

## In Vitro Activation of Ammonia Monooxygenase from *Nitrosomonas europaea* by Copper

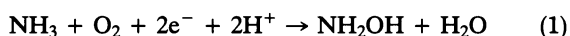
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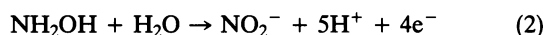
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The effect of copper on the in vivo and in vitro activity of ammonia monooxygenase (AMO) from the nitrifying bacterium *Nitrosomonas europaea* was investigated. The addition of  $\text{CuCl}_2$  to cell extracts resulted in 5- to 15-fold stimulation of ammonia-dependent  $\text{O}_2$  consumption, ammonia-dependent nitrite production, and hydrazine-dependent ethane oxidation. AMO activity was further stimulated in vitro by the presence of stabilizing agents, including serum albumins, spermine, or  $\text{MgCl}_2$ . In contrast, the addition of  $\text{CuCl}_2$  and stabilizing agents to whole-cell suspensions did not result in any stimulation of AMO activity. The use of the AMO-specific suicide substrate acetylene revealed two populations of AMO in cell extracts. The low, copper-independent (residual) AMO activity was completely inactivated by acetylene in the absence of exogenously added copper. In contrast, the copper-dependent (activable) AMO activity was protected against acetylene inactivation in the absence of copper. However, in the presence of copper both populations of AMO were inactivated by acetylene. [ $^{14}\text{C}$ ]acetylene labelling of the 27-kDa polypeptide of AMO revealed the same extent of label incorporation in both whole cells and optimally copper-stimulated cell extracts. In the absence of copper, the label incorporation in cell extracts was proportional to the level of residual AMO activity. Other metal ions tested, including  $\text{Zn}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Cr}^{3+}$ , and  $\text{Ag}^+$ , were ineffective at stimulating AMO activity or facilitating the incorporation of [ $^{14}\text{C}$ ]acetylene into the 27-kDa polypeptide. On the basis of these results, we propose that loss of AMO activity upon lysis of *N. europaea* results from the loss of copper from AMO, generating a catalytically inactive, yet stable and activable, form of the enzyme.

The chemolithoautotrophic ammonia-oxidizing bacterium *Nitrosomonas europaea* obtains all of its energy for growth from the oxidation of ammonia to nitrite (55). The initial oxidation of ammonia, which yields hydroxylamine as the product, is a reductant- and  $\text{O}_2$ -dependent reaction catalyzed by ammonia monooxygenase (AMO):



Hydroxylamine is further oxidized to nitrite by hydroxylamine oxidoreductase (HAO):



Two of the four electrons generated from hydroxylamine oxidation are used to support the oxidation of additional ammonia molecules, while the other two electrons enter the electron transfer chain and are used to support  $\text{CO}_2$  reduction and ATP biosynthesis (55).

Despite the key role AMO plays in initiating biological nitrification, the enzyme has not been purified, and consequently, little is known about its structure, enzymatic mechanism, or the identity and function of its cofactors. This can be largely attributed to the difficulty of working with and assaying the enzyme in cell-free systems. The most convenient assays for measuring AMO activity are to monitor ammonia-dependent  $\text{O}_2$  consumption or nitrite production. These assays require a high degree of coupling: the hydroxylamine formed upon ammonia oxidation must be further oxidized by HAO, and the electrons from hydroxylamine oxidation must be efficiently donated back to AMO to support the oxidation of additional ammonia. However, the

electron carrier(s) between AMO and HAO remains a mystery, as does the direct electron donor to AMO.

Much of what is known concerning the molecular properties of AMO has been deduced from whole-cell studies of the enzyme. One interesting property of AMO is its broad substrate specificity. AMO will oxidize a variety of alternate, nonphysiological substrates, including CO (30, 51), alkanes (22, 24, 31), and alkenes (22, 26) and cyclic (8), aromatic (23), and halogenated (5, 25, 40, 41, 54) hydrocarbons. Acetylene acts as a mechanism-based, irreversible inactivator of AMO (27-29). Inactivation of AMO by [ $^{14}\text{C}$ ]acetylene leads to the covalent labelling of a membrane-bound 27-kDa polypeptide which is believed to be the active site-containing subunit of the enzyme (20, 27).

Several lines of evidence suggest that copper may be a component of AMO (6). AMO activity in whole cells is sensitive to copper-selective chelating agents, including allylthiourea (14, 15, 32, 33), xanthates (52, 53), carbon disulfide (21),  $\alpha$ ,  $\alpha'$ -dipyridyl (15), and cyanide (15). Light is also a potent and specific inactivator of AMO (16, 42). On the basis of similarities between the photosensitive state of oxygenated AMO and that of the well-characterized copper enzyme tyrosinase, Shears and Wood have proposed a catalytic cycle for AMO which involves the binding and reduction of  $\text{O}_2$  at a binuclear copper site on the enzyme (42).

Similarities between AMO and the membrane-associated (particulate) methane monooxygenase (MMO) of methanotrophic bacteria also support a role for copper in AMO. Particulate MMO and AMO have a remarkably similar substrate range (35) and susceptibility to inhibitors, including copper-chelating agents (17, 44) and acetylene (38). Under conditions of copper limitation, a number of methanotrophs will express a soluble MMO (1), whereas in the

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presence of sufficient copper, only the particulate MMO is expressed (7, 39). Soluble MMO has been purified to homogeneity (9, 12, 37, 56) and has been shown to contain a binuclear iron center as a component of the active site of the hydroxylase component of the enzyme (10). The purification of a three-component particulate MMO system from *Methylosinus trichosporium* OB3b has been described, and one of the protein components of this system contained copper (50). However, subsequent attempts to reproduce this result have been unsuccessful (6). The solubilization of particulate MMO from the bacterial membrane of *Methylococcus capsulatus* (Bath) in a partially active state has recently been reported, but further attempts to purify the enzyme resulted in the irreversible loss of enzyme activity (43). Consequently, particulate MMO remains uncharacterized at the molecular level.

The most recent reports describing *in vitro* studies of AMO were published in the 1970s and early 1980s by Suzuki and coworkers. They described the preparation of *N. europaea* spheroplasts (45) and cell extracts (46, 48) capable of catalyzing ammonia-dependent O<sub>2</sub> uptake and nitrite production. In their cell-free system, active extracts were obtained only when bovine serum albumin (BSA), spermine, or Mg<sup>2+</sup> was added as a stabilizing agent, either before or after cell lysis (46, 48). Under optimal conditions, their extracts catalyzed ammonia-dependent O<sub>2</sub> uptake at rates close to 10% of the rates observed with whole-cell suspensions (48). The extracts were, however, quite unstable: upon storage at 4°C, the extracts lost 50% of their AMO activity in 4 h, and when frozen at 20°C, they were stable for only 1 to 2 days. The activity of the extracts was also variable and dependent upon the age and *in vivo* AMO activity of the *N. europaea* cells. Some cell suspensions yielded active extracts while others yielded inactive extracts (48).

