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Effect of Ammonia Starvation on Hydroxylamine Oxidoreductase Activity of *Nitrosomonas europaea*[1]

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A technique for detection of the activity of hydroxylamine oxidoreductase (HAO) involving denaturing SDS-polyacrylamide gels was developed. The activity of HAO of *Nitrosomonas europaea* was assayed using this technique, which revealed a single active band of 140 kDa. The HAO activity of other ammonia-oxidizers was also resistant to SDS, the molecular weights being identical to that of *N. europaea*. *N. europaea* cells starved of ammonia for up to 72 h retained a considerable amount of HAO, as detected on Western blot analysis, and a significant level of its activity, as found on assaying at the end of the starvation period. Only after 4 h incubation of starved *N. europaea* cells with 2.0 mM ammonia was some increase in the HAO level observed. The results indicate that HAO remains highly stable during ammonia starvation of *N. europaea*.

Key words: ammonia starvation, hydroxylamine oxidoreductase, *Nitrosomonas europaea*.

The chemolithotrophic ammonia-oxidizing bacteria obtain chemical energy by oxidizing ammonia to nitrite through a two-step process (*1*). The first step is the oxidation of ammonia to hydroxylamine catalyzed by the enzyme, ammonia monooxygenase (AMO), while the second step is the oxidation of hydroxylamine to nitrite by the enzyme, hydroxylamine oxidoreductase (HAO).

HAO is a complex hemoprotein of an $\alpha 2$ or $\alpha 3$ oligomer, and contains eight hemes per 63 kDa subunit (*2*). A molecular mass of 125-175 kDa has been reported for the active HAO (*3, 4*). The gene coding for the 63 kDa polypeptide has been cloned (*5*). Information on the regulation of HAO activity is limited. In a recent paper, Sayavedra-Soto *et al.* (*6*) reported that transcripts of both AMO and HAO are induced by ammonia, and that only 8 h ammonia starvation is needed for complete depletion of these transcripts from *Nitrosomonas europaea* cells. They also demonstrated that acetylene-treated *N. europaea* cells depleted of AMO and HAO transcripts showed increased AMO and HAO activity upon incubation for 3 h with increasing concentrations of ammonia. However, the HAO content and activity were not determined at the end of the starvation period, and the increased ammonia oxidation was attributed to *de novo* synthesis of AMO and HAO.

In this study, we have developed a technique which allows the detection of HAO activity *in situ* in denaturing polyacrylamide gels. In addition, antibodies against HAO from *N. europaea* have also been prepared to enable its detection by Western blot analysis. These two tools have enabled us to detect HAO activity and its level in a cell-free system. The results indicate that HAO is a stable protein and that *N. europaea* cells starved of ammonia for up to 72 h retain a significant level of active HAO.

MATERIALS AND METHODS

Bacteria and Culture Media—*N. europaea* ATCC 19718 was obtained from the American Type Culture Collection (Rockville, MD), and cultured in 100-ml batches of medium 221 (ATCC) at 26°C.

Purification of Hydroxylamine Oxidoreductase—Hydroxylamine oxidoreductase of *N. europaea* ATCC 19718 was prepared from cells harvested from 40 liters of a culture grown in a fermentor, by 3-4 cycles of quick freeze-thaw and then passage through a French press. After centrifugation at $10,000 \times g$ for 10 min, the supernatant was fractionated with ammonium sulfate, and the red precipitate which was pelleted between 60-70% saturation was dialyzed overnight against 10 mM Tris-HCl, pH 8.5. The purification of HAO from this preparation was performed as described by Yamanaka *et al.* (*4*). Antibodies against the purified HAO were prepared in rabbits (*7*).

Protein Analysis—For analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), bacterial cells were combined with sample buffer (*8*) without β-mercaptoethanol but with SDS as indicated. Proteins were subjected to 7.5% SDS-PAGE and then used directly for the detection of HAO activity. For immunoblot analysis, proteins were electrophoretically transferred to nitrocellulose filters, and then the blots were processed as described by Sambrook *et al.* (*9*). The transferred proteins were reacted with the rabbit antibodies against HAO, followed by with alkaline phosphatase-conjugated goat anti-rabbit antibodies. A mixture of 5-bromo-4-chloro-3-indolyl (BCIP) and nitro blue tetrazolium (NPT) was used as the substrates for

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Abbreviations: AMO, ammonia monooxygenase; HAO, hydroxylamine oxidoreductase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; PMS, phenazine methosulfate.

alkaline phosphatase (*9*).

