TERMINAL OXIDASES OF *MICROCOCCUS DENITRIFICANS**

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The cytochromes present in bacteria performing aerobic oxidations appear to function in electron transport in the bacterial cell in a manner analogous to the cytochrome system found in mammals. Cytochrome oxidase, the terminal enzyme in the classical electron transport mechanism involving cytochromes, has not been extensively investigated in the case of bacteria. Evidence for a bacterial cytochrome oxidase in *Pseudomonas aeruginosa* was obtained by YAMAGUTCHI[1] and in *Rhodospirillum rubrum* by KAMEN AND VERNON[2]. KEILIN AND HARPLEY[3] found no evidence for the presence of this enzyme in crushed cells of *Escherichia coli*, and SMITH[4] reported that of the eight bacterial species investigated by her, none demonstrated any cytochrome oxidase activity. However, experiments to date have generally involved the use of reduced mammalian cytochrome *c* as the electron donor when testing for the presence of cytochrome oxidase in bacteria, and the failure to demonstrate oxidase activity in such cases cannot be taken as conclusive evidence for the absence of the enzyme, since it has been shown that in some bacteria the bacterial cytochrome oxidase is specific for its own cytochrome *c*[5,6,7].

During an earlier investigation into the cytochrome content of *Micrococcus denitrificans*[5], it was observed that extracts of this organism had the ability to catalyze the oxidation by air of reduced mammalian cytochrome *c*. In this case the bacterial cytochrome need not be used as substrate for the bacterial cytochrome oxidase, and thus a ready means of assay for the enzyme was available. Cytochrome oxidase from *M. denitrificans* has been purified 17 fold from cell-free extracts, and was shown to be a particulate enzyme closely resembling mammalian cytochrome oxidase. Purification of DPNH*** oxidase from extracts of this bacterium was also accomplished, and a determination of the relative importance of these two oxidative pathways in the economy of the bacterium was made. Cell-free extracts of the bacterium were also shown to be capable of performing oxidative phosphorylation during the oxidation of reduced cytochrome *c*.

MATERIALS AND METHODS

M. denitrificans cells were grown and harvested as described previously[7]. Optical measurements were made with a Beckman DU spectrophotometer. Protein was determined by the method of GORNALL *et al.*[8].

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*** The following abbreviations are used: DPNH, dihydrodiphosphopyridine nucleotide; FMN, riboflavin-5'-phosphate; FAD, flavin adenine dinucleotide; Tris, tris(hydroxymethyl)aminomethane.

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The assay system for cytochrome oxidase contained 10 μmoles of phosphate buffer pH 7.0 and 0.04 μmole reduced horse heart cytochrome *c* in a final volume of 1.0 ml. One unit of enzyme activity was taken as the amount causing an optical density decrease at 550 mμ of 1.0 in the first 30 seconds after addition of the enzyme. Specific activity is defined as the number of enzyme units per mg of protein. Cytochrome *c* was reduced by adding an equivalent amount of ascorbic acid to a 1% cytochrome *c* solution at pH 7.0 and letting it stand for 30 min before freezing in small aliquots.

The assay system for DPNH oxidase activity contained 10 μmoles phosphate buffer pH 7.0, 1.0 μmole KCN and 0.12 μmole of DPNH in a final volume of 1.0 ml. One unit of enzyme activity was taken as that which would cause a decrease of 1.0 in optical density at 340 mμ in the first 3 min following addition of enzyme. In order to obtain stable solutions of DPNH, it was necessary to dissolve the DPNH in 0.1 *M* Tris buffer, pH 8.0. Such solutions were relatively stable for extended periods.

For these experiments the cytochrome *c* (horse heart), DPNH, FAD, FMN, alcohol dehydrogenase, and Tris buffer were obtained from Sigma Chemical Co., St. Louis, Mo. Other chemicals used were reagent grade chemicals commercially available.

