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Biochemical Basis of Obligate Autotrophy in *Nitrosomonas europaea*

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The specific activities of isocitric dehydrogenase, α-ketoglutaric dehydrogenase, succinic dehydrogenase, malic dehydrogenase, and reduced nicotinamide adenine dinucleotide (NADH) oxidase were determined in extracts of *Nitrosomonas europaea* and compared with the corresponding values for *Anacystis nidulans* and autotrophically grown *Hydrogenomonas eutropha*. In common with other obligate autotrophs and in contrast to facultative autotrophs, *Nitrosomonas* extracts lacked α-ketoglutaric dehydrogenase and KCN-sensitive NADH oxidase activity and had low succinic dehydrogenase activity. The *Nitrosomonas* NADH oxidase appeared to be of the peroxidase type.

Nitrosomonas europaea is an obligate autotroph which utilizes the oxidation of ammonia to nitrite as a source of energy and carries out carbon dioxide fixation by the reductive pentose pathway (15). Although growing *Nitrosomonas* cells are able to incorporate amino acids into cellular material (4, 5), they are unable to grow heterotrophically (18). Smith, London, and Stanier (17) recently showed that, in contrast to the facultative autotrophs *Thiobacillus intermedius* and *Hydrogenomonas eutropha*, the obligate autotrophs *Anacystis nidulans*, *Coccohloris peniocystis*, *T. thiooxidans*, and *T. thioparus* lack the enzymes α-ketoglutaric dehydrogenase and reduced nicotinamide adenine dinucleotide (NADH) oxidase. They proposed that the lack of these enzymes constitutes a general biochemical basis of obligate autotrophy.

Since an extremely low level of NADH oxidase was observed in *Nitrosomonas* cells, an attempt was made to assay the other enzymes of the Krebs citric acid cycle and terminal oxidation. *Nitrosomonas* was found to lack α-ketoglutaric dehydrogenase activity and to have low succinic dehydrogenase activity. Although it lacked KCN-sensitive NADH oxidase activity, it was found to possess KCN-insensitive NADH oxidase activity, which probably involved the combined action of a peroxide-generating NADH oxidase and a NADH peroxidase. In addition, extracts contained high NADH-ferricyanide reductase and NADH 2,6-dichlorophenol-indophenol (DCIP) reductase activity.

MATERIALS AND METHODS

Growth of cells. *Nitrosomonas* cells (culture kindly provided by E. L. Schmidt, University of Minnesota) were grown in batch culture as described previously (9). Lack of heterotrophic contamination of cultures was established by the procedures of Clark and Schmidt (4).

Preparation of extracts. For the preparation of cell-free extracts, a 0.2 g/ml suspension of *Nitrosomonas* cells containing a small amount of added pancreatic deoxyribonuclease (Worthington Biochemical Corp., Freehold, N.J.) was frozen three times at the temperature of a dry ice and acetone mixture, thawed, and centrifuged for 20 min at 20,000 × *g* to yield a clear dark-red supernatant fraction and a reddish-brown particulate fraction. The latter fraction was resuspended in 0.05 M phosphate solution (*p*H 7.5).

The absence of active whole cells in the extract was indicated by the fact that (i) neither the supernatant nor particulate fraction oxidized ammonia to nitrite and (ii) the supernatant fraction contained greater than 90% of the NH_2OH-dehydrogenase activity assayed as described previously (11) and had the ability to catalyze the aerobic oxidation of hydroxylamine to nitrite in the presence of catalytic amounts of phenazine methosulfate (see Table 1).

Subsequent centrifugation of the supernatant fraction at 100,000 × *g* for 2 hr yielded a very small pellet and a 100,000 × *g* supernatant fraction which contained greater than 90% of the hydroxylamine dehydrogenase and nitrite synthetase activity. Apparently, soluble enzymes are released by the freeze-thawing procedure, leaving large pieces of cell envelope in the 20,000 × *g* pellet.

Standard assay procedures. The following assays were carried out on the particulate and supernatant fractions. Protein was assayed by the Lowry procedure

TABLE 1. *Fractionation of Nitrosomonas cells*

Fraction	Vol	Protein	Enzyme activity	
			NH_2OH-cytochrome *c* reductase	$NH_2OH \longrightarrow HNO_2$[a]
	ml	*mg/ml*	*units/ml*	*units/ml*
20,000 × *g* supernatant.	6	18	13×10^{-3}	7.3×10^{-3}
20,000 × *g* particulate. .	3	8	3.0×10^{-3}	300×10^{-3}

