Volume 261, number 2, 441–444 FEBS 08190 February 1990

Reductive trapping of substrate to methylamine oxidase from *Arthrobacter* P1

Christa Hartmann* and Judith P. Klinman

Department of Chemistry, University of California, Berkeley, CA 94720, USA

Received 6 December 1989; revised version received 19 Janaury 1990

Methylamine oxidase (EC 1.4.3.6) from *Arthrobacter* P1 was inactivated by $NaCNBH_3$ in the presence of [^{14}C]benzylamine, leading to the incorporation of 1 mol of radiolabeled substrate/mol of enzyme subunit at complete inactivation. By contrast, no labeling of enzyme was observed using [^{3}H]$NaCNBH_3$ as reductant. These results are analogous to those previously reported for the eukaryotic enzyme, bovine serum plasma amine oxidase [(1987) J. Biol. Chem. 262, 962–965]. The observed pattern of labeling is consistent with the presence of dicarbonyl cofactor at the active site of methylamine oxidase. Further, these studies suggest that our reductive trapping technique, in which the pattern of radiolabeling of an enzyme is compared using C-14 substrate vs tritiated reductant, may serve as a general assay for covalently bound dicarbonyl structures.

Methylamine oxidase; Pyrroloquinoline quinone; Copper-containing amine oxidase

1. INTRODUCTION

In an earlier study, bovine serum amine oxidase was shown to be reductively inactivated in the presence of sodium cyanoborohydride and amine substrate [1]. This result was taken as evidence for a covalent adduct between substrate and cofactor via Schiff base formation, suggesting an aminotransferase mechanism for substrate oxidation. In contrast to pyridoxal phosphate chemistry, however, no tritium was observed to be transferred from [^{3}H]$NaCNBH_3$ into inactivated enzyme. This ability to trap ^{14}C-substrate, without a concomitant transfer of label from reductant, has been attributed to the presence of an active site dicarbonyl cofactor [1,2]. As reported originally by Lobenstein-Verbeek et al. [3] and Ameyama et al. [4], evidence exists for the α-dicarbonyl-containing species, pyrroloquinoline quinone (PQQ), at the active sites of numerous eukaryotic amine oxidases [5,6].

In the present study, methylamine oxidase (EC 1.4.3.6) from *Arthrobacter* P1 was chosen to examine the suitability of our double labeling methodology to a bacterial amine oxidase believed to contain covalently bound PQQ [7]. As described herein, methylamine oxidase can only be inactivated by $NaCNBH_3$ in the presence of substrate, leading to the incorporation of 1 mol of [^{14}C]benzylamine/mol of enzyme subunit at complete inactivation. Analogous to previous results obtained with bovine serum amine oxidase, no tritium was incorporated into protein upon reduction with [^{3}H]$NaCNBH_3$. These results suggest that our reductive trapping technique, in which the pattern of radiolabeling of an enzyme is compared using C-14 substrate vs tritiated reductant, may serve as a general assay for protein-bound dicarbonyl cofactors.

Correspondence address: J.P. Klinman, Department of Chemistry, University of California, Berkeley, CA 94720, USA

* *Present address:* Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94143, USA

2. MATERIALS AND METHODS

2.1. *Materials*

All materials were of reagent grade unless otherwise indicated. [UL-^{14}C]benzylamine-HCl (sp. act. 12–16 mCi/mmol), liver alcohol dehydrogenase (2 U/mg) and G-25 Sephadex (medium grade) were purchased from Sigma and [^{3}H]$NaCNBH_3$ was from Amersham (sp. act. 5.40 Ci/mmol). Bovine serum albumin and $NaCNBH_3$ were obtained from Aldrich and NADH was from P-L Biochemicals.

