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Roles of Bovine Serum Albumin and Copper in the Assay and Stability of Ammonia Monooxygenase Activity In Vitro

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We investigated the effects of bovine serum albumin (BSA) on both the assay and the stability of ammonia-oxidizing activity in cell extracts of *Nitrosomonas europaea*. Ammonia-dependent O_2 uptake activity of freshly prepared extracts did not require BSA. However, a dependence on BSA developed in extracts within a short time. The role of BSA in the assay of ammonia-oxidizing activity apparently is to absorb endogenous free fatty acids which are present in the extracts, because (i) only proteins which bind fatty acids, e.g., BSA or β-lactoglobulin, supported ammonia-oxidizing activity; (ii) exogenous palmitoleic acid completely inhibited ammonia-dependent O_2 uptake activity; (iii) the inhibition caused by palmitoleic acid was reversed only by proteins which bind fatty acids; and (iv) the concentration of endogenous free palmitoleic acid increased during aging of cell extracts. Additionally, the presence of BSA (10 mg/ml) or $CuCl_2$ (500 μM) stabilized ammonia-dependent O_2 uptake activity for 2 to 3 days at 4°C. The stabilizing effect of BSA or $CuCl_2$ was apparently due to an inhibition of lipolysis, because both additives inhibited the increase in concentrations of free palmitoleic acid in aging extracts. Other additives which are known to modify lipase activity were also found to stabilize ammonia-oxidizing activity. These additives included $HgCl_2$, lecithin, and phenylmethylsulfonyl fluoride.

Nitrosomonas europaea is a lithoautotrophic organism which obtains all of its energy for growth from the oxidation of ammonia to nitrite. Ammonia is initially oxidized to hydroxylamine (NH_2OH) by a membrane-bound monooxygenase enzyme, ammonia monooxygenase (AMO). The electrons required to support both AMO activity and ATP generation are provided by the further oxidation of hydroxylamine to nitrite by the complex multiheme enzyme hydroxylamine oxidoreductase (HAO) (25). HAO has been purified and studied in considerable detail, at both the biochemical and the genetic levels. In contrast, less is known about AMO, and it has not yet been purified with activity. There are two features of this enzyme system which have hindered progress in this area. First, the requirements and conditions for a suitable in vitro assay remain poorly defined. Second, ammonia-oxidizing activity in cell extracts is very unstable (5, 23). Purification and characterization of AMO will require that these issues are investigated and resolved.

In vitro activity of AMO in cell extracts was previously demonstrated to be highly dependent on assay conditions (5). Early studies by Suzuki's group demonstrated that in vitro assays of ammonia-dependent O_2 uptake activity required a variety of additives, including Mg^{2+}, spermine, or bovine serum albumin (BSA) (23). The most consistently effective additive was BSA (5). Recently, it was demonstrated that copper ions specifically activate AMO in cell extracts, although BSA was still required for enzyme activity (5). A high concentration of copper ions (250 μM) was required for maximal ammonia-oxidizing activity because of the copper-binding capability of BSA. However, it was also shown that other serum albumins were capable of replacing BSA and that ovalbumin, a nonserum albumin, was ineffective. Importantly, the effective serum albumins included proteins both with and without high-affinity copper-binding sites (5). These results suggest that the mechanism of action of serum albumins is independent of the copper-binding abilities of these proteins. However, the role of serum albumins in the assay was not established.

In contrast to the progress made with in vitro assays of ammonia-oxidizing activity, much less attention has been given to stabilization of activity in cell extracts. In general, ammonia-oxidizing activity is extremely labile and fresh extracts lose 50% of their activity within 2 h when stored at 4°C (22). Enzyme activity is also lost slowly over 1 to 2 days in extracts stored at −20°C. However, full activity can be retained for greater than 3 months when extracts are rapidly frozen and stored at −196°C (5). Although low-temperature storage allows for stockpiling of active extracts, stabilization strategies which are effective at higher temperatures will be needed before AMO can be purified. Because determination of stability requires measurement of activity, it is important to consider that all current in vitro assays of ammonia-oxidizing activity require the coupling of electron transfer from HAO to AMO. Instability of ammonia-oxidizing activity may result from the loss of catalytic competence of the individual enzymes. Alternatively, instability may result from a loss of coupling of electron transfer between HAO and AMO while the enzymes remain catalytically competent.

