hydroxylamine were as effective as KCN in the cytochrome *c* oxidation catalyzed by the *o*-type cytochrome from *Rsp. rubrum*. This distinct difference in inhibition facilitated the discovery that the terminal oxidase in light–anaerobically grown *Rps. spheroides* was the *o*-type cytochrome. It was further demonstrated that the *o*-type cytochrome was increased in dark grown *Rps. spheroides* cells when they were transferred from highly aerobic to semianaerobic incubation conditions. The appearance of the *o*-type cytochrome in these cells was confirmed by reduction of the cytochrome with the ascorbate–DCIP system (*cf.* ref. 9) and by the CO difference spectrum. However, there seems to be some difference between the *o*-type cytochromes in *Rsp. rubrum* and *Rps. spheroides* as judged from the K_m values for yeast cytochrome *c* (*cf.* Table I), but this point was not studied in detail.

Both the *a*-type and *o*-type cytochromes can function as the terminal oxidase in *Rps. spheroides*. The change of the functional terminal oxidase in *Rps. spheroides* evidently results from variation in the O_2 tension in the culture medium. It is unknown, however, whether the *o*-type cytochrome is a precursor of the *a*-type cytochrome in *Rps. spheroides*.

We may imagine from the results of this study that the *a*-type cytochrome is the terminal oxidase for organisms phylogenically well-adapted to aerobic condition, whereas the *o*-type cytochrome is that for the microorganisms poorly adapted to aerobic conditions. *Rsp. rubrum*, whose terminal oxidase is always the *o*-type cytochrome, may be one of the bacteria that are poorly adapted to aerobic conditions. *Rps. spheroides* may be the bacterium that has adapted fairly well but not completely to the aerobic condition, so that when the surrounding environment becomes anaerobic, the synthesis of the *a*-type cytochrome may be repressed and instead the *o*-type cytochrome may be synthesized. In this connection it is interesting to note the observations by MOK *et al.*[10] and SCHOLES AND SMITH[11]. MOK *et al.*[10] reported that cytochrome *o* existed in addition to cytochromes $a + a_3$ in *Saccharomyces cerevisiae* and *Candida utilis*, and the amount of cytochrome *o* seemed to increase in conditions where respiratory activity of the yeast was lowered. SCHOLES AND SMITH[11] observed that cytochrome *o* was detected besides cytochrome $a + a_3$ in anaerobically grown *Micrococcus denitrificans* and the content of the *a*-type cytochrome was lower in cells grown anaerobically than in cells grown aerobically. It will be of special interest to study the relations between cytochromes of the *a*-type and the *o*-type in relation to the biochemical evolution of the respiratory system.

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