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Purification and Characterization of a Copper-Containing Amine Oxidase from *Mycobacterium* Sp. Strain JC1 DSM 3803 Grown on Benzylamine

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A bacterial semicarbazide-sensitive amine oxidase (SSAO) was purified and characterized from *Mycobacterium* sp. strain JC1 DSM 3803 grown on benzylamine. During the purification procedures, the enzyme was tending to aggregate and exhibited heterogeneity in native PAGE. The heterogeneous forms having amine oxidase (AO) activity could be separated by their native molecular weights using gel-filtration chromatography. Most of the AOs behaved as dimers (M_r 150,000) composed of a 75-kDa subunit, but some aggregated to form tetramers (M_r 300,000). Besides their native molecular weight, subunit composition and V_{max} value, both forms (dimer and tetramer) have almost identical biochemical properties (*e.g.* subunit size, optimum pH and temperature, activation energy, K_m value on benzylamine, substrate and inhibitor specificities). When AO activity was observed by activity staining, the best-oxidized substrate was benzylamine, although the AO also oxidized tyramine and histamine. The AO was strongly inhibited by semicarbazide and isoniazid, but KCN did not affect its activity. The purified enzyme was shown to contain 2.39 mol of copper per mole of subunit, but there were no evidences of topaquinone co-factor involvement, when tested by absorption spectrum analysis and redox-cycling staining for quinoprotein detection.

Key words: amine oxidase, benzylamine, copper-containing, growth substrate, semicarbazide-sensitive.

Abbreviations: ADH, amine dehydrogenase; AO, amine oxidase; AS, ammonium sulfate; BA, benzylamine; CF, concentrated fraction; CO, carbon monoxide; CV, cadaverine; DB, 1,4-diamino-2-butanone; DP, dopamine; F-AO, fast-migrating amine oxidase; HT, histamine; INH, isoniazid; MAO, monoamine oxidase; NE, norepinephrine; PD, *o*-phenylenediamine; S-AO, slow-migrating amine oxidase; SC, semicarbazide; SMB, standard mineral base; SSAO, semicarbazide-sensitive amine oxidase; TR, tyramine.

The semicarbazide-sensitive amine oxidase (EC 1.4.3.6, SSAO) is a copper-containing enzyme and catalyses the oxidative deamination of both aromatic and aliphatic primary amines. The enzymes found mostly in mammalian cells exist in tissue-bound and soluble (plasma) forms, but there are wide species and tissue differences in their activities (*1*). SSAOs are usually defined by their sensitivity to inhibition by 0.1–1 mM semicarbazide. This distinguishes them from the monoamine oxidases (EC 1.4.3.4, MAOs), which are flavin-containing enzymes that demonstrate relatively weak effects at these semicarbazide concentrations (*2*).

Many bacteria can convert primary amines via an oxidative deamination step into products which can be utilized either as a carbon and/or energy source, as a nitrogen source or as both (*3*, *4*). Two types of enzymes, amine dehydrogenase (ADH) and AO, are normally implicated in this form of oxidation. ADHs catalyse the oxidative deamination of primary amines to their corresponding aldehyde and ammonia, but AOs yield hydrogen peroxide (H_2O_2) in addition to aldehyde and ammonia. Although many bacteria harbour these enzymes, their growth with some primary amines as a source of carbon and energy has been generally unsuccessful because of the toxicity corresponding to the aldehydes produced during the oxidation.

Benzylamine is a xenobiotic compound consisting of a benzyl group attached to an amine functional group. Although benzylamine is not a physiological substrate, it can be often used in amine oxidase assay because of its high reactivity as a substrate. Because benzylamine is often regarded as being the best substrate for the enzyme assay, therefore, SSAO has sometimes been referred to as benzylamine oxidase (*5*, *6*). However, benzylamine cannot be a supportive substance for bacterial growth because of the toxicity of benzaldehyde derived from the primary benzylamine oxidation. Indeed, there have been only two reports on the utilization of benzylamine as the sole carbon and energy sources for bacteria. *Pseudomonas putida* (*7*) and *Paracoccus denitrificans* IFO 12442 (*8*) could utilize benzylamine for their growth and they were found to possess a heme-containing ADH and a quinohemoprotein ADH, respectively. These results suggest that benzylamine-utilizing bacteria have adopted a dehydrogenase-type enzyme for their benzylamine oxidation.

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Mycobacterium sp. strain JC1 DSM 3803 has been known to be a facultatively chemolithotrophic bacterium with versatile metabolic activities, including a unique utilization of toxic compounds such as carbon monoxide (CO) and methanol (*9–12*). Recently, we observed that this bacterium could utilize benzylamine as a carbon and energy source (*13*). Studies with cell-free extract showed that the bacterium highly induced an oxidase-type enzyme for the benzylamine oxidation. In inhibitor studies with cell-free extracts, AO activity was strongly inhibited by a carbonyl group reagent, semicarbazide, and by a copper-chelating reagent, cuprizone (*13*), suggesting that this enzyme belongs to the class of copper-containing SSAOs.