RESULTS

Several methods for liberation of the bacterial cytochrome oxidase from the intact cell were tried. It was found that alumina grinding yielded extracts which consistently had the highest specific activity, being superior in this respect to extracts prepared by rupture with glass beads in a Waring blendor, autolysis, alternate freezing and thawing, or by aqueous buffer extraction of air or acetone dried cells. Accordingly, cell-free extracts were routinely prepared by adding Alcoa A-301 alumina to the wet cells in a weight ratio of 1:1. The resulting paste was frozen in a mortar and ground with a pestle as it was thawing, with the freezing and thawing being repeated five times. Following cell rupture, the paste was extracted with 0.1 *M* phosphate buffer pH 7.0 to obtain the cell-free extract which was fractionated further.

Purification of cytochrome oxidase

Ammonium sulfate was added to an ice cold cell-free extract of *M. denitrificans* cells to a final concentration of 200 g per liter. The supernatant fluid following centrifugation was discarded and the precipitate suspended in 0.1 *M* phosphate buffer pH 7.0 and centrifuged for 5 minutes at 20,000 $\times$ *g* to remove denatured protein. *Fraction 1.*

Calcium phosphate gel was added to fraction 1 until the gel to protein weight ratio was 2:1. After 30 minutes the mixture was centrifuged and the material adsorbed on the gel was differentially eluted with 0.5 *M* phosphate buffer pH 6.8. The oxidase activity was located in the eluant. *Fraction 2.*

Centrifugation of fraction 2 at 140,000 $\times$ *g* in a Spinco model L ultracentrifuge yielded a reddish-brown pellet which was dispersed in 0.1 *M* phosphate buffer pH 7.0. *Fraction 3.*

The purification procedure outlined above yielded preparations in which the specific activity of the cytochrome oxidase was increased by 15–20 fold, as shown by the data presented in Table I. Following the centrifugation at 140,000 $\times$ *g* there was little activity remaining in the supernatant fluid above the packed pellet, and what activity remained was concentrated in the zone immediately above the pellet. This indicated that the enzyme responsible for reduced cytochrome *c* oxidation was contained in a multi-enzyme particle, and in this respect resembles the cytochrome oxidase from *R. rubrum*[2]. In these two cases, then, the bacteria resemble mammalian cells in containing the enzymes concerned with electron transport in multi-enzyme units.

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TABLE I

PURIFICATION OF *M. denitrificans* CYTOCHROME OXIDASE

Fraction	% *Total activity*	*Specific activity*
Extract	100	0.25
Fraction 1	64	1.00
Fraction 2	23	2.9
Fraction 3	18	4.4

Spectrophotometric examination of a hydrosulfite-reduced *M. denitrificans* cell-free extract revealed absorption maxima at 417, 522 and 551 mμ, while fraction 3 demonstrated absorption maxima at 421, 522 and 553 mμ after reduction. In none of the preparations obtained to date was it possible to detect an absorption peak in the area of 605 mμ, which would be characteristic of cytochrome a_3. However, the low degree of purification of the bacterial cytochrome oxidase does not allow the conclusion that cytochrome a_3 is not the bacterial cytochrome oxidase in this case. The shifting of the absorption maxima during purification reflects the removal of *M. denitrificans* cytochrome *c* during the purification procedure and a concentration of cytochrome c_1 with the particulate material obtained. The presence of cytochrome c_1 in *M. denitrificans* has previously been postulated[7].

The effects of the classic cytochrome oxidase inhibitors on purified bacterial enzyme were tested. It was found that at a final concentration of $5 \cdot 10^{-4} M$, cyanide inhibited enzyme activity 98% while azide inhibited 55%. Saturation of the assay system with carbon monoxide resulted in 100% inhibition which was readily reversible by light. Thus the bacterial enzyme has an inhibition pattern similar to the mammalian enzyme, showing the bacterial enzyme to be a true cytochrome oxidase. The effect of added aluminum, magnesium and manganese ions (as the chloride salt) over a concentration range of $5 \cdot 10^{-6}$ to $5 \cdot 10^{-3} M$ revealed that both magnesium and manganese ion had no effect on enzyme activity while aluminum had a slight inhibitory effect at the higher concentration. A broad pH maximum from pH 7.0 to 7.4 was observed for enzyme activity, with no difference observed in phosphate or other buffers.