In the present report, we describe experiments which elucidate the role of BSA in the in vitro assay of ammonia-oxidizing activity. We have demonstrated that in vitro ammonia-oxidizing activity can be assayed by using freshly prepared cell extracts without BSA. However, the extracts subsequently developed a requirement for BSA. This acquired BSA dependence apparently arises from the need to absorb and remove inhibitory free fatty acids which accumulated as a result of lipolysis in the cell extracts. We also demonstrate for the first time that BSA and copper ions can independently stabilize ammonia-oxidizing activity for up to 2 or 3 days at 4°C. Our results suggest that the stabilizing effects involve the inhibition of the activity of an endogenous phospholipase. This understanding of the mechanism of stabilization of ammonia-oxidizing activity by BSA or copper ions has also led us to develop alternative stabilizing treatments which should be useful in future attempts at purification of active AMO. An irony of our results

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is that while copper ions and BSA serve different roles in the in vitro assays of ammonia-oxidizing activity, they play similar roles in the stabilization of ammonia-oxidizing activity.

MATERIALS AND METHODS

Materials. BSA and pig serum albumin (fraction V; 98%), ovalbumin (99%), β-lactoglobulin, myoglobin (98%), gamma globulin (99%), lactalbumin, L-α-egg yolk lecithin, spermine, phenylmethylsulfonyl fluoride (PMSF), and leupeptin were obtained from Sigma and were used without further purification. Ultrapure $CuCl_2$ (99.99%), $HgCl_2$ (99.5$^+$%), triethylamine, and 2,4′-dibromoacetophenone were obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. C_2H_2 was generated from calcium carbide (~80%; Aldrich) in a gas-generating bottle as previously described (11).

Growth of bacteria. *N. europaea* ATCC 19178 cultures were grown in a 100-liter carboy at 30°C with mixing resulting from forced aeration. Growth medium consisted of 25 mM $(NH_4)_2SO_4$, 3 mM KH_2PO_4, 750 μM $MgSO_4$, 200 μM $CaCl_2$, 10 μM $FeCl_3$, 16 μM EDTA, and 1 μM $CuSO_4$. The medium was buffered with phosphate (pH 8.0) consisting of KH_2PO_4 (18 mM) and NaH_2PO_4 (1.6 mM). The pH of the culture was maintained at 7.8 by the addition of a sterile solution of K_2CO_3 (20%, wt/vol) with a pH stat (Cole Palmer Instrument Co., Chicago, Ill.). When the nitrite concentration of the culture was 35 to 40 mM, the cells were harvested by filtration with a Pellicon Ultrafiltration Tangential Flow System (Millipore, Bedford, Mass.). The concentration cells were collected by centrifugation, washed twice with buffer (0.1 M KH_2PO_4 [pH 7.8], 1 mM $MgCl_2$), and resuspended in ~150 ml of the same buffer. The resuspended cells were rapidly frozen in liquid N_2 and stored at −80°C.

Preparation and storage of cell extracts. Frozen cells were thawed at 30°C and diluted twofold with buffer (0.1 M KH_2PO_4, pH 7.0). Typically, frozen cells (20 to 30 ml; 10 to 15 mg of protein per ml) were disrupted by one passage through a French pressure cell operated at 7,500 lb/in^2. Unbroken cells were removed by centrifugation (7,000 × *g*, 10 min). The extract was used immediately for experiments. For stability experiments, an extract was divided into several portions and stored with or without the various stabilizing agents either at 4°C with no agitation or at 30°C with vigorous agitation in a shaking water bath (170 cycles per min).

O_2 uptake measurements. Ammonia-dependent O_2 uptake was measured with a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) mounted in a water-jacketed reaction vessel (1.7- or 1.8-ml volume) maintained at 30°C. Except where indicated, BSA (10 mg/ml), $CuCl_2$ (230 μM), and $(NH_4)_2SO_4$ (2.8 mM) were routinely added to the buffer (0.1 M KH_2PO_4, pH 8.0) in the O_2 electrode chamber, and then the extract (200 μl; 2.2 to 2.7 mg of protein) was added. In order to measure BSA-independent, ammonia-dependent O_2 uptake activity, $CuCl_2$ (12 to 15 μM) and $(NH_4)_2SO_4$ (2.8 mM) were added to the buffer (0.1 M KH_2PO_4, pH 8.0) in the O_2 electrode chamber immediately before the extract was added. The HAO activity of the extract (200 μl; 2.2 to 2.7 mg of protein) was measured as hydrazine (1 mM)-dependent O_2 uptake after inactivation of AMO by acetylene in the presence of $(NH_4)_2SO_4$ (2.8 mM), $CuCl_2$ (14 μM), and C_2H_2 (0.23 mM).