To gain deeper insight into the bacterial AO-type enzyme responsible for benzylamine utilization, we purified the enzyme and characterized its biochemical properties. The results presented here reveal that *Mycoabcterium* sp. JC1 DSM 3803 truly possesses an inducible copper-containing AO sensitive to semicarbazide. We also discuss the reason why the enzyme shows heterogeneity and behaves as either dimers or tetramers having AO activity during the purification steps. To our knowledge, this is the first report demonstrating that bacterial utilization of benzylamine as a growth substrate can be mediated through a copper-containing AO.

MATERIALS AND METHODS

Bacterial Strain and Cultivation—Mycobacterium sp. strain JC1 DSM 3803 was cultivated at 37°C in standard mineral base (SMB) medium (*14*) supplemented with 0.5% (w/v) benzylamine. Cell growth was measured with a spectrophotometer by turbidity determined at 436 nm. Cells were harvested at the late exponential growth phase, washed once with 50 mM potassium phosphate buffer (pH 7.5, standard buffer), and used in purification procedures.

Protein Determination—Protein was determined using Bradford reagent (Sigma, USA) following the manufacturer's protocol, with bovine serum albumin as the standard.

Enzyme Assays—All assays were carried out at 25°C unless otherwise noted. A routine AO activity was assayed spectrophotometrically by measuring the benzylamine-dependent production of benzaldehyde ($\varepsilon_{250} = 12{,}800\ M^{-1}cm^{-1}$) in standard buffer at 250 nm as described previously (*15*). One unit (U) of AO activity was defined as the amount of enzyme required to oxidize 1 μmol benzylamine per minute

Purification of AO—All procedures were carried out at room temperature except when noted otherwise. Samples were concentrated, if needed, with a centrifugal filter device (Centricon YM-30) according to the manufacturer's protocol (Millipore, USA) at 4°C. Two and a half grams (wet weight) of benzylamine-grown cells were suspended in 10 ml of standard buffer and disrupted by sonication (20 s/ml). The disrupted cells were centrifuged at 12,000 *g* for 15 min. The resulting supernatant (crude extract) was treated with protamine sulphate to a final concentration of 0.054%, left on ice for 10 min, then centrifuged at 100,000 *g* for 90 min. The supernatant (soluble fraction) was collected and used in AO purification procedures performed using FPLC system (Pharmacia LKB, USA) equipped with the columns listed below. The soluble fraction was applied to a Mono-Q HR 5/5 column (Amersham-Pharmacia, USA) pre-equilibrated with standard buffer containing 0.2 M KCl. After the column was washed with 20 ml of the same buffer, elution was carried out with 30 ml of a linear 0.2–1.0 M KCl gradient in standard buffer at a flow rate of 0.5 ml/min. In a typical preparation, this step was carried out a total three times and the fractions with high AO activity from each run were pooled and concentrated to give a total sample volume of 2 ml. The concentrate was then applied to a Superose 12 HR 10/30 column (Amersham-Pharmacia, USA) pre-washed with standard buffer and eluted at a flow rate of 1 ml/min with standard buffer. Fractions containing AO activity were pooled and treated with ammonium sulphate (AS) to achieve a final concentration of 1 M. The AS-treated fraction was then applied to a HiTrap Phenyl HP column (5 ml, Amersham-Pharmacia, USA) pre-equilibrated with standard buffer containing 1 M AS. After the column was washed with 20 ml of the same buffer, elution was carried out with 40 ml of a linear 1.0–0 M AS gradient in standard buffer at a flow rate of 1 ml/min. Fractions with AO activity were combined, concentrated and desalted using a HiTrap desalting column (5 ml, Amersham-Pharmacia, USA) with standard buffer. The desalted fractions were concentrated and used in further AO characterization.

Electrophoresis—PAGEs without SDS (native-PAGE) and with SDS (denaturing-PAGE) were done on 7.5 and 10% polyacrylamide slab gels, respectively, by the method of Laemmli (*16*). After electrophoresis, the proteins were stained with Coomassie brilliant blue (CBB) R-250. Activity staining of AO was carried out after native PAGE as described previously (*17*). Briefly, the gel equilibrated twice for 20 min in standard buffer was incubated for 5 min in 50 ml of standard buffer containing 20 mg benzylamine and 10 mg 3-amino-9-ethylcarbazole. And then, 200 μl horseradish peroxidase (5 mg/ml, Sigma, USA) was added to the mixture and the gel was gently shaken in darkness at room temperature for 5–10 min until dark-red colored band appeared.