The specificity of some bacterial cytochrome oxidases for their own bacterial cytochrome *c* has been demonstrated[5,6,7]. In the present case the possibility exists that the cytochrome oxidase activity toward mammalian cytochrome *c* was an artifact, and the real substrate for the oxidase was some residual *M. denitrificans* cytochrome *c* contained in the bacterial electron transferring particle. If this be true, the mammalian cytochrome *c* would be oxidized by coupling with the bacterial cytochrome *c*, which would be possible since the potential of the bacterial cytochrome is close to that of mammalian cytochrome *c*[5]. Accordingly, the effect of adding purified *M. denitrificans* cytochrome *c* to the cytochrome oxidase test system was determined, with the results given in Table II. It is apparent from these data that addition of the bacterial cytochrome *c* did not increase the activity of the oxidase toward mammalian cytochrome *c*. Although not a conclusive experiment, it does support the concept that the bacterial cytochrome oxidase reacts directly with mammalian cytochrome *c*.

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TABLE II

EFFECT OF ADDED *M. denitrificans* CYTOCHROME *c* ON RATE OF MAMMALIAN CYTOCHROME *c* OXIDATION

μmoles M. denitrificans cytochrome c added to assay system	*Optical density decrease at 550 mμ in 30 sec*
none	0.120
0.002	0.112
0.01	0.107

Purification of DPNH oxidase

Examination of cell-free extracts of *M. denitrificans* cells revealed the presence of a DPNH oxidizing system other than the one involving cytochromes, since there was a disappearance of DPNH in the presence of bacterial extract and sufficient cyanide to completely inhibit cytochrome oxidase activity. Fractionation of cell-free extracts with ammonium sulfate and calcium phosphate gel according to the following procedure gave preparations with specific activity increased by approximately 70-fold.

Sufficient cold acetone was added to a cell-free extract to make the final concentration 50% in acetone by volume. After maintaining at —20° C for 20 minutes, the mixture was centrifuged and the residue discarded. Acetone was added to the supernatant fluid to make it 60% in acetone. The precipitate appearing after 5 minutes at —20° C was removed by centrifugation, dissolved in 0.1*M* phosphate buffer, pH 7.0, and the insoluble material removed by centrifugation. *Fraction 1.*

Ammonium sulfate was added to 200 g per liter, the precipitate separated by centrifugation and discarded. The supernatant fluid contained the majority of the activity. *Fraction 2.*

Ammonium sulfate was removed from fraction 2 by dialysis against 0.1*M* phosphate buffer pH 7.0 and calcium phosphate gel added to bring the gel to protein weight ratio to 1:2. After 10 minutes for establishment of equilibrium, the gel was removed by centrifugation and discarded. Repetition of the gel fractionation resulted in further purification, with the final supernatant fluid containing the purified DPNH oxidase. *Fraction 3.*

The results of a typical purification are given in Table III. By following the above procedure the purifications obtained were usually 60- to 70-fold. From the behavior of the DPNH oxidase during the purification procedure it appears that in contrast to the cytochrome oxidase, the DPNH oxidase was not linked to a multienzyme unit and was an individual, soluble protein. Thus, it was not precipitated

TABLE III

PURIFICATION OF *M. denitrificans* DPNH OXIDASE

Fraction	*% Total activity*	*Specific activity*
Extract	100	0.019
Fraction 1	82	0.41
Fraction 2	58	0.65
Fraction 3	44	1.30

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by low ammonium sulfate concentrations, could not be removed from solution by prolonged centrifugation at 20,000 × *g* and was not readily adsorbed on calcium phosphate gel. Earlier investigations of DPNH oxidases from non-bacterial sources indicated that in one case[9] the DPNH oxidase was particulate in nature, while the other appeared to be a soluble enzyme[10]. The DPNH oxidase purified from *Streptococcus faecalis* also appears to be a soluble protein[11].

