CELL-FREE AMMONIA OXIDATION BY NITROSOMONAS EUROPAEA EXTRACTS: EFFECTS OF POLYAMINES, Mg^{2+} AND ALBUMIN

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SUMMARY - Cell-free extracts of Nitrosomonas europaea prepared by a French Pressure Cell actively oxidized ammonia to nitrite only when protected or activated by bovine serum albumin, Mg^{2+} or polyamines such as spermine.

INTRODUCTION - Ammonia is oxidized to nitrite by Nitrosomonas europaea. Hydroxylamine, a known intermediate (1), is oxidized by cell-free extracts of the organism (2). Although recent reports (3, 4) suggest the involvement of an oxygenase in ammonia oxidation, so far attempts to obtain cell-free extracts capable of oxidizing ammonia have resulted in failure, hindering further study of the mechanism of ammonia oxidation

We earlier reported the oxidation of ammonia by spheroplasts of N. europaea after activation by hydroxylamine or Mg^{2+} (6). When the spheroplasts were lysed osmotically or by brief sonication, however, the resulting cell-free extracts did not oxidize ammonia even with an addition of hydroxylamine or Mg^{2+}.

In studying various methods and conditions for the preparation of cell-free extracts it was found that bovine serum albumin and Mg^{2+} protected the ammonia-oxidizing system during the passage of cells through a French Pressure Cell. The cell-free preparations obtained actively oxidized ammonia to nitrite at a rate of 10-20% of that by intact cells.

In this communication conditions required for the preparation of active, cell-free, ammonia-oxidizing system from N. europaea cells will be reported.

MATERIALS AND METHODS - The cells of Nitrosomonas europaea (Schmidt strain) were grown as described previously (6). The washed cells were suspended in 0.1 M potassium phosphate buffer (pH 7.5) at a concentration of 20 mg wet cells/ml.

Cell-free extracts were prepared by passage of the cell suspension through a French Pressure Cell at 7600 p.s.i., followed by centrifugation at 2000 x g for 20 min to remove intact cells. The extracts thus obtained (1∿1.2 mg protein/ml) did not oxidize ammonia.

For the preparation of active cell-free extracts, cells were suspended in a buffer containing 0.1 M potassium phosphate (pH 7.5), 10 mM $MgCl_2$ and 20 mg/ml bovine serum albumin (Sigma, Fraction V). Microscopic examination of either active or inactive extracts showed that the number of intact cells, if present, was less than 0.2% of that present in the cell suspension.

Oxidation of ammonia or hydroxylamine was followed in a Gilson Oxygraph (Clark Oxygen Electrode) at 22°C, the reaction vessel containing 1.5 ml of cell-free extract or cell suspension. All the additions were made in 10 μl volume, except albumin which was added as solid powder. Nitrite was determined as described previously (6).

RESULTS AND DISCUSSION - Cell-free extracts of N. europaea prepared in the presence of serum albumin and Mg^{2+} oxidized ammonia rapidly as shown in Fig. 1. The extracts prepared in the absence were inactive. The ammonia-oxidizing ability of active extracts was consistently 10-20% that of intact cells. Dipyridyl stopped the oxidation of ammonia in the cell-free system as well as in the intact cell system. Although allylthiourea (1) at 10^{-5} M stopped the oxidation also, there was a 1 min lag before the inhibition. Hydroxylamine oxidation was not inhibited by dipyridyl or allylthiourea.

Active cell-free extracts lost 50% of their activity after 4 hours at 4°C. When the extracts were centrifuged at 100000 x g for 1 hour neither the supernatant nor the precipitate oxidized ammonia. When the two fractions were combined ammonia was oxidized at 10% the rate of original extracts. A preliminary electron microscopic study of the precipitate indicated the association of membranes in a structure resembling chromatophores in photosynthetic bacteria. Such an association was absent in the precipitate from inactive extracts. These results are in agreement with our report on N. europaea