Molecular Weight Measurements—Molecular mass of the purified AO was measured by FPLC on a Sephacryl S-300 Hiprep HR 16/60 (Amersham-Pharmacia, USA) calibrated with a molecular weight standard marker kit (MW-GF-1000, Sigma, USA). Molecular weight of the AO subunit was determined by a SDS–denaturing PAGE. A pre-stained protein size marker kit (Elpis, Korea) was used for standards.

Effect of pH and Temperature on AO Activity—The optimal pH was determined by a routine AO assay method described in MATERIALS AND METHODS section, except the 1 ml reaction mixture contained 50 μmol of potassium phosphate buffer (pH 6.0, 7.0 and 8.0), 50 μmol of Tris–HCl buffer (pH 7.0, 8.0 and 9.0), or 50 μmol of glycine buffer (pH 9.0 and 10.0) instead of standard buffer (pH 7.5). Thermal stability was tested after incubation of the purified enzyme for 3 min at various temperatures (4, 25, 37, 45, 55 and 65°C), and then the remaining activity of the heat-treated enzymes was measured by a routine AO assay method. To determine effect of temperature on AO activity, the standard enzyme reaction was measured at various temperatures

using a spectrophotometer equipped with temperature controller (Beckman DU-70).

Determination of Kinetic Parameter—The apparent K_m and V_{max} values on benzylmine of the purified AO were determined by measuring initial velocities over a range of benzylamine concentrations (1–60 μM) in a routine AO reaction mixture, except that pH was 7.0 instead of 7.5. Apparent K_m and V_{max} values were calculated from Lineweaver–Burk plots.

Substrate and Inhibitor Specificity on AO Activity—To determine substrate and inhibitor specificity, the purified AO was separated in 7.5% native PAGE and the gel-strips were subjected to AO activity staining either in the presence of different substrates (20 mg of each substrate in 50 ml of staining buffer) or in the presence of different inhibitory reagents (2 mM each) as described above. The purified AO was also subjected to the routine AO activity assay in the presence of different inhibitory reagents (0.2 mM each).

RESULTS

Purification of AO—The purification of AO from *Mycobacterium* sp. JC1 is summarized in Table 1. AO was purified ~17.9-fold in five steps, with a specific activity of 2.33 U/mg of protein. During all the chromatographic purification procedures, AO activity resolved as a well-isolated peak for activity, which was coincident with a peak for protein content determined by the optical density at 280 nm (data not shown), suggesting a high degree of protein homogeneity. When the fractions were examined in native PAGE, however, the final phenyl–Sepharose fraction contained at least four proteins including two major bands and they all had AO activity (Fig. 1A and B, lane 5). Since one of the major proteins exists exclusively in both crude and soluble fractions (see Fig. 1B, lanes 1 and 2), we further purified the phenyl-Sepharose AO fraction by their native molecular weights using a sephacryl S-300 HiPrep HR 16/60 gel-filtration column chromatography to find the nature of the AO proteins.

Molecular Weights of Native Enzyme and Subunit—Throughout gel-filtration column chromatography, the AO proteins were broadly separated in fractions between #33 and 51 by their native molecular weights, when monitored the protein pattern by native PAGE (data not shown). Careful combination and concentration of the fractions by Centricon YM-30 gave three concentrated fractions (CF), designated as CF-1 (fraction #33 to #37), CF-2 (fraction #38 to 46) and CF-3 (fraction #47 to 51). As seen on Fig. 2A and B, CF-1 (lane 1) and CF-3 (lane 3) predominantly contained slow-migrating AO (S-AO) and fast-migrating AO (F-AO), respectively, according to their mobility on a native gel (R_M values 0.28 and 0.58 at pH 8.3, respectively; bromophenol blue = 1.0). Furthermore, CF-2 (lane 2) contained both S-AO and F-AO, although the F-AO was always the most prominent form. However, a SDS–PAGE analysis showed that all the CFs revealed a single band with apparent molecular mass of 75 kDa (Fig. 2C), indicating that the heterogeneous AOs had a same-sized subunit. Evaluation of the native molecular masses of S-AO and F-AO by gel-filtration chromatography gave values of 300 and 150 kDa, respectively (data not shown). Taken together, the results suggest that the two major bands (S-AO and F-AO) are size isomers and that the prominent dimers (F-AO) are associating into

Table 1. **Purification summary of AO from *Mycobacterium* sp. strain JC1 DSM 3803.**

Purification step	Total protein (mg)	Total activity (U)[a]	Sp act (U/mg)	Purification fold
Crude extract	68.9	8.96	0.13	1.0
Soluble fraction	65.2	10.43	0.16	1.2
Mono-Q	15.2	11.40	0.75	5.8
Suprose 12	13.6	15.64	1.15	8.9
Phenyl-Sepharose	7.8	18.17	2.33	17.9

[a]One unit (U) of AO is the amount catalysing the oxidation of 1 μmol benzylamine per minute.