2.2. *General methods*

Radioactivity was determined on a Beckman LS-8000 Liquid Scintillation spectrometer using a cocktail comprises of Chemfluor with surfactor X-100 (E&K Scientific Products) in toluene (30:70 v/v). Methylamine oxidase from *Arthrobacter* P1 was a kind gift from W.S. McIntire (Veterans Administration Hospital, San Francisco, CA). This enzyme, which is a dimer of 82 kDa subunits, indicated $\geq$95% purity by gel electrophoresis (W.S. MacIntire, personal communication). Activity was assayed spectrophotometrically on a Cary 118 spectrophotometer using 10 mM benzylamine as substrate in 100 mM phosphate buffer, pH 7.2; the increase in absorbance at 250 nm due to benzaldehyde formation was monitored, Δe = 12800 $M^{-1} \cdot cm^{-1}$ [8]. Protein concentration was determined spectrophotometrically at 280 nm, $E^{1\%}_{1cm}$ = 18.2 for methylamine oxidase [7].

2.3. *Phenylhydrazine titrations with methylamine oxidase*

Phenylhydrazine solutions were made immediately prior to use and were protected from light throughout the course of the experiments.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

A typical titration included 1 ml of enzyme (1–2.5 mg/ml) to which was added 1–2 μl aliquots of 1.7 mM phenylhydrazine. Reaction was allowed to proceed for 5–10 min between additions. The course of the titration was monitored by measuring the absorbance at 440 nm for methylamine oxidase. An approximate extinction coefficient for the phenylhydrazone of methylamine oxidase was determined by taking the final absorbance of the complex and dividing by the nmol of phenylhydrazine which had reacted.

2.4. *Inactivation of enzyme with [^{3}H]NaCNBH$_3$*

Reaction conditions were identical to those previously described [1], with the exception that 20 mM [^{3}H]NaCNBH$_3$ (1.1×10^9 cpm/μmol or 5.5×10^7 cpm/μmol) was used as reductant. Isolation and counting of the protein peaks and confirmation of the specific activity of the [^{3}H]NaCNBH$_3$ followed the earlier protocols of Hartmann and Klinman [1].

3. RESULTS AND DISCUSSION

3.1. *Phenylhydrazine titration of methylamine oxidase*

Prior to attempting reductive trapping of substrate to enzyme, it was important to determine the concentration of active sites. Phenylhydrazine is a potent irreversible inhibitor of methylamine oxidase. The titration of methylamine oxidase was monitored by the increase in absorbance at 440 nm (fig.1A). The complex formed between enzyme and phenylhydrazine has an approximate extinction coefficient of 30 $mM^{-1} \cdot cm^{-1}$. From the dependence of absorbance at 440 nm on the ratio of phenylhydrazine to methylamine oxidase, fig.1B, ~0.80 mol of phenylhydrazine were concluded to react with 1.0 mol of methylamine oxidase of specific activity 0.85 U/mg. It should be noted that a true tangent was not observed in fig.1B, such that the actual ratio of phenylhydrazine to enzyme may in fact be greater than 0.80:1.0. This result differs from previous titrations with methylhydrazine, which led to the conclusion of only one active site per dimer [7]. Recent anaerobic titrations of methylamine oxidase indicate two moles of product formed per enzyme dimer (Dooley et al., personal communication).

3.2. *^{14}C versus ^{3}H trapping of substrate with methylamine oxidase*

Methylamine oxidase was reacted with [^{14}C]benzylamine and NaCNBH$_3$, giving the time courses for inactivation and radiolabeling in fig.2A. These data are replotted in fig.2B, illustrating the correspondence between enzyme inactivation and radiolabeling and the extrapolation to one mol of ^{14}C-benzylamine per mol of enzyme subunit at 100% inactivation. Incubation of enzyme with radiolabeled substrate in the absence of reductant led to retention of enzyme activity and no incorporation of radioactivity into the protein.