The cell-free system of Suzuki and coworkers was further resolved into three components, a membrane fraction, a soluble fraction containing HAO, and a soluble fraction containing cytochromes, which could be recombined with the partial restoration of AMO activity (47). These studies were expanded to demonstrate that purified, reduced cytochrome *c*554 could serve as a reductant for the oxidation of ammonia or CO by the membrane fraction, leading to the suggestion that cytochrome *c*554 may serve as a direct electron donor to AMO (51). Recently, it has been shown that cytochrome *c*554 will also form a stable complex with and serve as an electron acceptor for purified HAO (3, 4).

Although some insights into the nature and function of AMO have been derived from the *in vivo* and *in vitro* studies discussed above, the further characterization of this novel enzyme will necessitate its purification to homogeneity in an active state. To date, a major barrier to the purification of AMO has been the difficulty in reproducibly obtaining active *in vitro* AMO preparations, and the extreme instability of these extracts. It is likely that loss of AMO activity results either from a loss of coupling integrity between AMO and accessory proteins required for assaying the enzyme or from the inherent instability of AMO *in vitro*. We have addressed these possibilities by attempting to define factors which could increase the activity of AMO in cell extracts. Given the evidence that copper may play a role in the catalytic mechanism and/or as a structural component of AMO, we have examined the effect of copper on the AMO-catalyzed oxidation of ammonia and ethane by cell extracts. In this report, we demonstrate that the *in vitro* activity of AMO is greatly stimulated by the addition of copper and present evidence that this stimulation is due to the activation of

catalytically inactive AMO. On the basis of these results, we propose that the *in vitro* instability of AMO is at least partially a function of labile copper being lost from the enzyme upon cell lysis.

## MATERIALS AND METHODS

**Materials.** Ethane (99.0%) was obtained from Liquid Carbonic, Chicago, Ill. [U-<sup>14</sup>C]acetylene was generated from Ba<sup>14</sup>CO<sub>3</sub> (specific activity = 56 mCi/mmol), obtained from Sigma Chemical Co., St. Louis, Mo., as described previously (19). Unlabelled acetylene was generated in a gas-generating bottle from calcium carbide (Aldrich Chemical Co., Milwaukee, Wis.) as described previously (18). Chelex-100 ion-exchange resin was obtained from Bio-Rad, Richmond, Calif. BSA (fraction 5, >98%) was obtained from United States Biochemical Corporation, Cleveland, Ohio. Ultrapure BSA and fatty-acid ultrafree BSA were obtained from Boehringer Mannheim, Indianapolis, Ind. Human, dog, pig, and rat serum albumins (fraction 5), ovalbumin (grade 5), gamma globulins (bovine fraction II, 99%), and spermine were obtained from Sigma. Ultrapure CuCl<sub>2</sub> (99.999%), ZnCl<sub>2</sub> (99.999%), CoCl<sub>2</sub> (99.999%), and CaCl<sub>2</sub> (99.99%) were obtained from Aldrich. Ultrapure NiCl<sub>2</sub>, CrCl<sub>3</sub>, and MnSO<sub>4</sub> were obtained from Johnson Matthey Chemicals, Royston, Hertfordshire, England. NADH (grade 3) was obtained from Sigma. Residual ethanol was removed by drying the NADH under a vacuum in a desiccator after dissolving in buffer (0.1 M KH<sub>2</sub>PO<sub>4</sub>, pH 7.8). After desiccating to dryness, no residual ethanol could be detected in the resuspended NADH by flame ionization detection-gas chromatography. All other chemicals used were of reagent grade and were used without further purification.

**Growth of bacteria.** *N. europaea* (ATCC 19178) cultures were grown in a 6-liter Microferm fermentor (New Brunswick Scientific Inc., New Brunswick, N.J.) at 30°C under forced aeration and with mixing from an overhead stirrer (300 rpm). The mineral salt growth medium used was as described previously (20) except that NaH<sub>2</sub>PO<sub>4</sub> (1.3 mM), KH<sub>2</sub>PO<sub>4</sub> (3 mM), and K<sub>2</sub>HPO<sub>4</sub> (12.6 mM) were substituted as phosphate sources. The pH in the fermentor was maintained at pH 7.8 by the addition of K<sub>2</sub>CO<sub>3</sub> (20% [wt/vol]) with a pH control system (Cole Parmer Instrument Co., Chicago, Ill.). Cells (5.2 liters) were drained from the fermentor daily when the nitrite concentration in the medium was approximately 35 mM; the corresponding optical density of the culture at 600 nm (1-cm path) was approximately 0.2. Fresh sterile medium was added to the remaining 0.5 liters of cells in the fermentor. Cells were collected by centrifugation, washed twice with buffer (0.1 M KH<sub>2</sub>PO<sub>4</sub>, pH 7.8), and resuspended in 6 ml of the same buffer containing glycerol (10% [vol/vol]) and MgCl<sub>2</sub> (1 mM). The resuspended cells were either used immediately or rapidly frozen and stored in liquid N<sub>2</sub>. In some cases, BSA (10 mg/ml) was added to cell suspensions prior to freezing.

**Preparation of cell extracts.** Frozen cell suspensions from individual collections were thawed in a 30°C water bath and pooled, and DNase I (10 µg/ml) was added. Typically, a 70-ml volume of thawed suspension containing 10 g (wet weight) of cells was disrupted by one passage through a French pressure cell operated at 7,500 lb/in<sup>2</sup>. Unbroken cells were removed by centrifugation at 7,000 × *g* for 10 min. Cell extracts were either used immediately or frozen and stored in liquid N<sub>2</sub> in 1-ml aliquots.

**O<sub>2</sub> uptake measurements.** Ammonia-dependent O<sub>2</sub> uptake measurements were performed in a Clark-type oxygen elec-

trode (Yellow Springs Instrument Co., Yellow Springs, Ohio) mounted in a water-jacketed reaction vessel (1.7-ml volume) maintained at 30°C. After preequilibrating a sample of whole-cell suspension or cell extract and buffer (0.1 M  $\text{KH}_2\text{PO}_4$ , pH 7.8) containing BSA (10 mg/ml) for 1 min,  $\text{O}_2$  uptake was initiated by the addition of  $(\text{NH}_4)_2\text{SO}_4$  (3 mM final concentration). Reactions were terminated by adding 10  $\mu\text{l}$  of a saturated aqueous solution of acetylene. For some assays,  $\text{CuCl}_2$  (final concentration = 350  $\mu\text{M}$ ) was added to the electrode chamber.

**Ammonia oxidation to nitrite.** Assays for measuring the production of nitrite from ammonia were performed in stoppered serum vials (9 ml) containing a sample of extract, buffer (0.1 M  $\text{KH}_2\text{PO}_4$ , pH 7.8), BSA (0 to 25 mg/ml), and  $\text{CuCl}_2$  (0 to 1 mM) in a total volume of 1 ml. Reactions were initiated by the addition of  $(\text{NH}_4)_2\text{SO}_4$  (5 mM final concentration). The vials were shaken (150 cycles per min) in a water bath at 30°C. At the desired times, samples (20 to 50  $\mu\text{l}$ ) were withdrawn and nitrite was quantified colorimetrically as described by Hageman and Hucklesby (13).

