A P-450-Like Cytochrome and a Soluble Terminal Oxidase Identified as Cytochrome O from Nitrosomonas europaea[1]

Michael Rees[2] and Alvin Nason
McCollum-Pratt Institute
The Johns Hopkins University

Received October 4, 1965

Nitrosomonas europaea, a strict chemoautotrophic bacterium, derives its energy from the aerobic oxidation of ammonia to nitrite as summarized by the following overall equation:

$$NH_3 + 1\ 1/2\ O_2 \text{ --------}\rightarrow NO_2^- + H_2O + 66 \text{ kcal} \qquad (1).$$

The reported presence of a particulate CO-sensitive terminal oxidase (2), (3) as well as the occurence of a small absorption peak in the 605 mμ region of the reduced difference spectra of concentrated preparations of impure cultures (4), (5), (6) have been largely responsible for considering the oxidase to be of the cytochrome a type.

The present report describes the results of experiments using cell-free preparations obtained by osmotic shock of lysozyme-treated, uncontaminated Nitrosomonas europaea cells. The data indicate, contrary to the findings of others that the terminal oxidase is soluble rather than particulate and that it is apparently of the cytochrome o type. Preliminary findings for the occurrence of a second CO-combining pigment, probably very similar or related to the cytochrome P-450 pigment of mammalian microsomes (7, 8), are also given. No evidence could be obtained to indicate that a cytochrome $(a + a_3)$ - type oxidase was present in pure cultures of the organism.

---

1. Contribution No. 452 of the McCollum-Pratt Institute. This investigation was supported in part by a research grant (No. 2332-14) from the National Institute of Health, United States Public Health Service.
2. Present Address: Laboratory of Physical Biochemistry, Massachusetts General Hospital, Boston, Massachusetts

Methods

Nitrosomonas europaea (kindly provided by Dr. E.L. Schmidt, University of Minnesota) was grown with forced aeration in 15-liter batches at 25° using the completely inorganic medium developed by Engle and Alexander (9). The bacterium was unable to grow in liquid Difco organic nutrient broth, and cultures were routinely examined for this characteristic. Any culture which demonstrated growth in the organic medium after 3 days at 25° was considered contaminated and discarded. When the stationary phase had been reached, the cells, still suspended in the original medium were stored for 24 hours at 4° (in order to allow inorganic precipitates to settle), collected by centrifugation of the supernatant fluid at 50,000 rpm using a Sharples refrigerated ultracentrifuge, and washed 3 to 5 times in several hundred ml of 0.1 M potassium phosphate, pH 7.4 - 7.8, 4°.

Spheroplasts were prepared by suspending the washed Nitrosomonas cells in 0.25 M sucrose-0.1 M potassium phosphate, pH 7.5, 1 x 10^{-3} M EDTA containing 50 mg of lysozyme (Worthington, 2x crystallized) in a final volume of 40 ml per gram wet weight, and the suspension incubated for 2 hours at 28° with gentle swirling. Subsequent centrifugation at 2,000 x g for 20 minutes at 0° yielded a precipitate consisting mostly of spheroplasts as confirmed by phase contrast microscopy. The spheroplast precipitate was resuspended to its original volume with 0.005 M potassium phosphate, pH 7.5, containing 1 x 10^{-3} M $MgCl_2$ and 1 µgm of DNase (Sigma, bovine pancreas, 1x crystallized) per ml of suspension and incubated with gentle swirling for 2 hours at 28° in order to reduce the viscosity of the solution (lysate 1). Any unruptured spheroplasts were removed by subsequent centrifugation at 2,000 x g for 20 minutes, 0°, resulting in a turbid supernatant solution (lysate 2). The lysate 2 fraction was centrifuged at 10,000 x g for 10 minutes, 0°, to yield a pellet (membrane fraction) and a supernatant solution (cytoplasmic fraction). The membrane fraction was washed twice with approximately 100 ml volumes of 0.25 M sucrose - 0.1 M potassium phosphate and resuspended in 1/20 of the original volume in 0.1 M potassium phosphate, pH 7.5. A portion of

the cytoplasmic fraction was further centrifuged for 1 hour at 144,000 x g at 0° and the minute precipitate discarded.

The enzymatic oxidation of reduced cytochrome c and the uptake of atmospheric oxygen as indicators of terminal oxidase activity were determined in 0.01 M potassium, pH 7.5, at room temperature by both spectrophotometric and recording polarographic assays as described in Table I. Absorption spectra were measured

Table I. Distribution of Terminal Oxidase Activity in Various Nitrosomonas europaea Fraction.