Extraction, derivatization, and quantitation of free fatty acids from extracts of *N. europaea*. Chloroform was chosen as the extraction solvent because it is both a poor solvent for complex lipids and a good solvent for simple lipids such as free fatty acids (3). An authentic fatty acid stock (0.5 to 3 mM) was prepared daily and diluted as needed in the same buffer used for the extract. Free fatty acids were extracted from an aliquot (500 μl) of either the extract or the stock solution of authentic fatty acid with $CHCl_3$ (300 μl) in the following manner. The samples were vigorously vortexed (10 s) and then subjected to microcentrifugation (14,000 × *g*, 2 min). The upper aqueous and interfacial layers were suctioned off, and an aliquot (100 or 50 μl) of the $CHCl_3$ layer was placed in a screw-cap vial. The $CHCl_3$ was removed in vacuo, affording a residue containing fatty acids. The free fatty acids were derivatized with 2,4′-dibromoacetophenone (4). Aliquots (25 μl each) of both triethylamine (10 mg/ml in acetone) and 2,4′-dibromoacetophenone (10 mg/ml in acetone) were added to the screw-cap vials. The samples were reacted in a heating block (100°C, 30 min) and then quenched with glacial acetic acid (3.5 μl) and heated further (100°C, 15 min). The acetone was removed in vacuo, and the *p*-bromophenacyl derivatives of the fatty acids were dissolved in acetonitrile. Because this derivatization procedure requires the fatty acid to be in the unesterified state, it is assumed that only free fatty acids were quantified and that abiotic hydrolysis of phospholipids did not occur. Not all of the free fatty acids present in the extract or in the fatty acid stock were extracted into the $CHCl_3$ layer. However, we assumed that a constant fraction of free fatty acids was extracted, as judged by the linearity of our standard curves.

The *p*-bromophenacyl-derivatized fatty acids were subjected to high-pressure liquid chromatography (HPLC). The samples were separated on an octadecyl-silica column (250 by 4.4 mm, 5-μm particle size; Vydac, Hesperia, Calif.) and eluted isocratically with 100% acetonitrile at a flow rate of 1 ml/min. The *p*-bromophenacyl-derivatized palmitoleic acid was detected at a wavelength of 254 nm. Palmitoleic acid in the extract was identified by comparison of the retention time with that of authentic palmitoleic acid which had been similarly derivatized with 2,4′-dibromoacetophenone. Palmitoleic acid was quantified by peak height on a chart recorder. To account for the effect of $CuCl_2$ (500 μM), BSA (10 mg/ml), or $HgCl_2$ (100 μM) on the extraction, derivatization, and quantitation processes, separate standard curves for authentic palmitoleic acid were prepared for each of these amended conditions.

Protein determination. The protein contents of cell suspensions or extracts were determined by using the biuret assay (9) after the cells or extracts were solubilized in 3 M NaOH (45 min, 75°C). BSA was used as the protein standard.

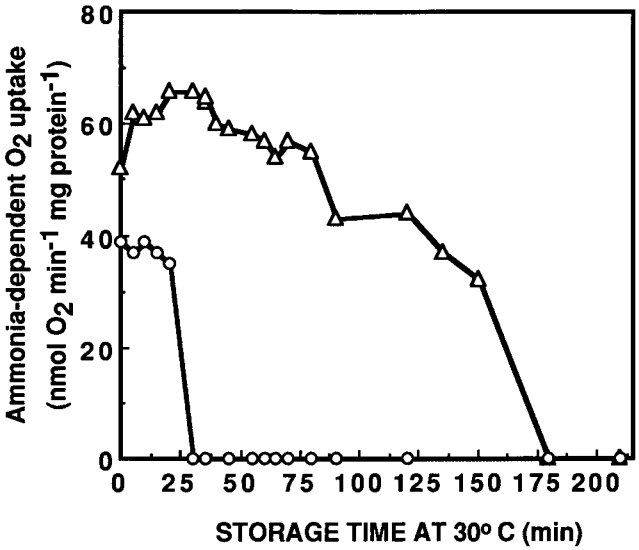

FIG. 1. Ammonia-dependent O_2 uptake activity measured in the presence and absence of BSA. The activity of an extract (200 μl; 2.2 mg of protein) was assayed at the indicated times as described in Materials and Methods in the presence of either $CuCl_2$ (14 μM) (○) or BSA (10 mg/ml) and $CuCl_2$ (230 μM) (△). Rates of ammonia-dependent O_2 uptake were recorded if they initiated within 5 min of addition of the extract to the O_2 electrode chamber.

RESULTS

The role of BSA in the assay of ammonia-dependent O_2 uptake activity. It was previously demonstrated that maximal ammonia-oxidizing activity in previously frozen cell extracts requires the presence of both BSA and copper ions (5). In the present study, we have found that ammonia-oxidizing activity can be assayed also in the absence of BSA. This BSA-independent activity is observed only in freshly prepared extracts with low protein concentrations (<15 mg of protein per ml). In general, these BSA-independent activities range from 60 to 90% of the activities of the same extracts assayed with BSA. Consistent with our previous observations, copper ions were still necessary for ammonia-oxidizing activity to be observed (5). However, in assays without the competitive binding of copper ions by BSA, maximal ammonia-oxidizing activity was observed with low concentrations of copper ions (13 to 18 μM).