**Ethane oxidation.** The oxidation of ethane to ethanol by *N. europaea* extracts was measured by using gas chromatography as described previously (22). Ethane oxidation assays were conducted in stoppered serum vials (9-ml volume) which contained buffer, BSA (10 mg/ml), *N. europaea* extract (1.8 mg of protein), and  $\text{CuCl}_2$  (0 or 200  $\mu\text{M}$ ) in a total volume of 1 ml. Ethane oxidation was initiated by the simultaneous addition of ethane (130  $\mu\text{mol}$ ) and hydrazine hydrochloride or NADH (0.5 or 2 mM). For time course measurements, samples (3  $\mu\text{l}$ ) of the liquid phase were removed at the indicated times and injected into the gas chromatograph. For fixed-time assays, reactions were terminated by the addition of acetylene (10  $\mu\text{mol}$ ) to the assay vials. Samples of the liquid phase (3  $\mu\text{l}$ ) were then analyzed by gas chromatography.

**In vivo inactivation of AMO by acetylene.** Cells harvested from the fermentor were suspended in 40 ml of buffer and placed in a stoppered serum bottle (150 ml). Acetylene gas (5 ml) was added to the headspace of the bottle, and the cells were incubated on a rotary shaker (200 rpm) at 30°C for 30 min. The cells were then sedimented by centrifugation, washed twice with buffer, and resuspended and frozen in liquid  $\text{N}_2$  as described above. Acetylene-treated cells were subsequently thawed and disrupted by French press as described above.

**In vitro inactivation of AMO by acetylene.** Acetylene gas (25  $\mu\text{l}$ ) was added to stoppered serum vials (8 ml) containing cell extract (3.6 mg), hydrazine hydrochloride (0.5 mM), and BSA (10 mg/ml) with or without  $\text{CuCl}_2$  (500  $\mu\text{M}$ ) in a total volume of 1 ml of buffer. The vials were incubated with shaking (60 cycles per min) in a 30°C water bath for 5 min, after which acetylene was removed by five cycles of evacuation and flushing with  $\text{N}_2$  on a vacuum manifold. The vials were then vented and reequilibrated with air, and the extracts were subsequently assayed for AMO activity. Control incubation vials were prepared and treated identically except in the absence of acetylene.

**In vivo and in vitro [ $^{14}\text{C}$ ]acetylene labelling of AMO.** Whole-cell suspensions and cell extracts were exposed to [ $^{14}\text{C}$ ]acetylene (15  $\mu\text{Ci}$ ; specific activity, 112 mCi/mmol) in stoppered glass serum vials (5 ml). The reaction vials contained whole-cell suspension (2.7 mg of protein) or cell extract (2.3 mg of protein) in buffer containing BSA (1 mg/ml) and the indicated concentrations of  $\text{CuCl}_2$ , in a total volume of 1 ml. The reactions were initiated by the addition of an aqueous solution of hydrazine hydrochloride (0.5 mM

final concentration). The reactions were conducted by incubating the vials at 30°C for 10 min in a shaking water bath. The reactions were terminated by transferring samples (900  $\mu\text{l}$ ) of the reaction mixture to a microcentrifuge tube and then diluting the [ $^{14}\text{C}$ ]acetylene by gently bubbling the reaction mixture with a 2,000-fold excess of unlabelled acetylene (5 ml). Samples (200  $\mu\text{l}$ ) of the terminated reaction mixture were then added to sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer (200  $\mu\text{l}$ ). Radiolabel incorporation into the 27-kDa component of AMO was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography, as described previously (20).

**Protein determination.** Protein concentrations were determined by the biuret assay (11) after solubilizing proteins in 3 M NaOH (30 min at 60°C) and sedimenting insoluble material by centrifugation (10,000  $\times g$  for 5 min). For reporting the protein concentration of suspensions and extracts which contained BSA, the concentration of BSA in the samples was subtracted so that the protein concentration reflected only *N. europaea* proteins.

## RESULTS

**Stimulation of AMO activity in *N. europaea* extracts by  $\text{CuCl}_2$ .** Suzuki and coworkers have described the preparation of extracts of *N. europaea* by French press, which catalyzed the ammonia-dependent uptake of  $\text{O}_2$  and production of nitrite (48). In their system, BSA was routinely included in the lysis and assay buffers to stabilize and optimize AMO activity. Cell extracts of *N. europaea* prepared in our laboratory in a similar manner with 10 mg of BSA per ml in the lysis and assay buffers have consistently exhibited very low rates of AMO activity. However, the AMO activity of these extracts can be dramatically increased by the addition of copper. As shown in Fig. 1, traces A and B, the addition of 350  $\mu\text{M}$   $\text{CuCl}_2$  to the assay buffer and extract prior to the addition of ammonia resulted in a ninefold increase in the subsequent rate of ammonia-dependent  $\text{O}_2$  uptake. When  $\text{CuCl}_2$  was added several minutes after the assay had been initiated with ammonia, the rate of  $\text{O}_2$  uptake was stimulated to the same maximal rate within 30 s of the addition of copper (trace C). The addition of acetylene to the reaction mixtures resulted in the complete inhibition of  $\text{O}_2$  uptake in both the absence (trace A) and the presence (traces B and C) of  $\text{CuCl}_2$ . Extracts prepared from *N. europaea* cells in which AMO had been selectively inactivated by acetylene before cell lysis had no ammonia-dependent  $\text{O}_2$  uptake activity either in the absence or in the presence of copper (trace D). The combination of assay buffer, ammonia, and  $\text{CuCl}_2$  alone also had no  $\text{O}_2$  uptake activity. In contrast to the stimulation of AMO by copper in vitro, there was no stimulation of ammonia-dependent  $\text{O}_2$  uptake by copper in whole-cell suspensions of *N. europaea*. The specific rate of ammonia-dependent  $\text{O}_2$  uptake catalyzed by extracts in the presence of copper (41 nmol of  $\text{O}_2$  consumed per min per mg of protein) was about 10% of the rate of that catalyzed by whole-cell suspensions.

A similar stimulatory effect of copper in vitro was observed when AMO activity was measured as ammonia-dependent nitrite production. Figure 2 shows the time course of nitrite production for *N. europaea* extracts assayed in buffer containing 10 mg of BSA per ml and various concentrations of copper. The initial rate of nitrite production was increased 7.3-fold in the presence of 200  $\mu\text{M}$   $\text{CuCl}_2$ , from 4.3 to 31 nmol of nitrite produced per min per mg of protein.