Fraction	Protein mg/ml	Volume (ml)	Polarographic Assay		Spectrophotometric Assay	
			O_2 Uptake μM/min/ml	% total activity	O_2 Uptake μM/min/ml	% total activity
Lysate 2	2.17	234	35.7	100	28	100
Cytoplasmic	0.83	234	31.0	87	23.3	83
Membrane	1.66	20	0	0	14.4	5
144,000 x g supernatant	0.63	234	34.7	97	22.1	79

In the spectrophotometric procedure, enzymatic activity of a 1.0 ml reaction mixture of 1.0 cm light path containing 1×10^{-4} M cytochrome c (Sigma, horse heart cytochrome c, type 111) reduced by the method of Smith (10) was measured in a Beckman DU spectrophotometer. The reaction was started by the addition of a suitable quantity of enzyme solution, and the decrease in optical density at 550 mμ followed at 30 second intervals for 5 minutes. The μmoles of cytochrome c oxidized per minute were calculated using the difference extinction coefficient, 1.9×10^{7} $M^{-1}Cm^{-1}$, of cytochrome c (11) and dividing by four in order to convert to μmoles O_2 uptake per minute. In the polarographic procedure, the enzymatic rate of oxygen uptake was measured using the Clark oxygen electrode (Yellow Springs Instrument Co., Antioch, Ohio) (12) operated for at least 3 minutes at a polarizing voltage of 0.6 volts and a final reaction volume of 3 ml consisting of enzyme, buffer, and 0.03 M p-phenylene diamine. In both types of assays, the enzymatic rate was linear with time and proportional to enzyme concentration. Fractions were assayed in a final dilution of 1/40 spectrophotometrically and 1/30 polarographically. The rates of corresponding control mixtures containing boiled enzyme (5 minutes at 100°) were also determined and in all cases showed no activity. Protein concentration was determined by the Lowry method as described by Layne (13) using crystalline bovine serum albumin as the standard.

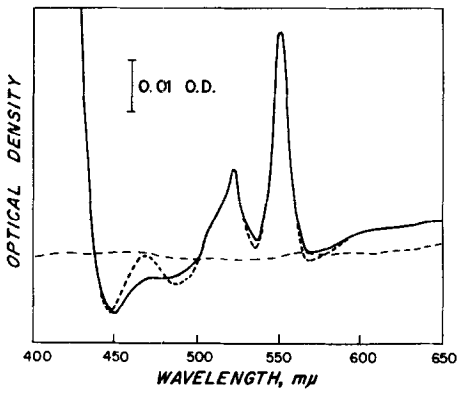

Fig. 1. Reduced Difference Spectrum and CO Spectrum of Cytoplasmic Fraction (0.65 mg protein/ml). After obtaining a baseline (— — — —) several small crystals of $Na_2S_2O_4$ were added to the sample cuvette (1.0 cm light path) and the reduced difference spectrum recorded (- - - - - - -). CO was then added by slowly bubbling the gas through the cuvette for 10 minutes in the dark and the resulting spectrum recorded (————).

---

at room temperature with a Cary Model 14 recording spectrophotometer as indicated in Figure 1. Acid-extractable haem was obtained by the addition of a mixture of 4 volumes of acetone and 1 volume of 1.5 N HCl per volume of Nitrosomonas fraction. After removal of precipitated protein by centrifugation, the haem was extracted into ether, the ether evaporated, and the residue dissolved in a volume of a 1:1 mixture of 0.2 N KOH and pyridine equal to the original volume of the extract (14). It was necessary to add a small amount of solid $Na_2S_2O_4$ to the pyridine-alkali solution in order to completely dissolve the ether residue.

Results

Distribution of the terminal oxidase activity. Table I summarizes the distribution of the terminal oxidase activity in the various Nitrosomonas fractions. Assuming lysate 2 to have 100% of the oxidase activity, the oxidase remained almost entirely (85%) in the cytoplasmic fraction. Further centrifugation of the cytoplasmic fraction at 144,000 x g for 1 hour resulted in a 5% decrease in oxidase activity as measured spectrophotometrically and a 10% increase in activity

as measured polarographically. Recombination of the cytoplasmic and membrane fractior gave a combined activity expected from addition of the activity of each fraction when assayed alone (not shown). The addition of boiled cytoplasmic fraction to membrane fraction did not affect the activity of the latter.

Nature of the CO-combining pigments. Fig. 1 presents the $Na_2S_2O_4$ - reduced difference spectrum of the cytoplasmic fraction. It is typically the spectrum of a fraction containing cytochromes of the b and c types with the exception of the small peak at 465 mμ, which, to our knowledge, has not been previously described. Fig. 1 also demonstrates that the addition of CO to the $Na_2S_2O_4$-reduced sample resulted in a depression of the peak at 465 mμ. Figs. 2, 3, and 4 present the reduced difference spectra and the CO difference spectra of lysate 2, cytoplasmic fraction, and membrane fraction, respectively. These fractions were the same as those whose oxidase activities are reported in Table I. It should be noted that the reduced difference spectra of the lysate 2 and cytoplasmic fractions are identical, but that the spectrum of the membrane fraction is different in that it lacks the peak at 465 mμ. The CO difference spectra of the lysate 2 and cytoplasmic fractions are also identical, and again the membrane fraction is notably different in that it lacks the absorption peak at 450 mμ of a presumed CO-combining pigment.