We have also observed that BSA-independent ammonia-oxidizing activity is transient and that over time extracts develop a requirement for BSA (Fig. 1). An aliquot of an extract was incubated at 30°C, and samples were assayed in either the absence or the presence of BSA (10 mg/ml). Ammonia-dependent O_2 uptake activity was lost within 30 min in the assay without BSA, but activity was observed for up to 2 h in the assay with BSA (Fig. 1). Once BSA-independent activity was lost, BSA (0.5 to 10 mg/ml) could reinstate activity. As the extract aged, greater BSA concentrations were required to reinstate activity. These results suggest that the composition of the extract undergoes at least two changes during storage. One change could be mitigated by adding BSA to the assay medium. Another change involved the loss of ammonia-oxidizing

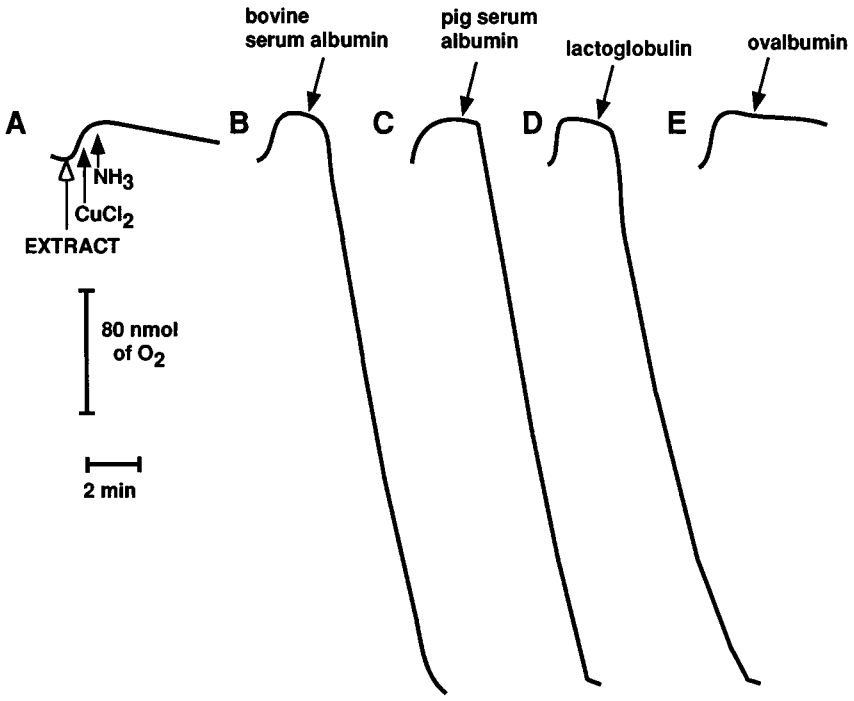

FIG. 2. Loss of ammonia-dependent O_2 uptake activity as measured in the absence of BSA was reversed by proteins which bind fatty acids. Ammonia-dependent O_2 uptake was measured as described in Materials and Methods except that BSA was not added prior to the addition of extracts. Extracts (200 μl; 2.2 mg of protein), $CuCl_2$ (13 μM), and $(NH_4)_2SO_4$ (2.8 mM) were added to each reaction mixture as shown in trace A, with no additional protein. At the indicated times, additional proteins were added (traces B through E), all at 2 mg/ml. Rates of ammonia-dependent O_2 uptake activities ranged between 38 and 42 nmol of O_2 min^{-1} mg of $protein^{-1}$.

activity which occurred at 2 h. This loss was not recoverable by the addition of more BSA (20 mg/ml).

To further investigate the role of BSA in the assay of ammonia-oxidizing activity, we considered whether other proteins could substitute for BSA. In mammalian systems, a major role for serum albumins other than metal binding is fatty acid binding (8, 21). Three fatty acid-binding proteins, BSA, pig serum albumin, and β-lactoglobulin (2 mg/ml), reinstated ammonia-dependent O_2 uptake activity in aged extracts (Fig. 2A to D). β-Lactoglobulin provides the first example of a protein other than serum albumins replacing BSA in the assay of ammonia-oxidizing activity. In contrast, neither ovalbumin (2 mg/ml; Fig. 2E) nor a higher concentration (8 mg/ml) of other proteins which do not bind fatty acids, such as myoglobin or lactalbumin (7, 17), was effective at reinstating ammonia-dependent O_2 uptake (not shown). Our results suggested that the ability to bind fatty acids is an important aspect of the role of BSA in the assay of ammonia-oxidizing activity.