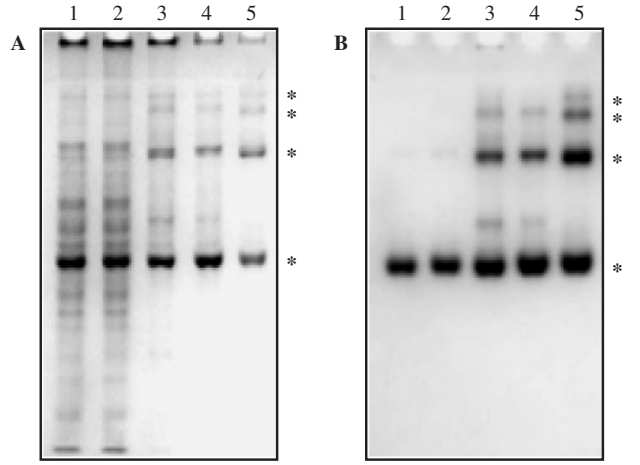

Fig. 1. **Non-denaturing PAGEs (7.5% separating gels) of amine oxidase (AO) preparation obtained from different purification steps.** The gels were stained for protein with CBB (A) and for AO activity (B) as described in text. Lanes 1 (crude cell extract, 40 μg of protein), 2 (soluble fraction, 40 μg of protein), 3 (Mono-Q fraction, 20 μg of protein), 4 (Suprose 12 fraction, 10 μg of protein) and 5 (Phenyl Sepharose fraction, 10 μg of protein). Asterisks indicate four proteins showing AO activity.

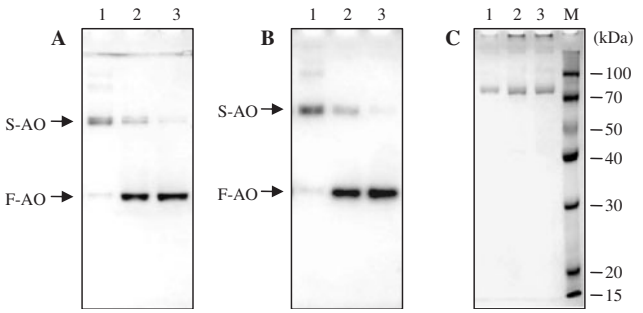

Fig. 2. **PAGE analyses of the heterogeneous forms of AO obtained from sephacryl S-300 gel-filtration chromatography.** The concentrated fractions (CFs) described in RESULTS section were subjected to either native PAGE [(7.5% separating gel; (A) and (B)] or SDS–PAGE [(10% separating gel; (C)], then the gels were stained for protein with CBB [(A) and (C)] and for AO activity (B). Lanes 1 (5 μg of CF-1), 2 (5 μg of CF-2), 3 (5 μg of CF-3) and M (10 μl of pre-stained protein size marker; Elpis EBM-1018, Korea). Abbreviations: S-AO (slow-migrating AO) and F-AO (fast-migrating AO).

tetramers (S-AO). SDS–PAGE analysis of S-AO under non-reducing condition (neither heat treatment at 100°C for 5 min nor β-mercaptoethanol treatment of CF-1 protein sample) showed a band near the top of the resolving gel (data not shown). But the band was dissociated into a 75-kDa band with only 5% (v/v) β-mercaptoethanol treatment (without heat treatment), suggesting that disulphide bond(s) may be involved in the association.

Some Biochemical Properties of S-AO and F-AO—Using CF-1 and CF-3 as each source of S-AO and F-AO, respectively, we further examined their biochemical properties. Both S-AO and F-AO had a sharp pH optimum at pH 7.0 in phosphate buffer (Fig. 3A). However, the specific activities of both AOs in Tris buffer at pH 7.0 were ~6-fold lower than those in phosphate buffer at same pH (Fig. 3A). When heat stability was investigated by measuring residual enzyme activity after incubating the AOs for 3 min at various temperatures, S-AO and F-AO retained 75 and 85% of the initial activity at the 65°C incubation condition (Fig. 3B), respectively, suggesting that both AOs have heat-stable properties. But F-AO showed it to be more stable (up to 15%) than S-AO. Activation energy of S-AO and F-AO calculating by Arrhenius plots was almost identical (27.8 and 27.1 kJ/mol, respectively) (data not shown), indicating that F-AO and S-AO have almost identical thermal kinetics.

The substrate kinetics on benzylamine of S-AO and F-AO were determined by measuring initial velocities over a range of benzylamine concentrations (1–60 μM). The apparent K_m values calculating from Lineweaver–Burk plots for S-AO and F-AO were 5.0 and 4.5 μM, respectively, which are almost identical. However, the apparent V_{max} value of F-AO (6.7 μmol/mg protein/min) was ~2-fold higher than that of S-AO (3.1 μmol/mg protein/min) (data not shown).