Detection of HAO Activity—HAO activity in SDS-polyacrylamide gels was detected by immersing the gels in a solution comprising 50 mM Tris-HCl, pH 8.0, 5 mM hydroxylamine, 0.1 mM phenazine methosulfate (PMS), and 0.2 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) obtained from Aldrich Chem. HAO activity was obtained as a dark precipitate over the HAO band after 30 min at room temperature due to the accumulation of formazan. The nitrite and ammonia concentrations were determined by standard methods, as previously described (*10*).

RESULTS AND DISCUSSION

Detection of HAO Activity in SDS-Polyacrylamide Gels—A procedure which allows qualitative detection of HAO activity within the matrix of SDS-polyacrylamide gels was developed. This method includes immersion of a gel in a medium containing hydroxylamine, as well as PMS and MTT, which have been shown to act as electron acceptors for hydroxylamine oxidoreductase (*11*). *N. europaea* proteins were fractionated by SDS-PAGE and HAO activity was examined (Fig. 1B). The gel and the running buffer both contained 0.1% SDS. Protein samples were treated with 0.1 or 2% SDS before loading. A band corresponding to an approximate molecular weight of 140 kDa was the only one that showed significant HAO activity (Fig. 1B). Coomassie Brilliant Blue staining revealed a distinct band of the same size (Fig. 1A). No HAO activity was observed when hydroxylamine or PMS was omitted from the reaction buffer. The addition of 2-mercaptoethanol to the sample buffer or boiling of the samples in SDS led to inactive high molecular weight aggregates (data not shown), as has been previously reported (*2*). Thus, the results are in close agreement with the molecular mass of the active HAO (125 kDa) determined by electrophoresis at high hydrostatic pressure (*3*). Removal of covalently bound heme by HAO treatment with nitrophenylsulfenyl chloride, and its boiling in the presence of SDS, resulted in the appearance of a single band of *ca.* 63 kDa (data not shown), as previously described (*2*). The proteins in the 63 kDa band, the 140 kDa band, and the high molecular weight aggregates have the same N-terminal amino acid sequence, DISTVPDETYD. This amino acid sequence is identical to that generated from the N-terminal of the 63 kDa protein by Arciero and Hooper (*2*). In addition, the 63 kDa protein and the HAO high molecular weight aggregates reacted with antibodies prepared against the active HAO (data not shown). The gene coding for HAO in *N. europaea* has been isolated and it codes for a protein of 64 kDa (*5*). Since the same molecular weight of approximately 140 kDa was obtained with native polyacrylamide gels (data not shown), the results support the possibility that the polypeptide structure of the active HAO from *N. europaea* is that of an $\alpha 2$ oligomer. The HAO activity of other ammonia-oxidizers, such as *N. europaea* LMD 86.25, *Nitrosomonas* spp., *Nitrosomonas eutropha* N904, and a *Nitrosolobus* spp., was also resistant to SDS, the molecular weights being identical to that of *N. europaea* (Fig. 2).

Stability and Induction of HAO Activity—Oxidation of ammonia to nitrite, through the sequential activities of the enzymes, AMO and HAO, provides the energy for the growth of ammonia-oxidizers. Sayavendra-Soto *et al.* (*6*) have shown that *N. europaea* cells starved of ammonia for 8 h at 4°C were completely depleted of RNA including the transcripts for AMO and HAO. However, the protein level and activity of HAO were not assayed during or immediately at the end of the starvation treatment. Since a protein and the mRNA for the protein may exhibit different degradation rates, it is possible that the HAO level is less affected by ammonia starvation than that of the transcript of the *hao* gene. To verify this possibility, the following experiments were conducted. *N. europaea* cells were concentrated (100-fold) from 2-week-old cultures, washed three times with the growth medium (see "MATERIALS AND METHODS") without ammonia, and then incubated in this medium for 24, 48, and 72 h at 4 or 26°C. Ammonia and nitrite were not detected in the incubation medium, and we assumed that if there was any trace of ammonia below the detection limit of the assay, it would be consumed by the cells within a short time (minutes-hours). The activity of HAO, on SDS-PAGE, and the HAO protein level, on