[a] The production of nitrite per milliliter of reaction solution was measured as described previously (11) in a reaction mixture containing 10^{-4} M NH_2OH, 5 μM phenazine methosulfate, enzyme, and 0.05 M tris(hydroxymethyl)aminomethane (*p*H 8.0).

as described previously (11). Nicotinamide adenine dinucleotide (NAD^+)- and nicotinamide adenine dinucleotide phosphate ($NADP^+$)-specific isocitric dehydrogenase were assayed by the procedure of Kornberg (14); malic dehydrogenase, by the procedure of Ochoa (16); and α-ketoglutaric dehydrogenase, by the procedure of Amarasingham and Davis (2). Succinic dehydrogenase was assayed by the method of Arrigoni and Singer (3) with the use of 0.033% phenazine methosulfate. Preincubation of the extract and the presence of KCN in the incubation mixture was required for full succinic dehydrogenase activity, indicating that the *Nitrosomonas* enzyme was activated in a manner similar to mammalian succinate dehydrogenase (13). NADH oxidase was assayed at *p*H 7.0 as described by Smith, London, and Stanier (17) and at *p*H 6.0 in 0.05 M citrate-phosphate solution in the presence of 7×10^{-5} M NADH. Hydrogen peroxide was added, when appropriate, at a concentration of 10 mM.

NADH-ferricyanide or NADH-DCIP reductase activity was measured as described by Dolin (6) in 0.05 M citrate-phosphate solution (*p*H 6.0) containing 6.6×10^{-5} M NADH and either 4×10^{-4} M potassium ferricyanide or 2×10^{-4} M DCIP.

All spectrophotometric enzyme assays were carried out by following absorbance changes at the appropriate wavelength with a Gilford model 2000 multiple sample absorbance recorder. The activity of each enzyme was determined under optimal conditions of assay (*p*H, and concentration of substrate, cofactor, or activator) and was proportional to the amount of extract solution added to the reaction mixture.

One unit of enzyme activity was defined as that amount of enzyme causing the oxidation or reduction of 1 μmole of substrate per min in a 1.0-ml reaction volume. Specific enzyme activity was expressed on the basis of activity per milligram of protein in the 20,000 × *g* particulate or supernatant fraction.

RESULTS

Krebs-citric acid cycle enzymes. In Table 2, the activities of several *Nitrosomonas* enzymes are compared with the activities reported by Smith, London, and Stanier (17) for the corresponding enzymes in *Anacystis nidulans* and *Hydrogenomonas eutropha*. *Nitrosomonas* cells had supernatant $NADP^+$-specific isocitric dehydrogenase activity and NAD^+-specific malic dehydrogenase activity in common with the obligate autotroph *Anacystis* and the facultative autotroph *Hydrogenomonas*. *Nitrosomonas* extracts did not contain NAD^+-isocitric dehydrogenase activity. *Nitrosomonas* extracts also had approximately the same succinic dehydrogenase activity as *Anacystis* and significantly lower activity than the facultative autotrophs. The low rate of enzymic NAD^+ reduction by succinate suggests that *Nitrosomonas* and other obligate autotrophs might, in fact, contain fumarate reductase. In *Escherichia coli*, fumarate reductase has been shown by Hirsch et al. (7) to differ from succinic dehydrogenase in that the maximal velocity is greater and the substrate K_m values are lower in the direction of fumarate reduction than in the direction of succinate oxidation. A *Nitrosomonas* "succinic dehydrogenase" with those properties would be similar to the glutamate dehydrogenase of *Nitrosomonas* which, because of the K_m and maximal velocity values and because glutamate oxidation is inhibited by reduced NADP (NADPH), functions almost exclusively in the direction of glutamate synthesis (10).

In common with the obligate autotrophs and in contrast to the facultative autotrophs, *Nitrosomonas* extracts did not contain α-ketoglutaric dehydrogenase activity. Enzyme activity was not observed at *p*H 8.5, 7.0, or 6.0 or at *p*H 7.0 in the presence of 25 mM ethylenediaminetetraacetate and 5×10^{-4} M flavin adenine dinucleotide, singly or in combination. Activity was not observed when the assay conditions of Holzer et al. (8) were used. Mixing *Nitrosomonas* extracts with extracts of *Micrococcus lysodeikticus* resulted in no inhibition of *M. lysodeikticus* α-ketoglutaric dehydrogenase activity and indicated that an enzyme inhibitor was probably not present in the *Nitrosomonas* extract.