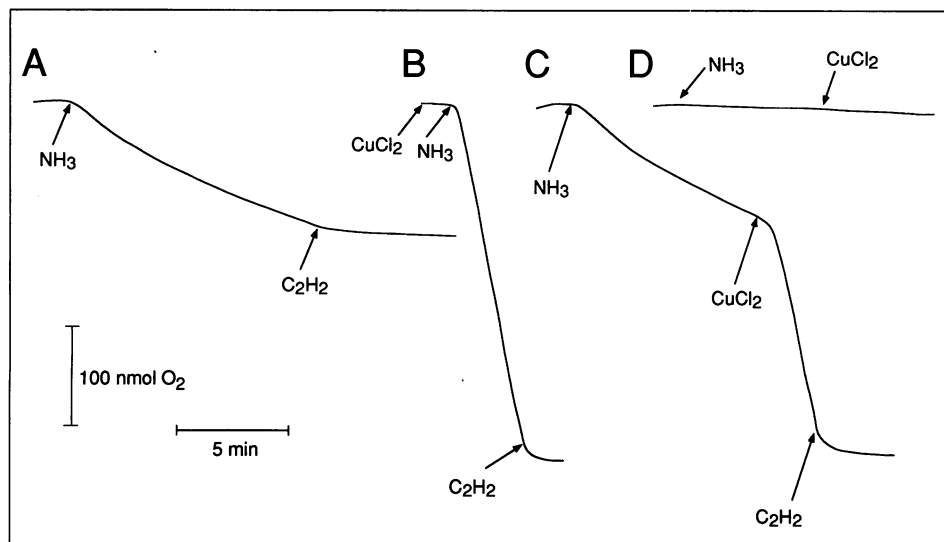


FIG. 1. Stimulation of ammonia-dependent  $O_2$  uptake by *N. europaea* cell extract upon addition of  $CuCl_2$ .  $O_2$  uptake was monitored as described in Materials and Methods. The arrows indicate the times of addition of ammonia [added as  $(NH_4)_2SO_4$ ],  $CuCl_2$ , or acetylene to the reaction vessel. Traces A to C show the time course of  $O_2$  consumption with extract (2.8 mg of protein) prepared from active cells; trace D shows the time course of  $O_2$  consumption with extract (2.5 mg of protein) prepared from cells in which AMO was inactivated with acetylene before cell breakage.

Additional copper did not significantly affect the total nitrite formed in the course of the assay. Decreasing the copper concentration to 50 or 100  $\mu M$ , however, substantially decreased the stimulation of nitrite production. No nitrite production was observed when assays were performed in the presence of acetylene or with extracts in which AMO had been inactivated by acetylene in vivo. As was observed for ammonia-dependent  $O_2$  uptake, there was no stimulatory effect of copper on nitrite production by whole-cell suspen-

sions of *N. europaea*, and the maximal rate of nitrite production catalyzed by the extracts was about 10% of the rate of that catalyzed by whole-cell suspensions.

The effect of copper on ammonia-dependent nitrite production by *N. europaea* extracts was further investigated by varying both the concentration of copper and that of BSA added to the assay buffer. As shown in Fig. 3, the concentration of copper required to maximize nitrite production in a fixed-time assay was influenced considerably by the concentration of BSA in the assay buffer. When assays were performed in buffer containing 1 mg of BSA per ml, optimal

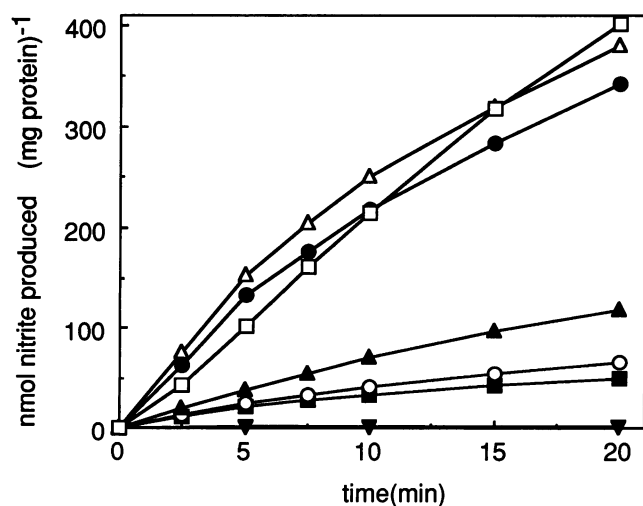


FIG. 2. Time course of nitrite production from ammonia by *N. europaea* cell extracts in the presence of various concentrations of  $CuCl_2$ . The production of nitrite from ammonia was measured as described in Materials and Methods. The nanomoles of nitrite produced per milligram of protein is plotted versus time for assays containing 1.8 mg of protein and 500  $\mu M$   $CuCl_2$  ( $\square$ ), 300  $\mu M$   $CuCl_2$  ( $\bullet$ ), 200  $\mu M$   $CuCl_2$  ( $\Delta$ ), 100  $\mu M$   $CuCl_2$  ( $\blacktriangle$ ), 50  $\mu M$   $CuCl_2$  ( $\circ$ ), no  $CuCl_2$  ( $\blacksquare$ ), and 350  $\mu M$   $CuCl_2$  and 4.5  $\mu mol$   $C_2H_2$  ( $\blacktriangledown$ ).

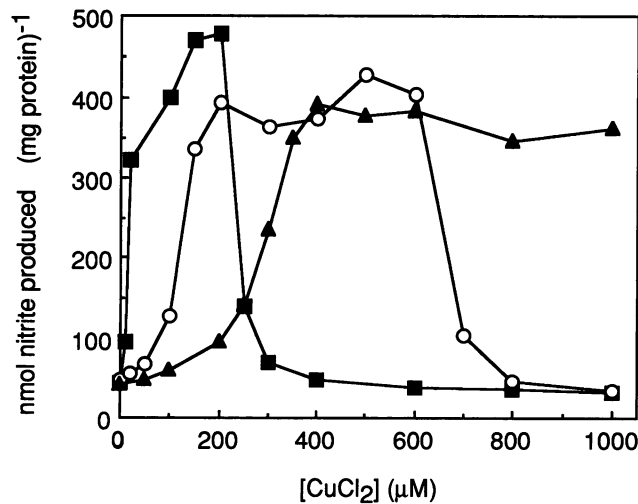


FIG. 3. Relationship between BSA concentration,  $CuCl_2$  concentration, and nitrite production from ammonia by *N. europaea* cell extracts. The nanomoles of nitrite produced per milligram of protein in a fixed-time (20-min) assay containing 1.8 mg of protein is plotted versus  $[CuCl_2]$ . The assay buffer (0.1 M  $KH_2PO_4$ , pH 7.8) contained 1 ( $\blacksquare$ ), 10 ( $\circ$ ), or 25 ( $\blacktriangle$ ) mg of BSA per ml.

AMO activity was observed between 20 and 200  $\mu\text{M}$  copper. Increasing the copper concentration from 200 to 250  $\mu\text{M}$  resulted in a sharp decrease in AMO activity. At higher BSA concentrations, the copper optimum was shifted to higher values, and maximal activity was observed over a wider range of copper concentrations (Fig. 3).