Specificity of S-AO and F-AO to Substrates and Inhibitors—To determine the substrate specificity of S-AO and F-AO, we used the AO activity staining method described in MATERIALS AND METHODS section instead of the routine spectrophotometrical AO assay method because the substrates used here (especially tyramine and dopamine) showed some optical interference at 250 nm in the assay condition. After the CF-2 containing both F-AO and S-AO was separated in 7.5% native polyacrylamide gels, each lane was cut and individually subjected to AO activity staining with the indicated substrates for 5 min. The results revealed that benzylamine and tyramine were the best substrates for both AOs (Fig. 4). Relatively weak enzyme activities were also detected with histamine, cadaverine and dopamine, but neither with norepinephrine (Fig. 4) nor methylamine (data not shown).

In inhibitor studies, semicarbazide and isoniazid at 0.2 mM concentration exerted profound inhibitory effect on both F-AO and S-AO activities (Table 2). *O*-phenylenediamine also inhibited both AO activities

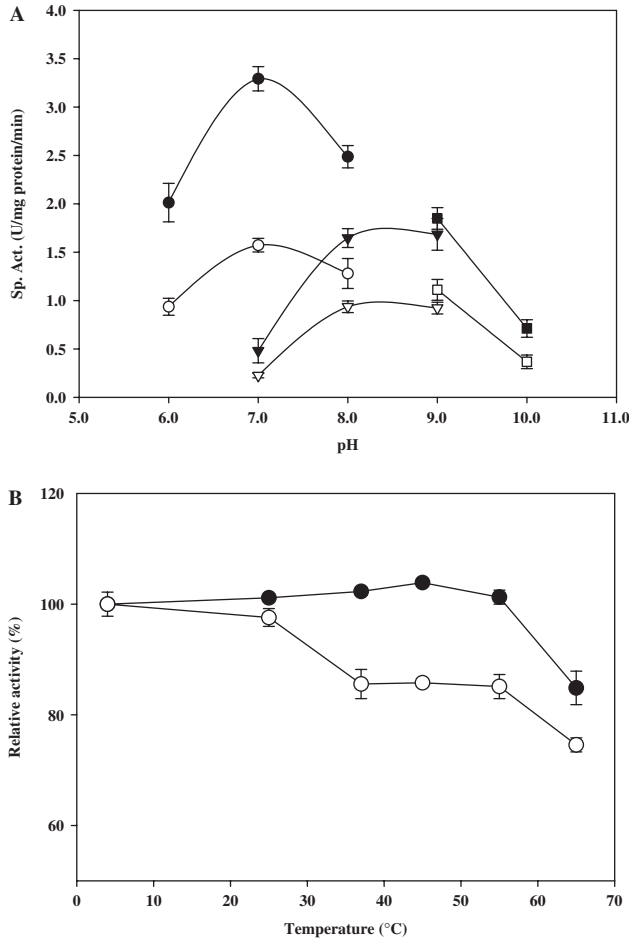

Fig. 3. **Effects of pH and temperature on S-AO and F-AO.** Sephacryl S-300 CF-1 and CF-3 proteins were used in each experiment as the enzyme sources of S-AO and F-AO, respectively. (A) Optimal pH was determined in different pH, as described in text. Open symbols denote S-AO activity, closed symbols F-AO activity. Symbols: ○, ● (phosphate buffer), ▽, ▼ (Tris–HCl buffer), □, ■ (glycine buffer). (B) Thermal stability was determined after incubation of each protein for 3 min at the indicated temperatures, as described in text. The initial activities at 4°C were set as a rate of 100% of S-AO (open circle) and F-AO (closed circle) and they corresponded to 2.27 U/mg of protein and 4.56 U/mg of protein, respectively.

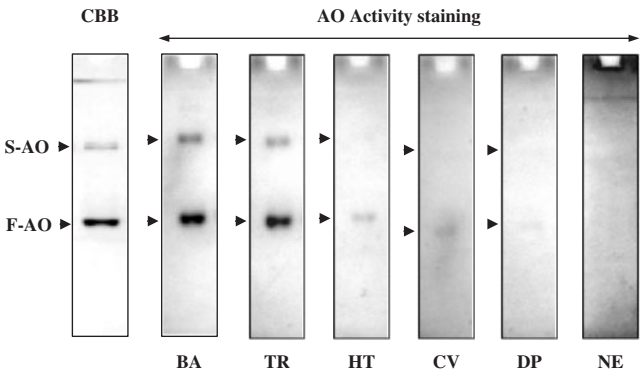

Fig. 4. **Substrate specificity of S-AO and F-AO.** After non-denaturing PAGE (7.5% separating gel) of CF-2 (4 μg each), the gel strips were stained for protein with CBB or for AO activity with different substrates, as described in text. The tested substrates (20 mg each per 50 ml reaction mixture) were as follows: benzylamine (BA), tyramine (TR), histamine (HT), cadaverine (CV), dopamine (DP) and norepinephrine (NE). Arrowheads indicate the positions of the actively stained S-AO (slow-migrating AO) and F-AO (fast-migrating AO).