NADH oxidase activity. In contrast to *Anacystis* and the other obligate autotrophs examined by Smith, London, and Stanier, *Nitrosomonas* contained NADH-oxidase activity. This activity was almost completely insensitive to inhibition by 10 mM KCN and was not stimulated by 5×10^{-5} or 5×10^{-4} M adenosine diphosphate. As shown in Table 3, enzymatic NADH oxidation took place at a higher rate at *p*H 6.0 than at *p*H 7.0 and was stimulated by the presence of hydrogen

TABLE 2. *Enzyme activities in extracts of obligate and facultative autotrophs*

Enzyme	Specific activity (units/mg of protein)			
	Nitrosomonas		*Anacystis nidulans*[a]	Autotrophically grown *Hydrogenomonas eutropha*[a]
	20,000 × g supernatant fraction	20,000 × g particulate fraction		
Isocitric dehydrogenase ($NADP^+$)	56.0×10^{-3}	0.0	36.0×10^{-3}	100.0×10^{-3}
α-Ketoglutaric dehydrogenase	0.0	0.0	0.0	1.0×10^{-3}
Succinic dehydrogenase	1.6×10^{-3}	9.5×10^{-3}	6.9×10^{-3}	90.0×10^{-3}
Malic dehydrogenase	99.0×10^{-3}	0.0	7.8×10^{-3}	8.3
NADH oxidase (*p*H 7.0)	0.6×10^{-3}	0.1×10^{-3}	0.0	22.0×10^{-3}

[a] Data of Smith, London, and Stanier (17).

TABLE 3. *Oxidation of NADH by Nitrosomonas 20,000 × g supernatant fraction*

Enzyme	Specific activity (units/mg of protein)
NADH oxidase, *p*H 7.0	0.6×10^{-3}
NADH oxidase, *p*H 6.0	11.0×10^{-3}
NADH oxidase + H_2O_2, *p*H 6.0	61.0×10^{-3}
NADH-ferricyanide reductase	1.8
NADH-DCIP reductase	1.9

peroxide. It thus appears that NADH oxidation in *Nitrosomonas* was catalyzed by the combined action of a peroxide-producing NADH oxidase and a NADH peroxidase, as observed by Dolin in *Streptococcus faecalis* (6). In addition, *Nitrosomonas* extracts contained NADH-ferricyanide reductase and NADH-DCIP reductase activity (Table 3).

DISCUSSION

Consistent with the generalization of Smith, London, and Stanier (17), *Nitrosomonas* is unable to grow heterotrophically because it lacks α-ketoglutaric dehydrogenase and a NADH oxidase system which is coupled to adenosine triphosphate (ATP) synthesis.

An enzyme which is repressed under conditions of autotrophic growth would not be observed in obligate autotrophs. The absence of an enzyme such as α-ketoglutaric dehydrogenase in cell extracts cannot, therefore, be taken as a definitive indication of a genetic lesion responsible for obligate autotrophy. Relevant to this point, Amarasingham and Davis (2) have shown that α-ketoglutaric dehydrogenase of *E. coli* is repressed in certain phases of aerobic metabolism. Smith, London, and Stanier found that α-ketoglutaric dehydrogenase was not repressed in autotrophically grown *H. eutropha*.

From the work of Aleem (1), it appears that in autotrophically growing *Nitrosomonas* the reduction of pyridine nucleotide by an inorganic nitrogen compound requires the concomitant hydrolysis of ATP. If so, pyridine nucleotide reduction in that manner would involve the expenditure of more chemical energy than would be captured by ATP synthesis coupled to the reoxidation of pyridine nucleotide. Hence, the existence of a coupled NADH oxidase with a low K_m would be disadvantageous from an evolutionary point of view. The existence of a coupled NADH oxidase in autotrophically growing *Nitrosomonas* would be possible if (i) NADH oxidation were efficiently coupled to ATP synthesis with a high P:2e ratio, (ii) the number of moles of ATP generated coupled to NADH oxidation was greater than the number required to reduce pyridine nucleotide with NH_2OH, and (iii) the rate of NADH oxidation was regulated so as to provide a balanced level of ATP and reduced pyridine nucleotide for biosynthetic reactions. In fact, given these conditions, it is possible for coupled NADH oxidation to be the only source of ATP synthesis in *Nitrosomonas*. A related scheme has been proposed by Kiesow for *Nitrobacter* (12).

It is interesting to note that facultative autotrophy might have been possible in *Nitrosomonas* if NADH oxidation occurred only in the presence of large amounts of glucose, pyruvate, or some other heterotrophic substrate. However, the rate of NADH oxidation in nature appears to be regulated simply by the availability of substrate, adenosine diphosphate, and orthophosphate.