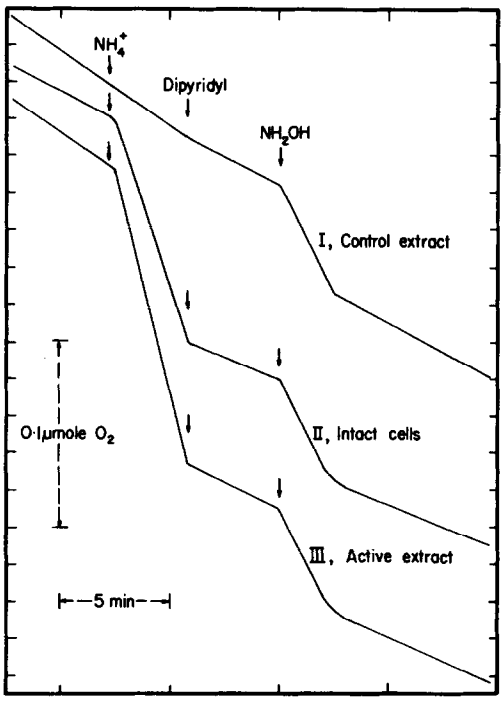

Fig. 1. Oxidation of ammonia by Nitrosomonas cells and French Pressure Cell extracts. Oxygen consumption was followed in a Gilson Oxygraph (reaction volume, 1.5 ml extract or cell suspension) as described in Materials and Methods. At the times indicated by arrows additions were made as follows: 1.7 mM $(NH_4)_2SO_4$, 0.3 mM α,α'-dipyridyl and 33 μM $NH_2OH.HCl$. Extracts were prepared in the absence (I, 1.1 mg extract protein/ml) and in the presence (III) of $MgCl_2$ and bovine serum albumin from a cell suspension of 20 mg/ml. In experiment II the original cell suspension was diluted 10-fold (2 mg cells/ml).

spheroplasts (6). Inactive spheroplasts became active for ammonia oxidation when incubated with Mg^{2+}, accompanied by an increase in membranous folds and invaginations. These electron microscopic studies will be reported elsewhere.

Although we prepared active extracts routinely in the presence of serum albumin and Mg^{2+}, the inactive extracts were found to be readily activated with the addition of either serum albumin or Mg^{2+} as shown in Fig. 2. Thus active ammonia-oxidizing system was reconstituted in vitro. The reconstituted activity was nearly as high as that of active extracts.

Since a structural requirement of membranes or particles seemed important in ammonia oxidation and the effect of Mg^{2+} seemed similar to the effect on

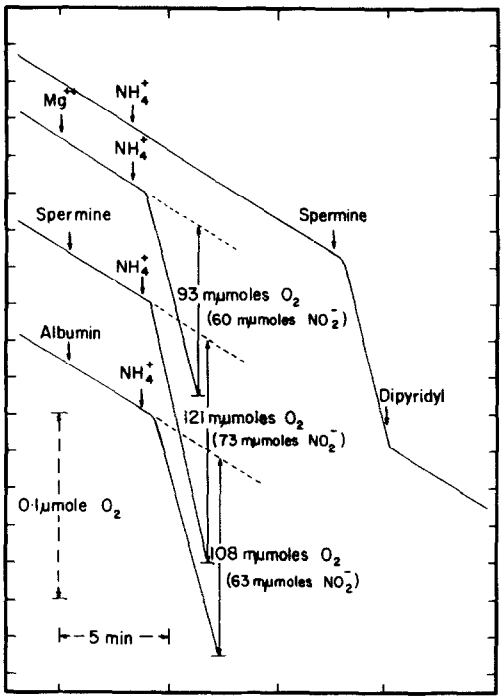

Fig. 2. Activation of Nitrosomonas extracts by Mg^{2+}, spermine and bovine serum albumin and stoichiometry of ammonia oxidation. The Oxygraph reaction vessel contained 1.5 ml of a cell-free extract (1.1 mg extract protein/ml) prepared in the absence of $MgCl_2$ and bovine serum albumin. Additions: 10 mM $MgCl_2$, 2 mM spermine, 20 mg/ml bovine serum albumin, 1.7 mM $(NH_4)_2SO_4$ and 0.3 mM α,α'-dipyridyl.

protein synthesis by ribosomes, polyamines reported to be able to replace Mg^{2+} in protein synthesis (7, 8, 9) were tested. As shown in Fig. 2 spermine at a concentration used for protein synthesis effectively replaced Mg^{2+} or serum albumin.

Stoichiometry of the reaction is also shown in Fig. 2. The amount of oxygen consumed due to ammonia oxidation and the amount of nitrite formed during the oxidation agreed with the equation: $NH_4^+ + 1\frac{1}{2}\ O_2 \longrightarrow NO_2^- + H_2O + 2\ H^+$.