up to 30% at 0.2 mM concentration, but 1,4-diamino-2-butanone, sodium azide (NaN_3) or KCN exhibited no significant inhibitory effect on S-AO and F-AO. Also, DTT or EDTA at 1 mM concentration had no effect on both AO activities (Table 2). The AO activity staining with 2 mM of each inhibitor clearly showed that SC and INH had strong inhibitory effect on the AOs (Fig. 5). As shown in Table 2, almost 90% of both AO activities were inhibited by adding 100 mM of salt (either KCl or NaCl) in the routine AO activity assay mixture. To find the nature of this salt inhibitory effect, both AO activities were measured in different concentrations of potassium phosphate buffer (pH 7.0) and potassium chloride (KCl). The results showed that variations of ionic strengths with potassium phosphate buffer up to 400 mM did not affect both AO activities (data not shown), suggesting that both potassium and phosphate ions have no inhibitory effect on the AO activities. However, the AO activities were inversely proportional to the logarithm of the chloride ion concentration in the assay mixture with the same potassium ionic strength (Fig. 6), indicating that the chloride ion is the cause for the AO inhibition.

As summarized in Table 3, S-AO and F-AO have almost identical biochemical properties, except of native molecular weight, subunit composition and V_{max} value on benzylamine.

Co-factor Studies—The visible absorbance spectrum of the purified AO (CF-2) showed a typical protein peak at 280 nm, but did not show any notable peaks at the visible light range (data not shown). Redox-cycling staining for identification of quinoproteins (*18*) showed no detectable quinone co-factor in the purified AO (data not shown). Atomic absorption analysis for determining copper content with X5 inductively coupled plasma-mass spectrometer (ICP-MS, ThermoElemental, UK) showed that the AO was found to contain 2.39 mol of copper per mole of subunit.

Table 2. **Effect of inhibitors on S-AO and F-AO activities.**

Compounds	Final concentration (mM)	Relative activity[a] (%)	
		S-AO	F-AO
None		100[b]	100[c]
Semicarbazide	0.2	55.3	41.3
1,4-Diamino-2-butanone	0.2	95.3	91.3
O-Phenylenediamine	0.2	73.6	66.8
NaN_3	0.2	94.4	87.9
KCN	0.2	95.7	93.9
Isoniazid	0.2	61.0	57.6
DTT	1	106	113
EDTA	1	116	116
KCl	100	12.1	12.4
NaCl	100	12.0	10.3

[a]Relative activity is expressed as percentage of the reaction rate obtained for benzylamine. [b]A rate of 100% corresponds to 2.87 U/mg of protein. [c]A rate of 100% corresponds to 5.04 U/mg of protein.

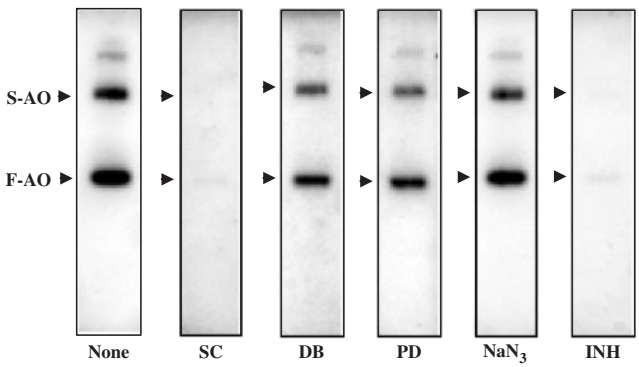

Fig. 5. **Inhibitor study of S-AO and F-AO.** After non-denaturing PAGE (7.5% separating gel) of CF-2 (5 μg each), the gel strips were incubated with 2 mM (final concentration) of each inhibitor for 5 min, and then subjected to AO activity staining with benzylamine, as described in text. The tested inhibitors were as follows; SC (semicarbazide), DB (1,4-diamino-2-butanone), PD (*O*-phenylenediamine) and INH (isoniazid). Arrowheads indicate the positions of the actively stained S-AO (slow-migrating AO) and F-AO (fast-migrating AO).