The decrease in optical density at 465 mμ that occurs when CO is added to reduced cytoplasmic fraction is thought tentatively to be due to the shift of the 465 mμ peak to 450 mμ by combination with CO. Since the membrane fraction is lacking both in the 465 mμ peak in the reduced difference spectrum and in the 450 CO-combining peak in the CO difference spectrum, this assumption seems reasonable. The pigment with the reduced peak at 465 mμ and the CO-combining peak at 450 mμ is assumed to be similar to the cytochrome P-450 pigment found in liver microsome. The CO difference spectrum of the latter is unusual in that it exhibits only a γ -peak (7, 8).

The CO difference spectrum of the acid-extractable haem of cytoplasmic fraction

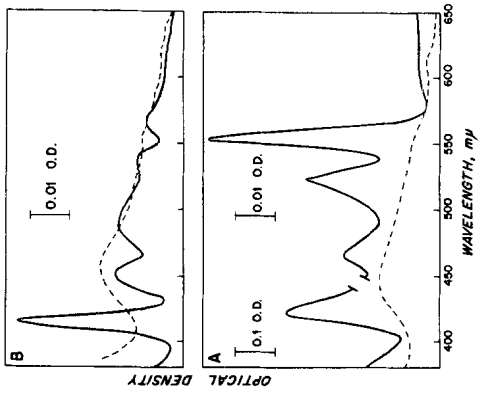

Fig. 2. Lysate

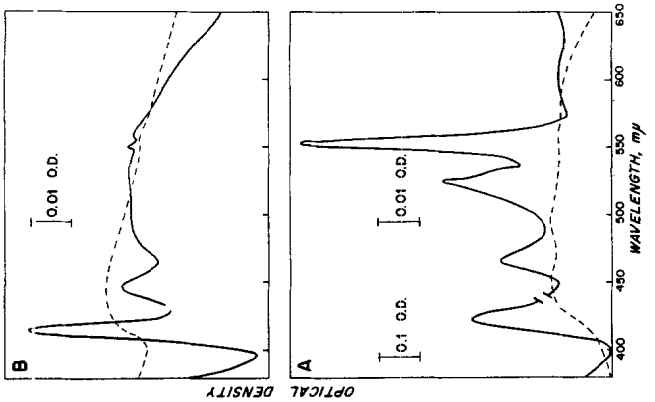

Fig. 3. Cytoplasmic Fraction

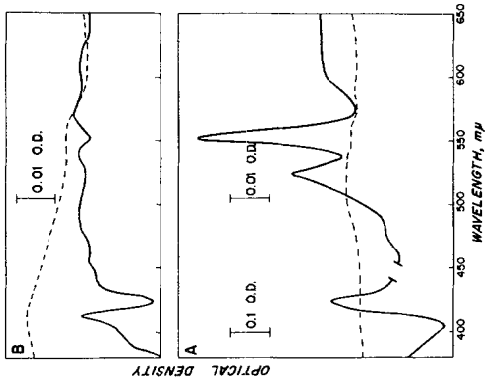

Fig. 4. Membrane Fraction

Fig. 2, 3, 4. Reduced Difference Spectrum (A) and CO Difference Spectrum (B) of Lysate 2 (0.54 mg protein/ml), Cytoplasmic Fraction (0.55 mg protein/ml) and Membrane Fraction (0.55 mg protein/ml), respectively. Same procedure as for Figure 1, except that for the CO difference spectrum $Na_2S_2O_4$ was also added to the reference cuvette and the baseline recorded (- - - -). CO was then added to the sample cuvette and the resulting CO difference spectrum recorded (———).