A reasonable explanation for the absence of a coupled NADH oxidase in *Nitrosomonas* is that the chemical properties of coupled NADH oxidase and the system coupling the oxidation of inorganic nitrogen compounds to ATP synthesis are mutually exclusive, so that the two reactions could not be carried out by the same electron transport system. The evolution of an effective ammonia-oxidizing system was therefore accompanied by the loss of NADH oxidase. In the absence of coupled NADH oxidase, α-ketoglutaric dehydrogenase would have become superfluous and been lost passively through mutation. If this hypothesis is correct one would expect to find that all ammonia-oxidizing autotrophs are obligately autotrophic. As far as is presently known, this is the case.

The absence of NADH oxidase is apparently not a universal basis for obligate autotrophy, since *Nitrosomonas* has that activity and *Nitrobacter* has a NADH oxidase which is coupled to the synthesis of ATP (12).

The function of NADH oxidase in *Nitrosomonas* is unknown. Perhaps it acts to regenerate NAD^+ for reactions such as the oxidative branch of the Krebs-citric acid cycle which generates α-ketoglutaric acid.

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LITERATURE CITED

1. Aleem, M. I. H. 1966. Generation of reducing power in chemosynthesis II. Energy-linked reduction of pyridine nucleotides in the chemoautotroph, *Nitrosomonas europaea*. Biochim. Biophys. Acta 113:216–224.
2. Amarasingham, C. R., and B. D. Davis. 1965. Regulation of α-ketoglutarate dehydrogenase formation in *Escherichia coli*. J. Biol. Chem. 240:3664–3668.
3. Arrigoni, O., and T. P. Singer. 1962. Limitation of the phenazine methosulphate assay for succinic and related dehydrogenases. Nature 193:1256–1258.
4. Clark, C., and E. L. Schmidt. 1967. Growth response of *Nitrosomonas europaea* to amino acids. J. Bacteriol. 93:1302–1308.
5. Clark, C., and E. L. Schmidt. 1967. Uptake and utilization of amino acids by resting cells of *Nitrosomonas europaea*. J. Bacteriol. 93:1309–1315.
6. Dolin, M. I. 1955. The DPNH-oxidizing enzymes of *Streptococcus faecalis*. II. The enzymes utilizing oxygen, cytochrome *c*, peroxide and 2,6-dichlorophenol-indophenol or ferricyanide as oxidants. Arch. Biochem. Biophys. 55:415–435.
7. Hirsch, C. A., M. Raminsky, B. D. Davis, and E. C. C. Lin. 1963. A fumarate reductase in *Escherichia coli* distinct from succinic dehydrogenase. J. Biol. Chem. 238:3770–3774.
8. Holzer, H., G. Hierholzer, and I. Witt. 1963. α-Ketoglutar atoxydase der Hefe. Biochem. Z. 337:115–119.
9. Hooper, A. B. 1968. A nitrite-reducing enzyme from *Nitrosomonas europaea*. Preliminary characterization with hydroxylamine as electron donor. Biochim. Biophys. Acta 162:49–65.
10. Hooper, A. B., J. Hansen, and R. Bell. 1967. Characterization of glutamate dehydrogenase from the ammonia-oxidizing chemoautotroph *Nitrosomonas europaea*. J. Biol. Chem. 242:288–296.
11. Hooper, A. B., and A. Nason. 1965. Characterization of hydroxylamine-cytochrome *c* reductase from the chemoautotrophs *Nitrosomonas europaea* and *Nitrosocystis oceanus*. J. Biol. Chem. 240:4044–4057.
12. Kiesow, L. 1964. On the assimilation of energy from inorganic sources in autotrophic forms in life. Proc. Natl. Acad. Sci. U.S. 52:980–988.
13. Kimura, T., J. Hauber, and T. P. Singer. 1967. Studies on succinate dehydrogenase. XIII. Reversible activation of the mammalian enzyme. J. Biol. Chem. 242:4987–4993.
14. Kornberg, A. 1955. Isocitric dehydrogenase of yeast, p. 705–709. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 1. Academic Press, Inc., New York.
15. Nicholas, D. J. D., and P. S. Rao. 1964. The incorporation of labelled CO_2 into cells and extracts of *Nitrosomonas europaea*. Biochim. Biophys. Acta 82:394–397.
16. Ochoa, S. 1955. Malic dehydrogenase from pig heart, p. 735–739. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 1. Academic Press, Inc., New York.
17. Smith, A. J., J. London, and R. Y. Stanier. 1967. Biochemical basis of obligate autotrophy in blue-green algae and thiobacilli. J. Bacteriol. 94:972–983.
18. Winogradsky, S. 1890. Recherches sur les organismes de la nitrification. II. Ann. Inst. Pasteur 4:257–275.