The composition of the lysis buffer used to prepare *N. europaea* extracts was varied to determine whether the ratio of copper-stimulated to copper-independent AMO activity would be affected. Extracts were prepared in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.8), MOPS (morpholinepropanesulfonic acid) buffer (pH 7.8) plus 10% glycerol, and 0.1 M potassium phosphate buffer which had been treated with Chelex-100 resin to remove trace contaminating metals. In each case, copper stimulated the AMO activity in the extracts. The specific activities of AMO in the presence of copper were similar for each extract. In individual preparations of extracts prepared in the standard lysis buffer (0.1 M  $\text{K}_2\text{HPO}_4$  [pH 7.8] containing 10% glycerol and 1 mM  $\text{MgCl}_2$ ), the maximal specific activity of AMO in the presence of copper varied at most by  $\pm 20\%$ . However, the residual activity in the absence of copper varied with the preparation, such that the increase in activity by the addition of copper varied from 5- to 15-fold.

**Effect of serum albumins and other stabilizing agents on AMO activity.** In order to determine whether stabilizing agents aside from copper are required to stimulate AMO in vitro, *N. europaea* extracts were prepared in the absence of BSA, and ammonia-dependent nitrite production was measured under various conditions. Figure 4 compares the amounts of nitrite produced from ammonia in a fixed-time assay in the presence of diverse stabilizing agents and over a range of copper concentrations. In agreement with the results of Suzuki and coworkers (46, 48), very little AMO activity was observed in the absence of a stabilizing agent. Under these conditions, there was a stimulatory effect of copper on AMO activity similar to the effect seen in the presence of BSA; however, the maximal activity, observed in the presence of 10  $\mu\text{M}$  copper, was only 5% of the maximal activity observed in the presence of BSA.

Other mammalian serum albumins (rat, pig, dog, and human albumins) were able to substitute for BSA in stimulating AMO activity (Fig. 4A). When these were added to the assay at 5-mg/ml concentrations, the relationship between copper concentration and stimulation of AMO activity was largely similar for the various serum albumins. There was, however, a marked difference in the stimulatory effect of copper concentrations under 100  $\mu\text{M}$ . There was a stimulation of activity at low concentrations of copper when the assays were performed in the presence of the pig and dog albumins. In contrast, when the assays were performed in the presence of the bovine, rat, and human albumins, the lowest concentrations of copper added did not stimulate AMO activity (Fig. 4A). As the concentration of serum albumin in the assay was increased, a higher threshold copper concentration was required to stimulate AMO activity (Fig. 3 and 4A). This result can be explained by differences in the copper-binding properties of the two types of albumins. Bovine, human, and rat serum albumins have a well-characterized high-affinity copper binding site at the amino terminus which is absent in the pig and dog albumins (2, 36). The lack of stimulation of AMO activity at low copper concentrations in the presence of the former albumins is likely due to the tight binding of copper at this high-affinity site, preventing copper from acting to stimulate AMO.

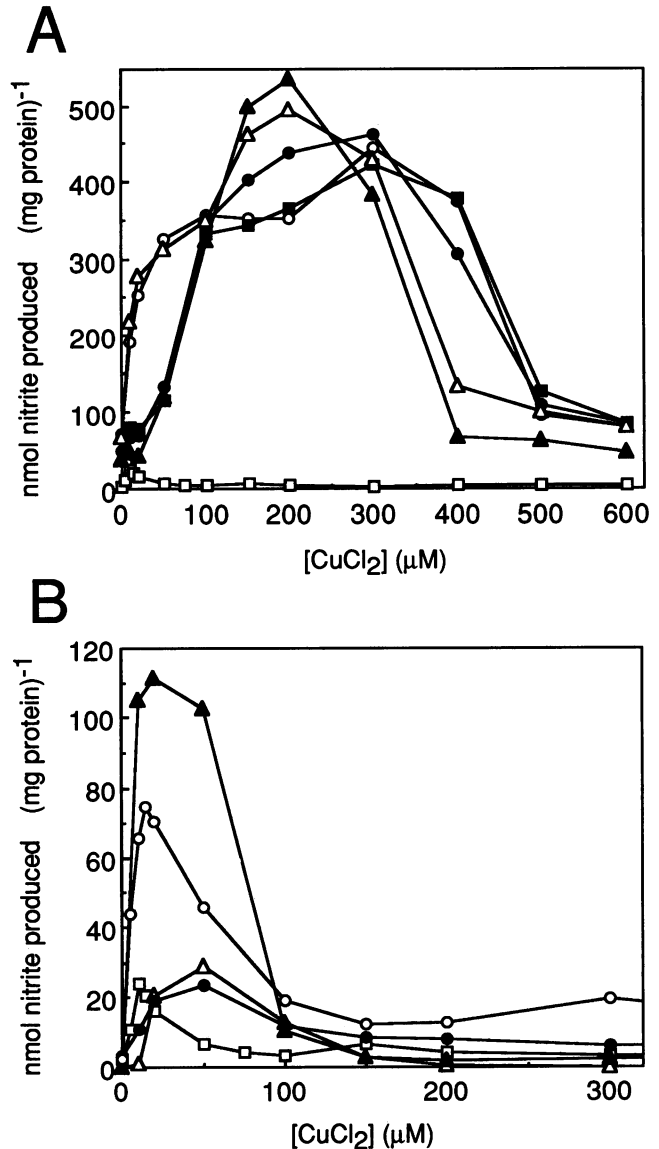


FIG. 4. Effect of albumins, gamma globulins, spermine, and  $\text{MgCl}_2$  on nitrite production from ammonia by *N. europaea* cell extracts in the presence of various concentrations of  $\text{CuCl}_2$ . The nanomoles of nitrite produced per milligram of protein in a fixed-time (20-min) assay containing 1.8 mg of protein is plotted versus  $[\text{CuCl}_2]$ . The assay buffer was 0.1 M  $\text{K}_2\text{HPO}_4$  (pH 7.8) with the indicated additions. Note the difference in the scale of the axes in plots A and B. (A) Effect of serum albumins on nitrite production from ammonia.  $\Delta$ , 5 mg of dog serum albumin per ml;  $\circ$ , 5 mg of pig serum albumin per ml;  $\blacktriangle$ , 5 mg of human serum albumin per ml;  $\bullet$ , 5 mg of rat serum albumin per ml;  $\blacksquare$ , 5 mg of BSA per ml;  $\square$ , no addition to the assay buffer. (B) Effects of spermine, ovalbumin, gamma globulins, and  $\text{MgCl}_2$  on nitrite production from ammonia.  $\blacktriangle$ , 2 mM spermine;  $\circ$ , 10 mM  $\text{MgCl}_2$ ;  $\Delta$ , 5 mg of ovalbumin per ml;  $\bullet$ , 5 mg of gamma globulins per ml;  $\square$ , no addition to the assay buffer.

Other potential stabilizers were examined for their effects on AMO activity in the absence and presence of copper. As shown in Fig. 4B, ovalbumin and gamma globulins were ineffective at stimulating AMO above the level obtained in buffer alone. In contrast, spermine or  $\text{Mg}^{2+}$ , which Suzuki

and coworkers reported could substitute for BSA in stimulating AMO in vitro (46, 48), was more effective at stimulating AMO (Fig. 4B). However, the maximal activity observed in the presence of spermine and  $Mg^{2+}$  was only 26 and 17%, respectively, of the activity in the presence of BSA. Imidazole (2 mM) was ineffective at stimulating AMO activity at copper concentrations of between 1 and 1,000  $\mu M$ , while histidine (2 mM) stimulated AMO to approximately the same maximal activity as  $Mg^{2+}$  (data not shown).