As with bovine serum amine oxidase, efforts were made to incorporate tritium into reductively trapped enzyme. Incubation of methylamine oxidase with high specific activity [^{3}H]NaCNBH$_3$ (1.1×10^9 cpm/μmol) alone gave rise to ~7% tritium incorporation which appeared to be time-independent. Incubation of enzyme

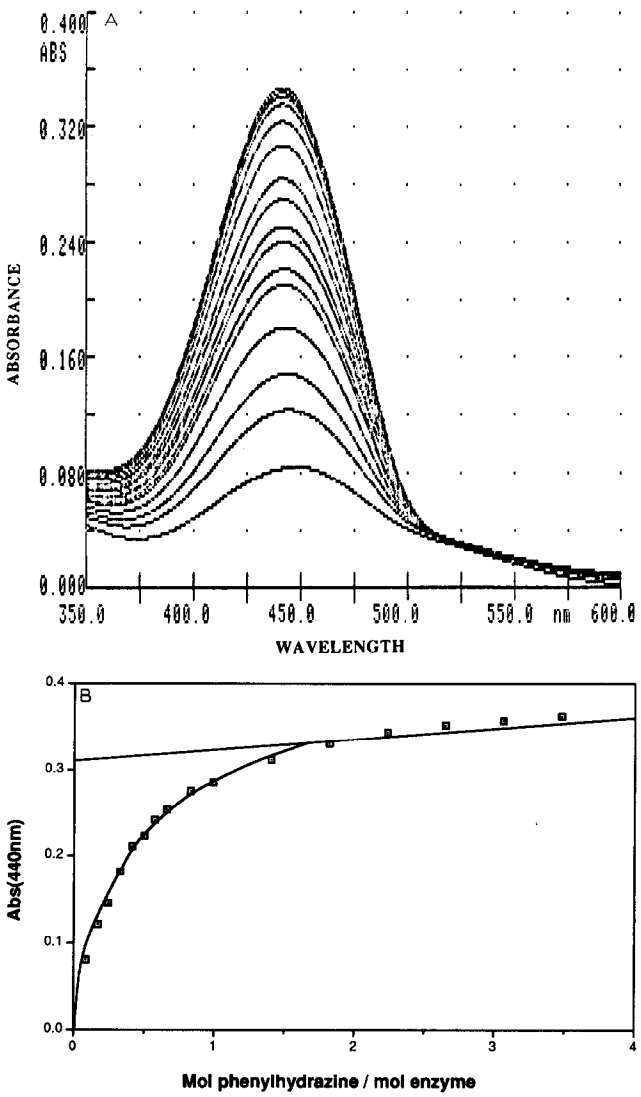


Fig.1. (A) Spectral changes induced by the addition of phenylhydrazine to methylamine oxidase. Methylamine oxidase (1.71 mg/ml) in 1 ml 0.10 M phosphate buffer, pH 7.2, was titrated with 1 μl aliquots of 1.7 mM phenylhydrazine, also in 0.10 M phosphate buffer, pH 7.2. (B) Stoichiometry of the reaction between methylamine oxidase (1.7 mg/ml) and 1.7 mM phenylhydrazine in phosphate buffer, pH 7.2, monitored at 440 nm.

in the presence of benzylamine, [^{3}H]NaCNBH$_3$, NADH and liver alcohol dehydrogenase to 89% inactivation yielded ≤6.8% incorporation of tritium. Since the amount of tritium incorporated ± substrate was identical, the percent incorporation in the presence of substrate is attributed to background for this enzyme.

It should be emphasized that the experiments in fig.2 were carried out in the presence of excess horse liver alcohol dehydrogenase (and NADH) to convert product aldehyde to alcohol. Reactions run in the absence of alcohol dehydrogenase indicated 100% incorporation of radiolabel at only 45% inactivation. This result, which suggests a non-specific condensation of product benzaldehyde with protein (lysyl) side chains [10], em-