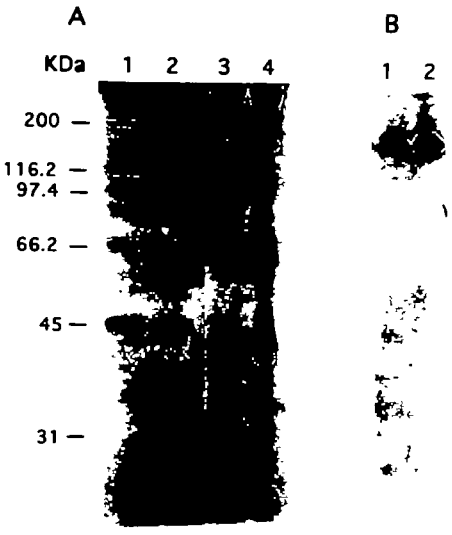

Fig. 1. **Coomassie Brilliant Blue staining of SDS-PAGE fractionated *Nitrosomonas europaea* proteins (A), and *in situ* detection of HAO activity (B).** (A) Lanes 1 and 2, high and low molecular weight standards, respectively. Lanes 3 and 4, *Nitrosomonas europaea* cells suspended in sample buffer containing 0.1 and 2% SDS, respectively. (B) Separate lanes from the same gel as in (A) were cut out and processed for the detection of HAO activity as described under "MATERIALS AND METHODS." The sample buffer in lanes 1 and 2 contained 0.1 and 2% SDS, respectively.

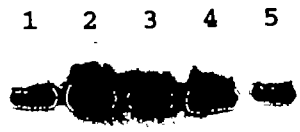

Fig. 2. **Analysis of HAO activity of several ammonia oxidizers in a SDS-polyacrylamide gel as described under "MATERIALS AND METHODS."** Lanes 1-5, extracts of *Nitrosomonas europaea* ATCC 19718, *Nitrosomonas europaea* LMD 86.25, *Nitrosomonas eutropha* N904, *Nitrosomonas* spp., and *Nitrosolobus* spp., respectively.

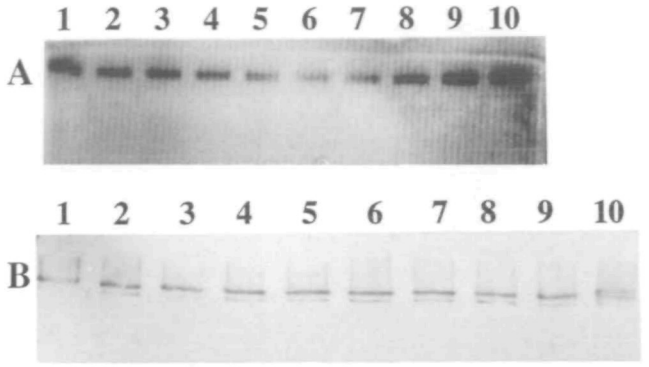

Fig. 3. **HAO activity (A) and level (B) in *N. europaea* cells starved of ammonia for 72 h.** (A) Cells were starved of ammonia and kept at 4°C (lanes 1–5) or 26°C (lanes 6-10), and then they were incubated in the presence of 0.2 mM ammonia at 26°C. Equal aliquots were taken for the detection of HAO activity in SDS-polyacrylamide gels as described under "MATERIALS AND METHODS." Lanes 1 and 6, 2 and 7, 3 and 8, 4 and 9, 5 and 10, samples taken after incubation for 0, 15, 30, 45, and 60 min, respectively. (B) An identical gel to that in A was processed as a Western blot for quantitative detection of the HAO protein using antibodies raised against the purified HAO from *Nitrosomonas europaea*.

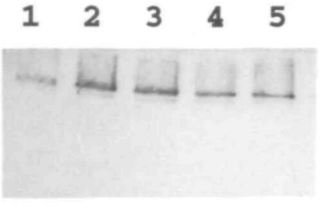

Fig. 4. **HAO level in *N. europaea* cells starved of ammonia for 72 h.** Cells were starved for ammonia and kept at 26°C, and then they were incubated in the presence of increasing concentrations of ammonia at 26°C. Equal aliquots were taken for detection of the HAO level by Western blot analysis using antibodies raised against the purified HAO from *N. europaea*. Lanes 1-4, 0, 0.2, 2.0, and 20.0 mM ammonia, respectively. Lane 5, 0.1 mM hydroxylamine.