Several grades of BSA were compared for their ability to stimulate AMO activity. The use of fraction 5 BSAs from several sources and ultrapure (>99%) or fatty-acid ultrafree BSA resulted in identical stimulation of AMO activity over a range of copper concentrations (data not shown).

**Effect of other metal ions on AMO activity.** A variety of other metal ions were tested for their ability to substitute for copper in stimulating AMO activity. The addition of  $Zn^{2+}$ ,  $Co^{2+}$ ,  $Ni^{2+}$ ,  $Fe^{2+}$ ,  $Fe^{3+}$ ,  $Ca^{2+}$ ,  $Mg^{2+}$ ,  $Mn^{2+}$ ,  $Cr^{3+}$ , or  $Ag^{+}$  to assays at a range of concentrations between 1 and 5,000  $\mu M$  resulted in no stimulation of AMO activity (data not shown). Not only were these metals unable to stimulate AMO activity, but a number of them substantially inhibited copper-dependent AMO activity. Ammonia-dependent nitrite production was measured in assays containing 200  $\mu M$   $CuCl_2$ , 10 mg of BSA per ml, and various concentrations of divalent metal ions (100 to 400  $\mu M$ ). The presence of  $Co^{2+}$ ,  $Zn^{2+}$ ,  $Ni^{2+}$ , and  $Fe^{2+}$  resulted in the inhibition of AMO activity in a concentration-dependent manner, with 35 to 80% inhibition of ammonia-dependent nitrite production observed at metal ion concentrations of 400  $\mu M$  (data not shown). In contrast, the presence of  $Ca^{2+}$  and  $Mn^{2+}$  and the presence of additional  $Cu^{2+}$  at a final concentration of up to 600  $\mu M$  had no inhibitory effect on AMO activity.

**Requirements for ethane oxidation by *N. europaea* extracts.** The previous in vitro studies of AMO were performed before it was known that AMO will oxidize a wide range of nonphysiological hydrocarbon substrates (22, 23, 24, 26). The ability to oxidize alternate substrates could prove beneficial towards further characterizing AMO in vitro. The products of hydrocarbon oxidations by AMO are usually water soluble, highly stable, and not further oxidized by AMO or other *N. europaea* enzymes and can be readily quantified by gas chromatography (22). However, the oxidation of hydrocarbons by AMO requires the input of reducing equivalents which cannot be regenerated by the further oxidation of the product of the reaction as is the case with hydroxylamine generated from ammonia oxidation. To circumvent this problem, we have investigated the possibility of using indirect reductants to serve as electron donors for the oxidation of ethane to ethanol by AMO in cell extracts. Logical candidates for this role are (i) hydroxylamine, the physiological substrate for HAO and the source of reductant for AMO in vivo; (ii) hydrazine, which serves as an alternate substrate for HAO both in vivo and in vitro (34); and (iii) ammonia, which can stimulate hydrocarbon oxidations as a cosubstrate of AMO in whole cells (22). NADH, which has been shown to serve a priming role in initiating ammonia-dependent  $O_2$  uptake by *N. europaea* extracts (48, 49), was also tested for its ability to sustain ethane oxidation.

Table 1 shows the amount of ethanol formed from ethane by *N. europaea* extracts in fixed-time assays containing 10 mg of BSA per ml and 0 or 200  $\mu M$  copper and in the absence or presence of various reductants. No ethanol production was observed in the absence of an added reductant. Maximal ethanol production was obtained when hydrazine was used as the reductant. In the presence of 2 mM hydrazine, ethanol

TABLE 1. Effect of  $CuCl_2$  and reductant on the oxidation of ethane to ethanol by *N. europaea* extracts<sup>a</sup>

Reductant (mM)	Nmol of ethanol produced mg of protein <sup>-1</sup> 30 min <sup>-1</sup>	
	Without $CuCl_2$	With $CuCl_2$
None	ND <sup>b</sup>	ND
Ammonia (0.5)	ND	ND
Ammonia (2)	ND	ND
Hydrazine (0.5)	88	380
Hydrazine (2)	220	1,240
Hydroxylamine (0.5)	22	81
Hydroxylamine (2)	19	69
NADH (0.5)	61	180
NADH (2)	100	470

<sup>a</sup> Ethane oxidation assays were performed as described in Materials and Methods with and without  $CuCl_2$  (200  $\mu M$ ) present in the assays. No activity was observed when acetylene (10  $\mu mol$ ) was added to the assays.

<sup>b</sup> ND, no detectable activity (<4 nmol of ethanol produced mg of protein<sup>-1</sup> 30 min<sup>-1</sup>).

production was further increased sixfold by the addition of 200  $\mu M$  copper to the assay buffer. A similar stimulation of ethane oxidation by copper was observed in the assays with hydroxylamine or NADH present as the reductant. The maximal ethanol produced with hydroxylamine and NADH as reductants was 6.5 and 38%, respectively, of the maximal ethanol produced with hydrazine. Ammonia was ineffective at sustaining ethane oxidation in vitro. This is in contrast to the effect of ammonia on the oxidation of ethane by AMO in whole cells, where the addition of 1 to 10 mM ammonia can stimulate the rate of ethanol production by three- to ninefold over the rate of ethanol production supported by endogenous reductants alone (22).

Figure 5 shows the time course for ethane oxidation catalyzed by extracts in the presence of 2 mM hydrazine and

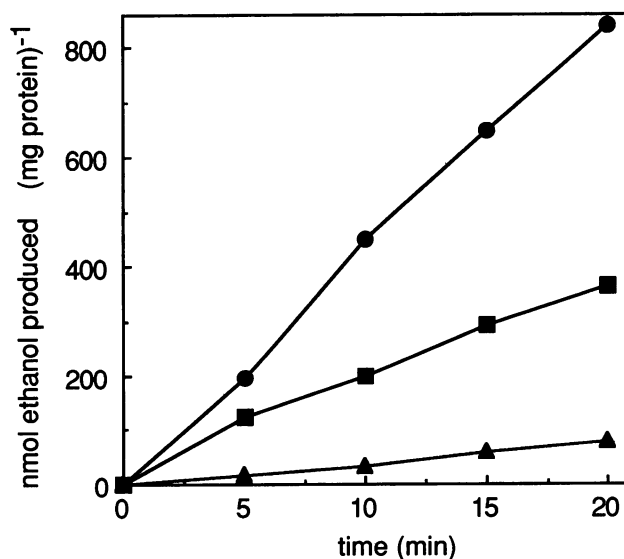


FIG. 5. Time course of ethane oxidation to ethanol by *N. europaea* cell extracts with hydrazine as a reductant. Ethane oxidation was measured as described in Materials and Methods. Assay vials contained extract (1.8 mg of protein), BSA (10 mg/ml),  $CuCl_2$  (200  $\mu M$ ), and ethane (130  $\mu mol$ ). ●, extract alone; ■, extract plus  $(NH_4)_2SO_4$  (0.5 mM); ▲, extract plus hydroxylamine (1 mM).