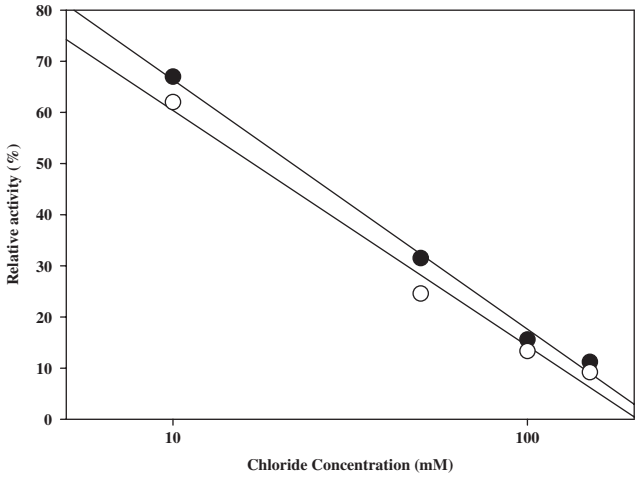

Fig. 6. **Inhibitory effect of chloride ion on the S-AO and F-AO.** Sephacryl S-300 CF-1 and CF-3 proteins were used in each experiment as the enzyme sources of S-AO and F-AO, respectively. The residual AO activity was measured in the AO assay mixture having different concentrations of potassium chloride (KCl) ion, as described in text. The AO activities without KCl were set as a rate of 100% of S-AO (open circle) and F-AO (closed circle) and they corresponded to 2.87 U/mg of protein and 5.04 U/mg of protein, respectively.

Table 3. **Comparison of biochemical properties between S-AO and F-AO.**

Properties	Slow-migrating AO (S-AO)	Fast-migrating AO (F-AO)
Molecular weight (kDa)	300	150
Native	75 (tetramer)	75 (dimer)
Subunit (composition)	7.0	7.0
pH Optimum	27.8 kJ/mol	27.1 kJ/mol
Activation energy		
Kinetics (benzylamine)		
K_m (μM)	5.0	4.5
V_{max} (μmol/mg protien/min)	3.1	6.7
Optimal substrates[a]	Benzylamine, tyramine	Benzylamine, tyramine
Strong inhibitors	Semicarbazide, isoniazid	Semicarbazide, isoniazid

[a]The results were determined by AO activity staining.

DISCUSSION

Though some microorganisms can utilize benzylamine as a nitrogen source for growth (*19*, *20*), their growths with benzylamine as a sole source of carbon and energy have been generally unsuccessful, presumably due to the possible toxicity of benzylamine and its metabolites (benzaldehyde, H_2O_2 and ammonia). To date, only *P. putida* and *P. denitrificans* IFO 12442 (*7*, *8*) have been demonstrated to utilize benzylamine as a sole carbon and energy source. They have been known to adapt a dehydrogenase-type enzyme for their benzylamine oxidation. We reported previously that *Mycobacterium* sp. JC1 DSM 3803 was capable of growth on benzylamine ($t_d = 7.7$ h) as a sole source of carbon and energy after being subjected to several consecutive cultures in benzylamine-containing medium. Because its primary deamination of benzylamine was mediated by an inducible oxidase-type enzyme (*13*), it has been proposed that bacteria also adapt an oxidase-type enzyme for benzylamine utilization.

In this study, we apparently purified an AO from the benzylamine-grown cells. During the purification procedures, however, the AO showed heterogeneity in its native composition. Further separation of the heterogeneous AOs by their native molecular weights revealed the two major active bands (F-AO and S-AO) are size isomers and the tetrameric form (S-AO) is composed of two tightly associated dimers (F-AO). The association behaviour of *Mycobacterium* sp. JC1 AO is closely resembled by methylamine and benzylamine oxidases found in the yeast *Candida boidinii* (*20*). Both enzymes consisted of similar-sized subunit of about 80 kDa. The benzylamine oxidase behaved usually as a dimer (M_r 136,000), but under certain conditions, it aggregated to a tetramer (M_r 288,000). Also, the methylamine oxidase was mainly in the form of an octamer (M_r 510,000), and it was readily dissociated into active dimers (M_r 150,000) or tetramers (M_r 286,000) (*20*). These association–dissociation phenomena have been also reported in beef plasma amine oxidase (*21*) and pig kidney diamine oxidase (*22*), suggesting that it may be common in some amine oxidases. It is unclear why the dimers itself tend to dimerize during protein concentration steps. Under the usual isolation conditions, the association of a protein generally involves some moderate environmental changes such as high salts, hydrophobic interaction environment and/or high protein concentration. In most cases, the association is bonding of the non-covalent linkages, although some proteins have been found to be made up of several polypeptide chains covalently linked by disulphide bridges (*21*). In the case of *Mycobacterium* sp. JC1 AO, the association-phenomena of the AO may be due to the fact that with its hydrophobic nature and at high protein concentrations the active dimers become slowly associated into the active tetramers. Also, possible intermolecular disulphide bonds may be involved in the AO association because 5% (v/v) β-mercaptoethanol showed almost complete dissociation of S-AO (a tetrameric form) into the 75-kDa subunit.