The DPNH oxidases examined to date have some form of riboflavin as the prosthetic group. Likewise the enzyme mediating electron transfer from DPNH to cytochrome *c*[12] is a flavoprotein. Spectroscopic examination of the purified *M. denitrificans* DPNH oxidase fractions gave no information concerning the nature of the prosthetic group of the enzyme. There was general absorption in the blue region, but no maximum was observed in the 450 mμ region where flavins would be expected to absorb. Repetition of ammonium sulfate fractionation or use of organic solvents resulted in a loss of enzymic activity, indicating a possible loss of prosthetic group during the procedures. Accordingly, the ability of flavins to reactivate such fractions was examined, with the results given in Table IV. Addition of FMN to partially inactivated fractions does cause reactivation of the enzyme, being much more effective in this regard than FAD of the same concentration. This reactivation by FMN is presumptive evidence for the occurrence of FMN as the prosthetic group of the enzyme, and would identify it with the other DPNH oxidases which contain some form of flavin as the prosthetic group.

TABLE IV

REACTIVATION OF DPNH OXIDASE

Treatment	*Specific activity*
Fraction 2	0.65
Fraction 2 precipitated by ammonium sulfate	0.36
Fraction 2 precipitated by ammonium sulfate + 1 μmole FAD	0.39
Fraction 2 precipitated by ammonium sulfate + 1 μmole FMN	0.52
Fraction 2 precipitated by ammonium sulfate + 5 μmoles ascorbate	2.73
Fraction 2 precipitated by ammonium sulfate + 5 μmoles ascorbate and 1 μmole FMN	3.60

KERN AND RACKER[13] have reported a stimulation of the DPNH oxidase from yeast by added ascorbic acid, and a similar response of a DPNH oxidase from peas has been reported by NASON *et al.*[14]. Table IV reports the effect of added ascorbic acid on the DPNH oxidase from *M. denitrificans*, and shows the marked stimulation caused by addition of ascorbate. This stimulation can be demonstrated on enzyme fractions of all degrees of purity, including the cell-free extract, so it is doubtful if ascorbate replaces some factor lost during the purification procedure. Previous investigators[13,14] have postulated that some oxidation product of ascorbic acid (semiquinone) acts as an electron acceptor in the DPNH oxidase system, thus increasing the rate of DPNH oxidation.

DOLIN[11] has reported the presence of a DPNH peroxidase in *Streptococcus faecalis*. In testing *M. denitrificans* extracts for the presence of this enzyme, 10 μmoles of added H_2O_2 caused no increase in the rate of DPNH oxidation, indicating the absence of a DPNH peroxidase in this case. The difference in physiology of the two

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bacteria, coupled with DOLIN's explanation of the peroxidase function in *S. faecalis*, militate against its presence in *M. denitrificans*, so its absence was to be expected.

The activation energy of the DPNH oxidase was calculated by determining the rate of oxidation over a temperature range from 0 to 39° C. By means of an Arrhenius plot[15] the activation energy was determined to be 14,900 cal per mole, placing it in the ordinary range of respiratory enzymes. By means of the Lineweaver-Burk plot[16], shown in Fig. 1, the Michaelis constant of a purified DPNH oxidase fraction was determined. The K_m for DPNH was found by this procedure to be $1.5 \cdot 10^{-6}$ moles per liter, as compared to a value of $1.5 \cdot 10^{-5}$ moles per liter found by MACKLER *et al.*[9] for beef heart DPNH oxidase.