The effect of various concentrations of bovine serum albumin, Mg^{2+} and spermine is shown in Fig. 3 as double reciprocal plots. The concentration of each activator required for half-maximum activation was estimated as 2.7 mg/ml serum albumin, 5 mM Mg^{2+} and 0.75 mM spermine (the higher value, 0.1 mM for the lower value). Egg albumin, casein and lysozyme did not replace bovine serum

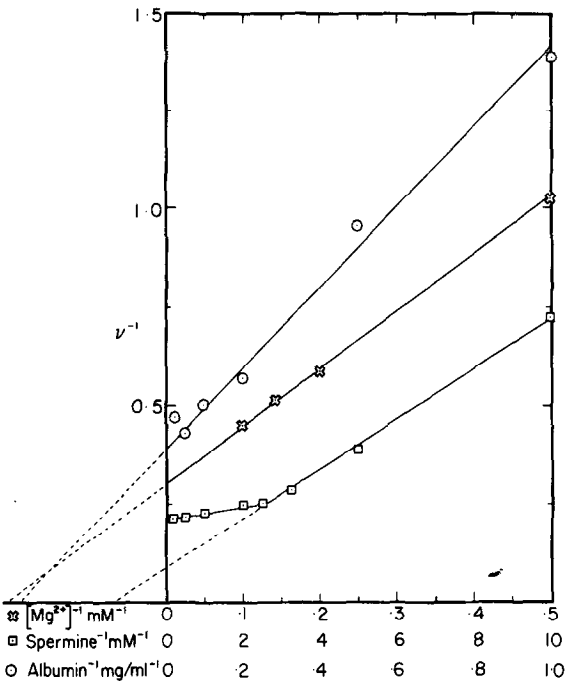

Fig. 3. Effect of Mg^{2+}, spermine and bovine serum albumin concentrations on the rate of ammonia oxidation. v, mμmoles O_2 consumed/min. The reaction vessel contained 1.5 ml of Nitrosomonas extract (1.0 mg protein/ml) prepared in the absence of $MgCl_2$ and albumin. Mg^{2+}, spermine or albumin was added before the initiation of reaction with 1.7 mM $(NH_4)_2SO_4$.

albumin. Ca^{2+} and Mn^{2+} did not replace Mg^{2+} probably because of the precipitation that occurred. Spermidine was less effective than spermine requiring 10 mM for attaining the effect of 0.2 mM spermine. Poly-L-lysine (MW = 140,000) had the same effect as 0.3 mM spermine at a concentration of 100 μg/ml. Glycerol or sucrose (20%) had no effect and mercaptoethanol or dithiothreitol was ineffective.

It is not clear at this stage whether the effect of serum albumin, Mg^{2+} or spermine on cell-free ammonia oxidation is identical in nature. At relatively low concentrations the addition of two activators produced an activity higher than that observed with either activator alone. At saturating concentrations, however, the activating effect was not additive. In fact the activating effect of 2 mM spermine, 10 mM $MgCl_2$ and 20 mg/ml serum albumin together was less than that of 2 mM spermine alone. As is obvious in Fig. 3 spermine was the

most effective activator.

The results reported in this paper conclusively demonstrate that ammonia oxidation can be carried out in a cell-free system and suggest a possibility of eventual purification and characterization of the ammonia-oxidizing system of N. europaea.

ADDENDUM - After the submission of this paper Watson et al.(10) reported the preparation of ammonia-oxidizing cell-free extracts from a halophilic marine bacterium, Nitrosocystis oceanus, in the presence of seawater, Tris, phosphate, ATP and Mg^{2+}. In our Nitrosomonas system ATP had no effect.

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REFERENCES

1. Hofman, T. and Lees, H., Biochem. J., 54, 579 (1953).
2. Falcone, A.B., Shug, A.L. and Nicholas, D.J.D., Biochim. Biophys. Acta, 77, 199 (1963).
3. Rees, M. and Nason, A., Biochem. Biophys. Res. Commun., 21, 248 (1965).
4. Rees, M. and Nason, A., Biochim. Biophys. Acta, 113, 398 (1966).
5. Peck, Jr., H.D., Ann. Rev. Microbiol., 22, 489 (1968).
6. Suzuki, I. and Kwok, S.C., J. Bacteriol., 99, 897 (1969).
7. Cohen, S.S. and Lichtenstein, J., J. Biol. Chem., 235, 2112 (1960).
8. Takeda, Y., Biochim. Biophys. Acta, 179, 232 (1969).
9. Takeda, Y. and Igarashi, K., Biochem. Biophys. Res. Commun., 37, 917 (1969).
10. Watson, S.W., Asbell, M.A. and Valois, F.W., Biochem. Biophys. Res. Commun., 38, 1113 (1970).