(Fig.5) is identical to that seen in the membrane fraction, both of which very closely resemble the CO difference spectrum that Taniguchi and Kamen (15) have reported for the cytochrome o occurring in the membrane fraction of heterotrophically-grown Rhodospirillum rubrum (CO-combining peaks at 419-420, 540, and 572 mμ and troughs at 432-433 and 560 mμ as compared to peaks at 418, 540, and 565 mμ and troughs at 425 and 552 mμ in the CO difference spectra of Nitrosomonas membrane

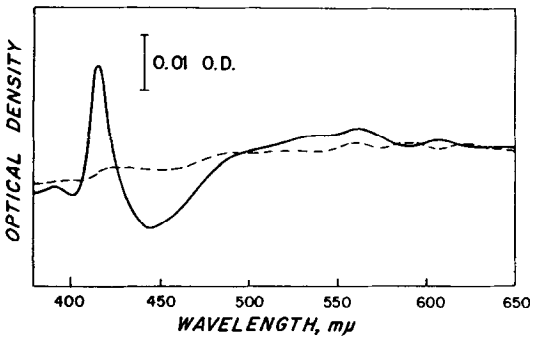

Fig. 5. CO Difference Spectrum of Acid-Extractable Haem of Cytoplasmic Fraction. Baseline (- - - - —); plus CO (————).

---

and acid-extractable haem fraction). If the contribution of P-450 is subtracted from the CO difference spectra of the lysate 2 and cytoplasmic fractions, then the resulting spectra are identical to that of the membrane and acid-extractable haem fractions. The considerably smaller total quantity of cytochrome o in the membran fraction as compared to the cytoplasmic is commensurate with the little or no observable terminal oxidase of the former fraction.

Discussion

Although several workers, using extracts prepared by sonic oscillation of whole Nitrosomonas cells, found that the terminal oxidase activity of Nitrosomonas appeared mainly in the precipitate after centrifugation for 1 hour at 144,000 x g (2, 6), the results presented here indicate that the oxidase is soluble. It seems likely that the oxidase may be loosely associated with the recently

described peripheral cytoplasmic membranes of the intact Nitrosomonas cell (15), and is separated from them during the preparative procedures described in the present paper. Probably sonication caused vesicularization of the membranes and resulted in sedimentation of entrapped material (including the terminal oxidase) that might otherwise have been liberated into the supernatant solution. No evidence could be found for the presence of a cytochrome of the $(a + a_3)$ type, and the small peak in the 605 mμ region seen by others probably represented contamination of the cultures with an organism containing either a cytochrome $(a + a_3)$ or chlorophyll (14). From the close similarity between the CO difference spectra seen here with that reported by Tanaguchi and Kamen for cytochrome o, it is tentatively concluded that the oxidase of Nitrosomonas is of the cytochrome o type.

Finally, the presence in Nitrosomonas of a cytochrome P-450-like pigment, which in mammalian cells is considered to be functional in certain oxygenase reactions (i.e., activation of atmospheric oxygen for substrate hydroxylation), raises the speculation as to whether this pigment may be involved as an oxygenase in one of the several presumed steps in the aerobic oxidation of ammonia to nitrite by this organism. The recent report from this laboratory (17) that at least one of the oxygen atoms in the nitrite arising from the oxidation of ammonia by Nitrosomonas is in fact derived from atmospheric oxygen, thereby implying participation of an oxygenase system, would be in keeping with such a proposed role for cytochrome P-450 in Nitrosomonas.

References

1. Hofman, T. and Lees, H., Biochem. J., 54, 579 (1953).
2. Anderson, J.H., Biochem. J., 91, 8 (1964).
3. Nicholas, D.J.D. and Jones, O.T.G., Nature, 165, 512 (1960).
4. Falcone, A.B., Shug, A.L., and Nicholas, D.J.D., Biochim. Biophys. Acta, 77, 199 (1963).
5. Aleem, M.I.H. and Lees, H., Can. J. Biochem. and Physiol., 41, 763 (1963).
6. Hooper, A.B. and Nason, A., J. Biol. Chem., In press (1965).
7. Omura, T. and Ryo Sato, J. Biol. Chem., 239, 2370 (1964).
8. Cooper, D.Y., Levin, S., Narasimhulu, S., Rosenthal, O., and Estabrook, R.W., Science, 147, 400 (1965).

9. Engle, M.S. and Alexander, M.J., J. Bact., 76, 217 (1958).
10. Smith, L., In S.P. Colowick and N.O. Kaplan (Editors), Methods in Enzymology, Vol. II, p. 735, Academic Press, New York, 1955.
11. Margoliash, E. and Frohwirt, N., Biochem. J., 71, 570 (1959).
12. Cooper, D.Y., Estabrook, R.W. and Rosenthal, O., J. Biol. Chem., 238, 1320 (196
13. Layne, E., in S.P. Colowick and N.O. Kaplan (Editors) Methods in Enzymology VIII, p. 448. Acad. Press, New York, 1955.
14. Lemberg, R. and Benson, A., Nature, 183, 628 (1959).
15. Taniguchi, S. and Kamen, M.D., Biochim. Biophys. Acta, 96, 395 (1965).
16. Murray, R.G.E. and Watson, S.W., J. Bact., 89, 1594 (1965).
17. Rees, M. and Nason, A., Biochim. Biophys. Acta. In Press.