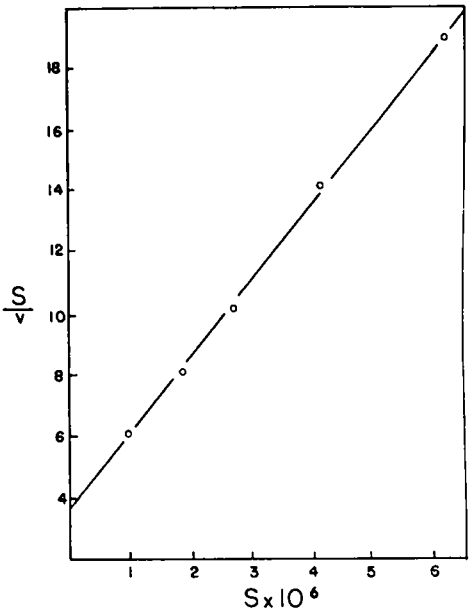

Fig. 1. Lineweaver and Burk plot for calculation of the Michaelis constant with respect to DPNH for purified *M. denitrificans* DPNH oxidase.

The oxygen uptake of a cell-free extract of *M. denitrificans* functioning as a cytochrome oxidase and as a DPNH oxidase was measured in a Warburg respirometer. In the cytochrome oxidase test system cytochrome *c* was maintained in the reduced state by addition of ascorbic acid. The DPNH of the DPNH oxidase test system was maintained in the reduced state by alcohol-alcohol dehydrogenase. The oxygen uptake while the cell-free extract was functioning as a cytochrome oxidase was 7.5 μliters per minute, compared with a value of 3.52 μliters per minute for the same amount of extract functioning as a DPNH oxidase in the presence of cyanide. Thus when operating maximally the DPNH oxidase system could account for at least one-third of the oxygen taken up during aerobic respiration.

Oxidative phosphorylation

Cell-free extracts of *M. denitrificans* were tested for their ability to cause oxidative phosphorylation during the oxidation of reduced mammalian cytochrome *c*. The results of a series of such experiments are given in Table V. The assay system used was essentially that of MALEY AND LARDY[17] except that phosphate acceptor was not added since sufficient acceptor was present in the cell-free extract. Inorganic phosphate was determined by the method of LOWRY AND LOPEZ[18]. The average P:O ratio was found to be 0.39, with the range being from 0.32 to 0.47. Using mammalian preparations MALEY AND LARDY[17] and COOPER AND LEHNINGER[19] obtained values from 0.55 to 1.0 and 0.36 to 0.72 respectively for this ratio. This again indicates a similarity of the bacterial system to the mammalian system investigated, with one phosphorylation occurring during the oxidation of reduced cytochrome *c* by oxygen via the particulate cytochrome oxidase system.

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TABLE V

OXIDATIVE PHOSPHORYLATION DURING THE OXIDATION OF REDUCED MAMMALIAN CYTOCHROME *c* BY CELL-FREE EXTRACTS OF *M. denitrificans*

The complete system consisted of 30 μmoles phosphate buffer pH 7.4, 40 μmoles sodium fluoride, 30 μmoles magnesium chloride, 20 μmoles ascorbic acid, 0.5 μmole mammalian cytochrome *c*, 30 μmoles Tris buffer pH 7.4 and cell-free extract in a final volume of 3.0 ml.

	μ atoms oxygen consumed	*μ moles phosphate esterified*	*P/O ratio*
Complete system, range for 20 runs	12.8–15.4	4.6–6.3	0.32–0.47
Complete system, average value	14.6	5.42	0.40
Complete system + 32 μmoles KCN	0	0	-

DISCUSSION

From the evidence cited in this investigation it appears that the cytochrome oxidase from *M. denitrificans* is in most respects similar to mammalian cytochrome oxidase. The failure to observe an absorption maximum at 605 mμ in the purified preparations does not rule out cytochrome a_3 as the bacterial cytochrome oxidase, since the most pure bacterial preparations were only about one-sixth as active on a protein basis as crude mammalian cytochrome oxidase preparations[20]. The bacterial enzyme also resembles the mammalian analogue in the inhibition pattern obtained, and in its occurrence on a multi-enzyme particle.