More-direct evidence that the role of BSA in the assay involved fatty acid binding was established by considering the effects of free fatty acids on the assay of ammonia-dependent O_2 uptake. Palmitoleic acid is the predominant fatty acid in *N. europaea* (1). The addition of palmitoleic acid (240 μM) to the assay medium completely inhibited BSA-independent, ammonia-dependent O_2 uptake (Fig. 3A and B). However, the inhibition of ammonia-dependent O_2 uptake by palmitoleic acid was reversed by 100% by either BSA or pig serum albumin (5 mg/ml) and reversed by 70% by β-lactoglobulin (5 mg/ml; Fig. 3C to E). In contrast, the inhibition was not reversed by ovalbumin (5 mg/ml; Fig. 3F), myoglobin, gamma globulin, or lactalbumin (5 mg/ml; not shown).

In addition to palmitoleic acid, both palmitic and oleic acid also completely and reversibly inhibited ammonia-dependent O_2 uptake (not shown). The concentration of fatty acid required for complete inhibition of ammonia-dependent O_2 uptake varied from 20 to 400 μM among different extracts. This variability may reflect differences in the age and preparation of extracts. Neither palmitoleic acid (400 μM) nor BSA (10 mg/ml) affected the rate of hydrazine-dependent O_2 uptake (not shown), which suggested that the inhibition by fatty acids was specific for a component(s) associated with AMO activity.

Stabilization of ammonia-dependent O_2 uptake activity by either BSA or $CuCl_2$. A high concentration of either BSA (10 mg/ml) or $CuCl_2$ (500 μM) stabilized ammonia-dependent O_2 uptake activity for up to 2 days at 4°C (Table 1). In contrast, extracts stored without either BSA or $CuCl_2$ lost all ammonia-dependent O_2 uptake activity within 8 h (Table 1). Partial stabilization was achieved by using a lower concentration of either BSA or $CuCl_2$, but storage with both BSA and $CuCl_2$ did not have an additive effect on stability (Table 1). However, the lack of an additive effect may reflect the binding of Cu^{2+} ions by BSA. Pig serum albumin (10 mg/ml) also stabilized ammonia-dependent O_2 uptake activity (not shown), but ovalbumin (10 mg/ml) did not (Table 1). Our results with BSA and $CuCl_2$ provide both the longest recorded stabilization of ammonia-oxidizing activity to date and the first report of an effect of $CuCl_2$ on stability.

Free palmitoleic acid was detected in our extracts (Table 2). Consistently, lower concentrations of free palmitoleic acid were observed in extracts that had been stored with either BSA or $CuCl_2$ than in extracts stored without BSA or $CuCl_2$. The concentrations of free palmitoleic acid in the extracts were determined by HPLC for each of the extracts stored with or without either BSA or $CuCl_2$ at the start and at the end of the