Both S-AO and F-AO revealed almost identical biochemical properties such as sharp pH optimum at pH 7.0, thermal kinetics, K_m value and substrate and inhibitor specificities. Moreover, mass spectrum analysis of both S-AO and F-AO subunits after trypsin digestion showed that they were identical (data not shown). Taken together, these results clearly indicate that the two major active bands (F-AO and S-AO) are size isomers rather than two different forms of enzyme, and that the tetrameric form (S-AO) is composed of two tightly associated dimers (F-AO). It is quite interesting that the specific activity and V_{max} value of F-AO were ~2-fold higher than those of S-AO (see Table 3). We have not investigated further the cause of these differences but propose that the association of the AO dimers (F-AO) into tetramers (S-AO) may cause a half reduction of the AO activity.

In substrate-specificity studies, benzylamine and tyramine were the optimal substrates for the purified AO and relatively low activities were recorded with dopamine and histamine, but not with norepinephrine, as similarly shown in previous results from crude extract (*13*). The purified AO had a high specific activity (2.33 U/mg of protein) on benzylamine. This value is almost 10-fold higher than membrane-bound SSAO (0.25 U/mg of protein) from bovine lung (*23*). The K_m value of F-AO (4.5 μM) is much lower than membrane-bound bovine lung SSAO (50 μM) and bovine plasma SSAO (730 μM) (*23*, *24*), suggesting that *Mycobacterium* sp. JC1 AO is highly specific towards benzylamine than other mammalian SSAOs. It is interesting that the purified AO could not oxidize methylamine, which is a substrate for SSAO, but not for monoamine oxidase (*25*, *26*). For primary methylamine oxidation, this bacterium has been known to possess a dehydrogenase-type of enzyme (*10*). Though we could not examine the AO kinetics on tyramine because of optical interference of it at 250 nm, the AO seems to have substrate specificity on primary aromatic amines.

SSAOs are characteristic sensitivity to inhibition by carbonyl-group reagents, such as semicarbazide, results from the presence of a topaquinone (6-hydroxydopa; 2,4,5-trihydroxyphenylalanine) as the redox co-factor (*27*) that is formed by a copper-dependent post-translational modification of a peptidyl tyrosine residue (*28*). This distinguishes them from the flavin-containing MAOs which are not inhibited by semicarbazide. The antitubercular drug isoniazid is also known to inhibit plasma and tissue-bound SSAOs at concentrations up to 1 mM, with relatively little effect upon MAO (*2*). The purified AO described here was strongly inhibited by low concentrations (0.2–2 mM) of semicarbazide and isoniazid. Also, the enzyme was strongly inhibited by hydroxylamine and cuprizone, as a carbonyl reagent and a copper-chelating agent, respectively (data not shown, and see also in ref. 13). Moreover, atomic absorption analysis for determining copper content showed that the purified AO was found to contain 2.39 mol of copper per mole of subunit, proposing that *Mycobacterium* sp. JC1 AO truly possesses a copper-containing AO. It is noteworthy that the AO activity was markedly inhibited by the presence of chloride ions. Caeruloplasmin, a copper-containing oxidase in human serum, possessed *p*-phenylenediamine oxidase activity and halide ions such as fluoride or chloride ions inhibited its activity presumably by binding to reduced copper atoms and preventing their oxidation (*29*, *30*). Also, chloride ion

was found to be a non-competitive inhibitor of copper-containing apple polyphenol oxidase (PPO) and it could interact with enzyme–substrate complex in the protonated form (*31*). Our results thus correlate with a suggestion that chloride ions can bind to reduced copper atoms and inhibit the copper-containing oxidase activity. This result further confirms that *Mycobacterium* sp. JC1 AO is truly a copper-containing enzyme. In particular, the AO showed the highest specific activity in phosphate buffer at pH 7.0, but the activity was greatly reduced in Tris buffer at the same pH (seen in Fig. 3A). This reduction of the AO activity to Tris buffer suggests that the amino group in Tris may compete with benzylamine for the active site of the AO and this competition results in the inhibition of the AO activity. Indeed, the AO activity was significantly inhibited in proportion to Tris concentrations in the AO assay mixture (data not shown).

The visible absorbance spectrum of the purified AO showed a typical protein peak at 280 nm, but it did not show any notable peaks for other possible co-factor(s) at the visible light range (data not shown). Also, a redox-cycling staining (*18*) showed the absence of quinone co-factor in the purified AO (data not shown), suggesting that the AO may not contain topaquinone as a co-factor, not likely as other copper-containing AOs (*27*). Because mass spectrum analysis of the AO after trypsin digestion showed no significant homologies in peptide mass to other known AOs (data not shown), it remains to be clarified whether *Mycobacterium* sp. JC1 AO possesses topaquinone as a co-factor. Since a consensus sequence flanking the topaquinone co-factor structure has been established in copper AOs (*32*, *33*), it would be necessary to obtain amino acid sequence data to definitively establish the presence of topaquinone.

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