With the availability of purified bacterial cytochromes of the *c* type, it has become apparent that in the main, mammalian cytochrome oxidase does not oxidize the reduced bacterial cytochromes, and likewise cell-free extracts of the corresponding bacteria do not oxidize reduced mammalian cytochrome *c*[2,5,6,7,21]. Thus, a considerable degree of species specificity exists in reduced cytochrome *c* oxidation with cytochrome oxidase. In the case of *M. denitrificans*, however, the oxidase exhibits no specificity for its own cytochrome, since it will oxidize both its own reduced cytochrome, the reduced cytochrome *c* of *R. rubrum*[5], and reduced mammalian cytochrome *c*. Furthermore, *M. denitrificans* cytochrome *c* is distinguished from other bacterial cytochromes of the *c* type in its ability to be oxidized by mammalian cytochrome oxidase[5]. Thus, this bacterium is uniquely non-specific as regards cytochrome interactions with cytochromes of other bacterial or mammalian species, and represents a departure from the general trend found to date.

The occurrence of a direct mechanism of DPNH oxidation by oxygen mediated by flavoprotein enzymes is common in many tissues. The availability of two pathways for DPNH oxidation in a bacterial cell is probably implicated with adjustment to environmental conditions and allows the cell a more precise control over its respiratory economy. Thus, only one of the systems may carry out oxidative phosphorylation, thus allowing the cell to manipulate oxidation and phosphorylation separately. From its behavior during purification the DPNH oxidase described in this investigation appears to be a soluble enzyme and apparently does not involve cooperation with a DPNH peroxidase as is the case with *S. faecalis*. Although direct evidence that the DPNH oxidase from *M. denitrificans* is a flavoprotein is lacking, reactivation obtained with added FMN is indicative of this fact and would characterize this enzyme as a flavoprotein along with the other DPNH oxidases examined to date.

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The oxidation of DPNH via this pathway would result in the production of H_2O_2, which would be readily dismuted by means of the catalase present in the cell.

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SUMMARY

1. A cytochrome *c* oxidase was partially purified from the facultative anaerobe, *Micrococcus denitrificans*. This enzyme was shown to be associated with the particulate matter of the cell and was capable of oxidizing reduced mammalian cytochrome *c*. A procedure for purification of this oxidase is given.

2. The optimum pH for the bacterial cytochrome oxidase was found to be from 7.0 to 7.4. Activity was not increased by the addition of aluminum ion to the assay system, and magnesium and manganous ions had no effect upon the activity. The oxidase activity was 98% inhibited by $5 \cdot 10^{-4} M$ cyanide, while a similar concentration of azide inhibited only 55%. Carbon monoxide affected 100% inhibition which was light-reversible.

3. The reduced spectrum of a purified cytochrome oxidase fraction had maxima at 421, 522 and 553 mμ, while the corresponding maxima for a cell-free extract were at 417, 522 and 551 mμ, indicating removal of cytochrome *c* during the purification procedure and concentration of cytochrome c_1 in the particulate matter containing the cytochrome oxidase. Cell-free extracts had ability to perform oxidative phosphorylation, with an average P:O ratio of 0.39 when oxidizing reduced cytochrome *c*.

4. A cyanide-insensitive DPNH oxidase, which appeared to be a soluble flavoprotein, was partially purified from cells of *M. denitrificans*. The Michaelis constant with respect to DPNH was determined to be $1.5 \cdot 10^{-6}$ moles per liter. The activation energy was found to be 14,900 cal per mole. A comparison of these two oxidases as a terminal oxidative enzyme was determined, showing that the DPNH oxidase can accommodate approximately one-half as much oxygen uptake as the cytochrome oxidase system.

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