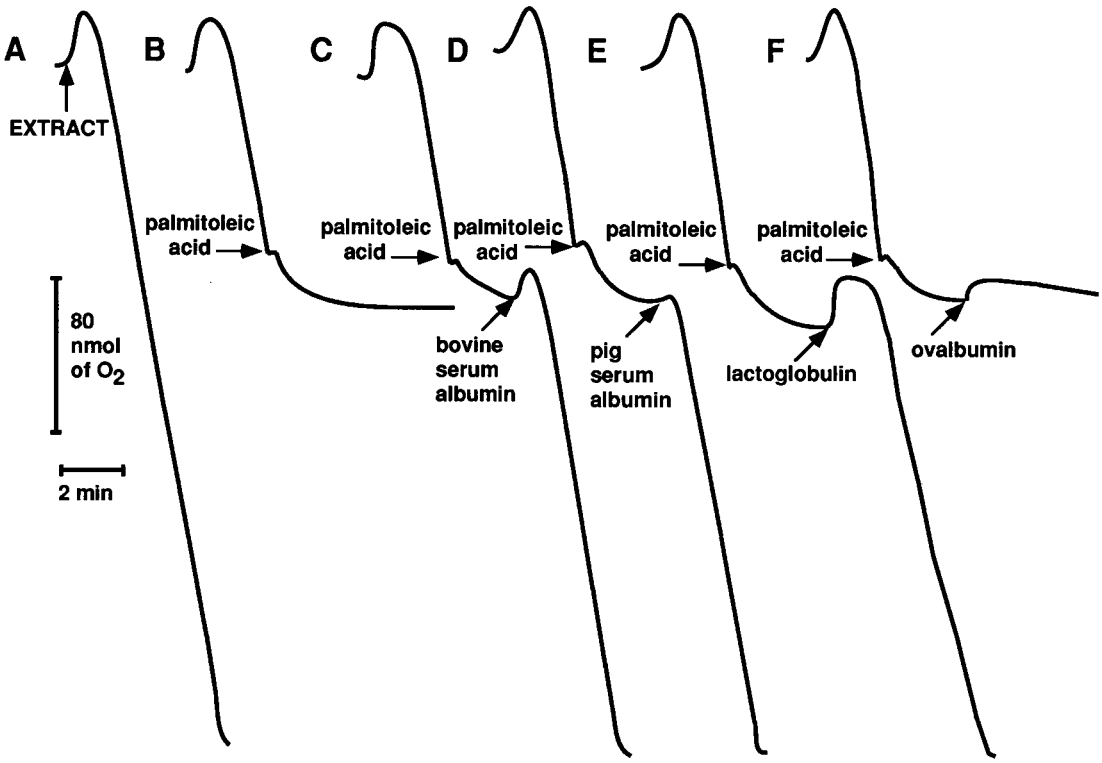

FIG. 3. Inhibition of ammonia-dependent O_2 uptake by exogenously added palmitoleic acid and relief of the inhibition by either serum albumins or β-lactoglobulin. Uptake was measured as described in Materials and Methods except that BSA was not added prior to the addition of extracts. The O_2 electrode chamber contained cell extracts (200 μl; 2.2 mg of protein), $CuCl_2$ (13 μM), and $(NH_4)_2SO_4$ (2.8 mM). Ammonia-dependent O_2 uptake was measured in the absence of BSA (trace A). Palmitoleic acid (240 μM) was added to traces B to F at the indicated times. Proteins (5 mg/ml) were added as indicated (traces C through F).

1.75-h incubation at 30°C (Table 2). The free palmitoleic acid concentrations were 2.3 to 3 times higher in the unamended extract than in the amended extracts (Table 2). Additionally, the ammonia-dependent O_2 uptake activity of each extract was measured at the start and at the end of the 1.75-h incubation. The extracts stored with either BSA or $CuCl_2$ retained nearly 100% of their original ammonia-dependent O_2 uptake activity, while the unamended extract had lost all of its activity after 1.75 h (Table 2). The inhibition of the accumulation of free palmitoleic acid by BSA or $CuCl_2$ was also observed in extracts stored at 4°C, indicating that this process was not associated solely with storage at 30°C.

Because these results suggested that the stabilizing effect of $CuCl_2$ was not due to the catalytic requirement of AMO for $CuCl_2$ but instead to the inhibition of lipolysis (Table 2), we tested various other metals as stabilizing agents. Of the metals tested, only $HgCl_2$ (100 μM) stabilized the ammonia-dependent O_2 uptake activity (Table 2). Additionally, $HgCl_2$ (100 μM) inhibited lipolysis (Table 2). Because PMSF is reported to inhibit lipases (16), it was tested as a potential stabilizing agent.

TABLE 1. Stabilization of ammonia-dependent O_2 uptake activity by either $CuCl_2$ or BSA[a]

Agent(s) added (concn)	Ammonia-dependent O_2 uptake (nmol of O_2 min^{-1} mg of $protein^{-1}$) after the indicated storage time (h)[b]										
	0	4	8	12	21	28	36	44	50	62	72
None	65	75	0	0	0	0	0	0	0	0	0
$CuCl_2$											
50 μM	59	65	52	41	0	0	0	0	0	0	0
500 μM	73	75	67	58	57	62	60	54	54	0	0
BSA											
1 mg/ml	63	73	56	52	0	0	0	0	0	0	0
10 mg/ml	63	69	62	62	58	50	49	43	41	34	0
$CuCl_2$ (500 μM) + BSA (10 mg/ml)	71	78	65	71	62	67	65	60	58	0	0
Ovalbumin (10 mg/ml)	59	65	49	0	0	0	0	0	0	0	0

[a] Samples of an extract were stored at 4°C in the absence or presence of potential stabilizing agents. At the indicated times, an aliquot of extract (200 μl; 2.2 mg of protein) was assayed for ammonia-dependent O_2 uptake in the presence of BSA (10 mg/ml) and $CuCl_2$ (230 μM) as described in Materials and Methods. For a given sample, the rates are reproducible to within 5%. Ammonia-independent O_2 uptake rates ranged from 2 to 4 nmol of O_2 min^{-1} mg of $protein^{-1}$.

[b] The onset of activity for all storage conditions was characterized by the development of a lag which increased as the extracts aged. If activity was not initiated within 5 min following addition of the extract to the O_2 electrode chamber, then a value of 0 was recorded.