TABLE 2. Effect of  $\text{CuCl}_2$  on suicidal inactivation of AMO by acetylene in vitro<sup>a</sup>

Preincubation conditions		AMO activity (nmol of nitrite produced mg of protein <sup>-1</sup> )	
Acetylene	$\text{CuCl}_2$	Without $\text{CuCl}_2$	With $\text{CuCl}_2$
-	-	58	450
-	+	NA <sup>b</sup>	390
+	-	0.6	330
+	+	NA	0.3

<sup>a</sup> *N. europaea* extracts were preincubated with and without acetylene and copper as described in Materials and Methods. AMO activity, assayed in the absence and presence of 500  $\mu\text{M}$   $\text{CuCl}_2$ , is reported as nanomoles of nitrite produced in a 20-min assay.

<sup>b</sup> NA, not applicable.

200  $\mu\text{M}$  copper and the effects of ammonia and hydroxylamine on this activity. The rate of ethanol production was constant for at least 20 min under these assay conditions and corresponded to a specific activity of 43 nmol of ethanol produced per min per mg of protein. This value is comparable to the initial rate of nitrite production from ammonia (31 nmol of nitrite produced per min per mg of protein; Fig. 2). Hydroxylamine (1 mM) was a potent inhibitor of hydrazine-dependent ethane oxidation, a result which helps to explain why hydroxylamine is ineffective as a reductant for ethane oxidation despite being the physiological substrate for HAO (Table 1). Possibly, this inhibition results from a feedback interaction between hydroxylamine and AMO. The presence of ammonia (1 mM) also resulted in the inhibition of hydrazine-dependent ethane oxidation, presumably because ammonia and ethane are competitive substrates for AMO.

**Effect of copper on the inactivation of AMO by acetylene in vitro.** There are two explanations which could account for the in vitro stimulation of AMO activity by copper described in this paper. One possibility is that copper is required to activate a copper-deficient, catalytically inactive pool of AMO which is generated during cell lysis. Alternatively, copper could be simply stimulating the rate of activity of a homogeneous pool of catalytically competent AMO. The AMO-specific inhibitor acetylene was used as a probe for discriminating between these two possibilities. Acetylene is a suicide substrate and irreversible inactivator of AMO (20, 27). Apparently, AMO attempts to oxidize acetylene, generating a reactive intermediate which covalently modifies and inactivates AMO (20, 27). The time-dependent inactivation of AMO by [<sup>14</sup>C]acetylene results in a progressive and saturable incorporation of <sup>14</sup>C label into the 27-kDa polypeptide of AMO (20). Importantly, acetylene inactivates AMO only under conditions in which the enzyme is catalytically active (21). Accordingly, if a catalytically inactive pool of AMO is generated by cell lysis, then the majority of AMO should be protected against acetylene inactivation in the absence of exogenously added copper. Conversely, if copper is simply stimulating the rate of catalysis of active AMO, all of the AMO should be inactivated by acetylene regardless of whether copper is present.

To test the arguments discussed above, extracts were preincubated in assay vials with acetylene and 0.5 mM hydrazine in the absence or presence of 500  $\mu\text{M}$  copper. Acetylene was then removed from the assay vials, and the ammonia-dependent nitrite production activity of the extracts was determined in the absence and presence of copper. The results of this experiment are presented in Table 2. The AMO activity of control extracts which were not

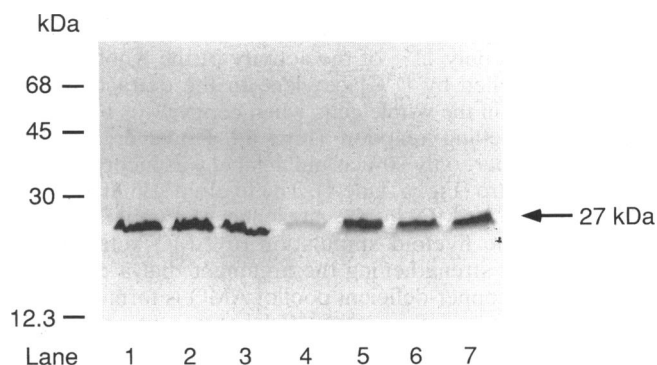


FIG. 6. Comparison of in vivo and in vitro labelling of the 27-kDa polypeptide of AMO by [<sup>14</sup>C]acetylene in the presence and absence of  $\text{CuCl}_2$ . Whole-cell suspensions and cell extracts of *N. europaea* were treated with [<sup>14</sup>C]acetylene as described in Materials and Methods. Each lane of the fluorogram represents 200  $\mu\text{g}$  of protein from the [<sup>14</sup>C]acetylene labelling. Lane 1, whole cells with no  $\text{CuCl}_2$  added; lane 2, whole cells plus 150  $\mu\text{M}$   $\text{CuCl}_2$ ; lane 3, whole cells plus 600  $\mu\text{M}$   $\text{CuCl}_2$ ; lane 4, extract with no  $\text{CuCl}_2$  added; lane 5, extract plus 50  $\mu\text{M}$   $\text{CuCl}_2$ ; lane 6, extract plus 150  $\mu\text{M}$   $\text{CuCl}_2$ ; lane 7, extract plus 600  $\mu\text{M}$   $\text{CuCl}_2$ .

exposed to acetylene or copper in the preincubation period was stimulated eightfold by the subsequent addition of copper to the assays. Essentially no AMO activity was present in extracts pretreated with acetylene without copper when the assay was performed in the absence of copper. However, when copper was added to the assay, AMO activity was stimulated to approximately the same degree as the extract which had not been pretreated with acetylene. AMO was completely inactivated in the extract which was pretreated with acetylene in the presence of copper. These results support the idea that, in the absence of exogenous copper, there is a pool of AMO which is catalytically inactive and, accordingly, is protected against acetylene inactivation.

**Comparison of in vivo and in vitro labelling of AMO by [<sup>14</sup>C]acetylene.** [<sup>14</sup>C]acetylene was used as a probe to quantitate the extent of modification of the 27-kDa component of AMO by acetylene in whole-cell suspensions versus cell extracts and to further investigate the requirement of copper for inactivating and labelling AMO with acetylene in vitro. A portion of freshly harvested cells was set aside for labelling with [<sup>14</sup>C]acetylene and measuring AMO activity, while the remainder of the suspension was lysed by French press. The AMO activity of the whole cells and corresponding cell extract was then measured in buffer containing 1 mg of BSA per ml and 0, 50, or 200  $\mu\text{M}$   $\text{CuCl}_2$ . Samples of the whole-cell suspension and extract were also labelled with [<sup>14</sup>C]acetylene in the presence of several copper concentrations in the same assay buffer used to measure AMO activity. The <sup>14</sup>C label incorporated into AMO in the whole cells and extracts was quantitated by densitometric scan of the fluorogram shown in Fig. 6.