Western blot analysis, were determined at the end of the starvation period and after ammonia induction. The results showed that after 72 h starvation at both temperatures (4 or 26°C), *N. europaea* cells retained both detectable HAO activity (Fig. 3A) and HAO protein (Fig. 3B). The HAO level showed no significant change when the cells were incubated with 0.2 mM ammonia for 60 min (Fig. 3B) regardless of whether the starved cells were kept at 4 or 26°C. However, the results may indicate that the recovery of HAO activity differs between the two treatments (Fig. 3A). Similar results were obtained after 24 and 48 h ammonia starvation (data not shown).

In the following experiments, starved *N. europaea* cells (72 h) were incubated for 4 h in the presence of increasing concentrations of ammonia and hydroxylamine. The HAO level (Fig. 4) and nitrite production (Table I) were determined. The results show that the starved *N. europaea* cells retained a detectable amount of HAO in the absence of ammonia, and an increase in the HAO level, which was optimal at 2.0 mM ammonia, which can be attributed to *de novo* synthesis, was observed. The starved cells oxidized both ammonia and hydroxylamine (Table I), indicating the viability of the cells after the starvation treatment and the

TABLE I. **Ammonia and hydroxylamine oxidation by starved *N. europaea* cells.**

Ammonia (mM)	Hydroxylamine (mM)	μg NO_2/sample[a]
0.2	–	4.4±0.8[b]
2.0	–	13.2±1.9
20.0	–	14.8±1.2
–	0.01	2.9±1.5
–	0.10	10.5+1.5
0.2	0.01	5.2±0.9
0.2	0.10	17.9±1.1

[a] *N. europaea* cells were incubated without ammonia for 72 h at 26°C. Equal aliquots were incubated in the basal medium with the addition of ammonia and/or hydroxylamine, as indicated. After 4 h, the cells were removed and nitrite was determined. [b] These values are the means±standard deviation for three experiments.

presence of both enzymes AMO and HAO. The effects of ammonia and hydroxylamine were additive with low concentrations of ammonia and higher concentrations of hydroxylamine (1 mM) were toxic to the cells, as indicated by the sharp decrease in nitrite production (data not shown). These results are in agreement with the results reported by de Bruijn *et al.* (*12*), who showed that *N. europaea* is capable of growing mixotrophically on ammonia and hydroxylamine. In separate experiments, starved *N. europaea* cells (72 h) were preincubated for 30 min in the presence of 25 μg/ml of chloramphenicol, and then 10 mM ammonia was added. The inclusion of chloramphenicol had no effect on nitrite production by the cells for up to 4 h incubation (data not shown). These findings are further support for the presence of the key enzymes involved in ammonia oxidation in *N. europaea* cells starved of ammonia for 72 h.

The results reported in this paper indicate that although the HAO transcript in *N. europaea* cells is totally degraded within 8 h ammonia starvation (*6*), HAO is a relatively stable protein and active HAO could still be detected after 72 h ammonia starvation (Figs. 3 and 4). Protein degradation is a cellular tool for modulating the levels of specific proteins and for eliminating damaged ones. Some proteins are degraded within minutes while others are very stable. Regulatory proteins and enzymes usually have fast turnover rates, thus their levels rapidly change in response to stimuli (*13*). The selectivity and regulation of protein degradation in both eukaryotes (*14*) and prokaryotes (*15*) are due to ATP-dependent and ATP-independent mechanisms. It is tempting to speculate that HAO degradation proceeds through an ATP-dependent mechanism (*14, 15*). AMO and HAO are the key enzymes involved in ammonia oxidation and hence energy generation in the obligate autotroph, *N. europaea* (*1*). The stability of HAO and possibly AMO (not examined in this study) ensures a renewed energy supply to cells subjected to fluctuations in the availability of ammonia in their growth environment. The coupling of HAO and possibly AMO degradation to an ATP-dependent system allows their limited degradation during ammonia starvation, and normal turn-over rates when ammonia is available and the energy supply is not limited.

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