TABLE 2. Stabilization of ammonia-dependent O_2 uptake activity and the inhibition of lipolysis by $CuCl_2$, BSA, or $HgCl_2$

Storage time (h)	Ammonia-dependent O_2 uptake (nmol of O_2 min^{-1} mg of $protein^{-1}$) stored with the following addition[a]:				Palmitoleic acid concn (mM) stored with the following addition[b]:					
	None	$CuCl_2$ (500 μM)	BSA (10 mg/ml)	$HgCl_2$ (100 μM)	None	$CuCl_2$ (500 μM)	None	BSA (10 mg/ml)	None	$HgCl_2$ (100 μM)
0	59	63	53	57	0.3	0.2	0.4	0.3	0.2	0.2
1.75	0	58	63	57	1.8	0.5	2.8	0.9	1.6	0.5
Ratio[c]					6	2.5	7	3	8	2.5

[a] Ammonia-dependent O_2 uptake rates were determined as described in Materials and Methods. The extracts were stored with or without the various additions at 30°C with vigorous shaking.

[b] Averages for duplicate samples are shown. The deviation from the mean fell between 0 and 16% of the mean, with an average of 5%. To account for the effects of additions on experimental procedures, $CuCl_2$, BSA, or $HgCl_2$ was added to extracts stored with no additions prior to $CHCl_3$ extraction, and separate standard curves were prepared for each of these amended conditions. The concentration of palmitoleic acid varied among the samples stored with no additions as a result of using separate standard curves. The palmitoleic acid concentrations reported for extracts with no additions are for the purpose of comparison with the respective concentration of the amended samples.

[c] Ratio of the concentration of palmitoleic acid at 1.75 h to that at 0 h.

PMSF (0.5 mM) stabilized ammonia-dependent O_2 uptake activity as effectively as either $CuCl_2$ (500 μM) or BSA (10 mg/ml) (Table 3). Because PMSF is also a well-known protease inhibitor, another protease inhibitor, leupeptin, was tested for stabilization. However, leupeptin did not stabilize ammonia-dependent O_2 uptake activity (not shown). Lecithin was reported to stabilize mitochondrial activities by protecting membranes from lipolysis by providing an alternative substrate for phospholipases (18). Ammonia-dependent O_2 uptake activity was stabilized by egg yolk lecithin (10 mg/ml); however, lecithin was less effective than BSA, $CuCl_2$, or PMSF (Table 3). Stabilization of ammonia-oxidizing activity by lecithin may result from protection of *N. europaea* membranes rather than protection from the products of phospholipid hydrolysis. Consistent with this mechanism, incubation of an extract with exogenous palmitoleic acid (1.4 mM) did not induce a loss of ammonia-oxidizing activity.

DISCUSSION

Role of BSA in the in vitro assay of ammonia-oxidizing activity. Our experiments have demonstrated four important points with regard to the in vitro assay of ammonia-oxidizing activity. First, BSA is not essential for substantial rates of ammonia-dependent O_2 uptake in freshly prepared extracts. However, during aging of extracts, BSA becomes a necessary additive for ammonia-oxidizing activity to be observed (Fig. 1). Second, only proteins capable of binding fatty acids support ammonia-dependent O_2 uptake (Fig. 2). Third, exogenous palmitoleic acid completely and specifically inhibited ammonia-dependent O_2 uptake activity, and the inhibition was reversed only by proteins capable of binding fatty acids (Fig. 3). Fourth, an increase in the concentration of endogenous palmitoleic acid occurred within a time frame compatible with the onset of activity becoming dependent on BSA (Fig. 1 and Table 2). On the basis of these results, we propose that the role of BSA in the in vitro assay of ammonia-oxidizing activity is to bind inhibitory free fatty acids and that endogenous lipolysis is responsible for the release of free fatty acids during aging of the extract. In addition, we conclude that the requirement for proteins which bind fatty acids is realized once lipolysis has generated sufficient fatty acid concentrations to be inhibitory when the fatty acids are subsequently transferred, along with extracts, into enzyme assays.

Although we demonstrated reversible inhibition of ammonia-oxidizing activity by exogenous fatty acids, the exact mode of inhibition by fatty acids was not determined. A variety of inhibitory mechanisms are possible. For example, fatty acids alter membrane fluidity and therefore could alter protein associations and are also reported to uncouple energy-dependent processes (19, 20). Additionally, fatty acids may bind at the active site of AMO, since AMO is known to oxidize straight-chain hydrocarbons such as *n*-octane (12) and is inactivated by acetylenic fatty acids such as undecynoic acid (10a).