The whole-cell suspension catalyzed the formation of 3,100 nmol of nitrite per mg of protein in a 10-min assay, regardless of whether copper was present in the assay buffer. Likewise, the 27-kDa polypeptide of AMO was labelled by [<sup>14</sup>C]acetylene to the same extent in the whole-cell suspension regardless of whether copper was present (Fig. 6, lanes 1 to 3). Under the same assay conditions, the extract catalyzed the formation of 68, 320, and 340 nmol of nitrite per mg of protein at copper concentrations of 0, 50, and 200

$\mu\text{M}$ , respectively. Although the maximal AMO activity of the extract was only 11% of the activity of the whole cells, AMO was labelled by [ $^{14}\text{C}$ ]acetylene in the extract to the same extent as in the whole cells when copper was included during the labelling reaction (Fig. 6). However, in the absence of copper, only 20% as much label was incorporated into AMO in vitro (Fig. 6, lane 4). The fivefold stimulation of  $^{14}\text{C}$  labelling of AMO in the presence of copper agrees exactly with the fivefold stimulation of AMO activity by copper, further strengthening the argument that a catalytically inactive, copper-deficient pool of AMO is formed upon cell lysis. The same amount of  $^{14}\text{C}$  label was incorporated into AMO in the extract when the labelling was performed in the presence of a concentration of copper high enough to significantly inhibit AMO activity (compare Fig. 3 and 6, lanes 5 to 7). The lower rates of AMO activity observed at high copper concentrations are thus not due to the irreversible inactivation or denaturation of AMO molecules by excess copper but evidently result from a slower rate of catalysis due to inhibition of AMO, HAO, or accessory electron carrier proteins.

The possibility exists that other metals can substitute for copper in activating AMO in vitro, albeit at a sufficiently reduced rate that no stimulation can be seen by conventional AMO assays. Since the labelling of AMO by acetylene is a much more sensitive assay for measuring enzyme turnover, we investigated whether other transition metals would facilitate the incorporation of  $^{14}\text{C}$  label from acetylene into AMO. The copper-independent (residual) AMO activity of a sample of extract was first inhibited with unlabelled acetylene. The acetylene was then removed, and the extract was incubated with [ $^{14}\text{C}$ ]acetylene and hydrazine with no added metal or with  $\text{Cu}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Zn}^{2+}$ , or  $\text{Co}^{2+}$  present. While the 27-kDa polypeptide of AMO was labelled to the expected degree in the presence of copper, no label was incorporated into AMO in the absence of copper or in the presence of the alternate transition metals (data not shown). This result further demonstrates the specificity of copper in activating AMO in vitro.

**Stability of *N. europaea* extracts.** The *N. europaea* extracts described in this paper could be stored on ice or at  $4^\circ\text{C}$  for 8 h with no loss of ammonia-oxidizing or hydrazine-dependent ethane-oxidizing activity. However, prolonged storage resulted in the time-dependent loss of AMO activity. Loss of activity was more rapid in extracts which were prepared in the absence of BSA or glycerol. Notably, the addition of copper to the extracts had no effect on AMO stability. The extracts were, however, stable for at least 6 months when frozen and stored in liquid  $\text{N}_2$ . The activity of the extracts was also stable in regard to freeze-thawing: after seven repeated cycles of freezing in liquid  $\text{N}_2$  followed by thawing at  $30^\circ\text{C}$ , no loss of AMO activity was observed.

## DISCUSSION

In this paper, we demonstrate that the activity of AMO in cell extracts of *N. europaea* is dramatically increased by the addition of copper. The protection of copper-dependent AMO activity against inactivation and covalent labelling by the mechanism-based inactivator acetylene in the absence of exogenous copper (Table 2 and Fig. 6) suggests that copper is required to activate a pool of copper-deficient AMO which is generated during cell lysis. Although copper has previously been proposed to play a catalytic role in the reaction(s) catalyzed by AMO (42, 55), these results provide the first

direct evidence for the involvement of copper as a component of AMO.

Since AMO has not yet been purified, the stoichiometry and role(s) of copper in the enzyme remain unknown. The specificity of copper in stimulating AMO activity and facilitating the incorporation of  $^{14}\text{C}$  label from [ $^{14}\text{C}$ ]acetylene into the 27-kDa polypeptide of AMO suggests, however, that the role of copper is catalytic. If copper were simply playing a structural or effector role, other metals such as nickel, iron, or zinc could be expected to substitute for copper, yielding a fully or partially active enzyme. To the contrary, these metals strongly inhibited copper-dependent AMO activity, suggesting that they may compete for binding to the putative copper site, thereby yielding an inactive enzyme.

It is unclear why copper is lost from AMO upon cell lysis, if that is indeed the reason why copper is required in the in vitro system. Suzuki and coworkers have described extracts prepared by French press which catalyzed ammonia-dependent  $\text{O}_2$  uptake at rates close to 10% of whole-cell rates (46, 48). However, for unknown reasons, they were only able to obtain active extracts from cells grown and harvested under very specific conditions. Other cell suspensions, while exhibiting whole-cell AMO activity, yielded inactive extracts after cell lysis (48). Possibly, the *N. europaea* growth conditions, the age of cells at the time of harvest, or some other undefined factor dictates whether copper is lost from AMO upon cell lysis, and to what extent. Although Suzuki and coworkers apparently did not investigate the effect of copper on their inactive extracts, the possibility exists that the extracts could have been activated by the addition of the proper concentration of copper.

It is important to note that although our cell extracts exhibit at best only 10 to 15% of the corresponding whole-cell activity, AMO in the extracts is still labelled to the same extent as in whole cells by [ $^{14}\text{C}$ ]acetylene when copper is present in the assay buffer (Fig. 6). This demonstrates that, in the presence of copper, all of the AMO in vitro is catalytically competent to undergo reduction, bind and reduce  $\text{O}_2$ , and oxidize substrate. The reduced rate of catalysis is thus not due to the denaturation or inactivation of the majority of AMO upon cell lysis. Rather, the decreased rate of catalysis in vitro is probably due to a loss of coupling efficiency among AMO, HAO, and accessory proteins.

In addition to catalyzing the oxidation of the physiological AMO substrate ammonia, the extracts described in this paper also catalyzed the oxidation of the alternate AMO substrate ethane to ethanol with hydrazine or NADH as a reductant (Table 1 and Fig. 5). Although hydrazine and NADH are apparently not direct reductants for AMO but require the mediation of other enzymes (i.e., HAO, NADH dehydrogenase, and electron carrier proteins), this assay presents several advantages over measuring the ammonia-dependent production of nitrite which could facilitate the further fractionation and purification of AMO. Whereas ethanol can be directly quantified, nitrite production requires that hydroxylamine formed by AMO be further oxidized by HAO, both to provide a detectable product and to provide reductant for AMO. This introduces an additional degree of complexity to the already complex system. The loss of AMO activity which occurs during the storage of extracts is likely due to the loss of coupling integrity among AMO, HAO, and the as-yet-unidentified accessory protein(s) involved in electron transfer between the two enzymes. The discovery of an electron donor which can directly donate electrons to AMO would greatly facilitate the purification and characterization of the enzyme.



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