Stabilization of ammonia-oxidizing activity. Results presented in this study have demonstrated for the first time that either BSA or copper ions stabilize ammonia-oxidizing activity in cell extracts of *N. europaea*. Under appropriate conditions,

TABLE 3. Stabilization of ammonia-dependent O_2 uptake activity by either PMSF or lecithin[a]

Agent added (concn)	Ammonia-dependent O_2 uptake (nmol of O_2 min^{-1} mg of $protein^{-1}$) after the indicated storage time (h)[b]										
	0	4	8	12	24	32	37	48	58	72	96
None	74	73	62	0	0	0	0	0	0	0	0
$CuCl_2$ (500 μM)	72	82	82	68	78	60	72	70	68	64	0
BSA (10 mg/ml)	76	76	82	78	76	78	72	68	64	56	0
Lecithin (10 mg/ml)	56	70	62	50	50	41	37	0	0	0	0
PMSF (0.5 mM)	70	72	74	74	72	70	68	54	62	56	0
Dimethyl sulfoxide (0.14 M)[c]	66	68	52	0	0	0	0	0	0	0	0

[a] Samples of an extract were stored at 4°C in the absence or presence of various compounds. At the indicated times, an aliquot of extract (200 μl; 2.2 mg of protein) was removed and assayed for ammonia-dependent O_2 uptake in the presence of BSA (10 mg/ml) and $CuCl_2$ (230 μM) as described in Materials and Methods. For a given sample, the rates are reproducible to within 5%. Ammonia-independent O_2 uptake ranged between 2 and 4 nmol of O_2 min^{-1} mg of $protein^{-1}$.

[b] The onset of activity for all storage conditions was characterized by the development of a lag which increased as the extracts aged. If activity was not initiated within 5 min following addition of the extract to the O_2 electrode chamber, then a value of 0 was recorded.

[c] Solvent for PMSF stock solution.

enzyme activity can be maintained for 2 to 3 days at 4°C (Tables 1 and 3). The present increase in stability represents an approximately 20-fold improvement in stability over that reported by Suzuki et al. (23). This increased level of stabilization may provide sufficient time for future attempts at purification of AMO.

In addition to the stabilizing effect of BSA on ammonia-oxidizing activity, the presence of BSA during storage inhibited an increase in the concentration of free palmitoleic acid (Table 2). Furthermore, exogenous palmitoleic acid did not induce instability of ammonia-oxidizing activity. Both these results suggest that the stabilizing effect produced by BSA is due to inhibition of lipolysis rather than an indirect effect in which BSA simply absorbs the product of lipolysis.

Inhibition of lipolysis by BSA has been observed in other biological extracts (6, 10). It is also well documented that BSA can activate and/or protect many other membrane-associated enzymatic activities (2, 13). In particular, BSA has been added to numerous subcellular preparations involving membrane-bound enzyme complexes and electron transfer systems. Accordingly, BSA is generally regarded as essential for the production of fully active mitochondria and chloroplasts (6, 15). In the case of mitochondria, BSA had two effects on membrane-bound ATPase activity. First, BSA stimulated activity when added to the assay. Second, BSA attenuated the loss of activity during aging of the mitochondria (2). The first effect was concluded to result from the binding of free fatty acids by BSA. The second effect may have resulted from either the binding of free fatty acids or the inhibition of lipolysis (6). The dual effects of BSA on ATPase activity and stability are similar to the dual effects of BSA on ammonia-oxidizing activity and stability in cell extracts.

Our results also demonstrate for the first time that $CuCl_2$, $HgCl_2$, lecithin, or PMSF can stabilize ammonia-oxidizing activity in cell extracts. In view of previous studies demonstrating the activating effect of Cu^{2+} on in vitro AMO activity (5), the simplest interpretation of this result is that Cu^{2+} exerts its stabilizing effect directly on AMO. However, as was the case for BSA, our results demonstrate that either $CuCl_2$ or $HgCl_2$ inhibits the accumulation of fatty acids during storage of extracts (Table 2). Moreover, both Hg^{2+} and Cu^{2+} ions are known inhibitors of lipases (14, 24); therefore, a more reasonable interpretation is that these metal ions, like BSA, stabilize enzyme activity by their inhibition of lipolysis.

The idea that stabilization of ammonia-oxidizing activity in extracts can be achieved by the inhibition of lipolysis led us to consider alternative procedures which might be useful in future attempts at purification of AMO. The partial stabilizing effect of lecithin (Table 3) suggests that some degree of stabilization can be achieved by adding an alternative lipase substrate. Lecithin is thought not to inhibit lipase activity but to divert the activity of these enzymes away from the cell membranes (18). Treatment of cell extracts with PMSF is probably another example of direct inhibition of lipase activity, given that PMSF is known to inhibit lipases (16), in addition to having a more traditionally recognized role as a protease inhibitor. However, we did not unequivocally eliminate proteolysis as a cause for the loss of activity. Certainly the use of PMSF is the most promising stabilizing treatment we have developed, since PMSF stabilizes as well as either BSA or Cu^{2+} ions (Table 3) and does not involve the use of additional proteins, metals, or lipids in potential purification schemes.

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