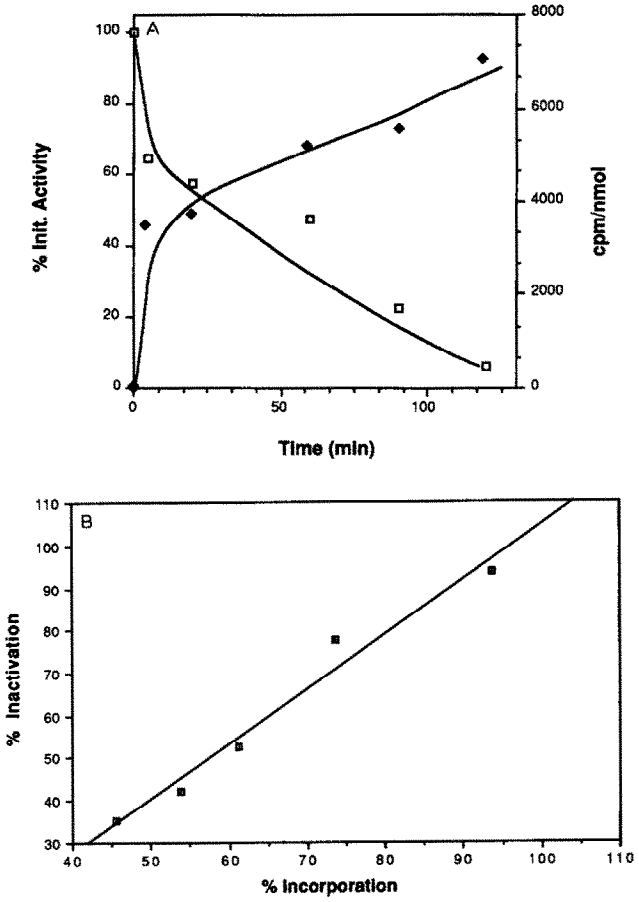
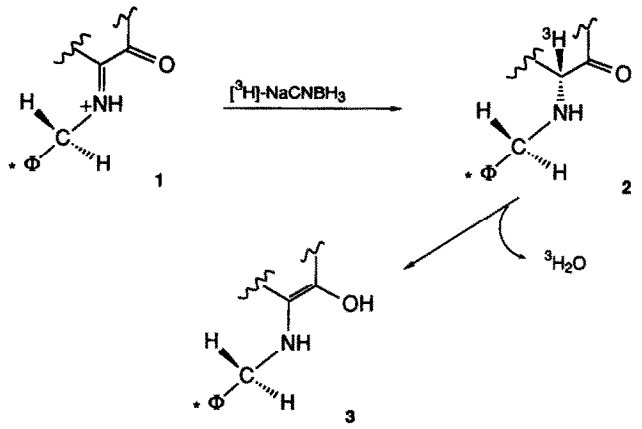


Fig.2. (A) Time course for inactivation and labeling of methylamine oxidase. Enzyme at a concentration of 2–3 mg/ml was incubated with [^{14}C]benzylamine and $NaCNBH_3$ in 50 mM/Pi, pH 6.04, at 4°C. Activities (□—□) and ^{14}C content (◆—◆) were determined following gel filtration; enzyme activity was corrected for some loss following incubation of enzyme with $NaCNBH_3$ alone (10% at 120 min). Substrate concentration was maintained at approximately 20 mM throughout the course of enzyme inactivation; initial reductant concentration was 20 mM. (B) A replot of the data in (A) showing the correspondence between enzyme inactivation and radiolabeling and the extrapolation to one mol of [^{14}C]benzylamine per mol of enzyme subunit at 100% inactivation.

phasizes the importance of the use of a coupled assay system in experiments aimed at correlating enzyme inactivation with the incorporation of radiolabeled substrate.

A mechanistic explanation which will account for our results is shown in scheme 1. According to this scheme, covalent adduct formation between substrate and cofactor (**1**) precedes reduction by $NaCNBH_3$. Although tritiated reductant is predicted to initially label the enzyme (**2**), subsequent (possibly base catalyzed) enolization to generate the more stable form of the cofactor (**3**) leads to loss of tritium. As originally proposed by Suva and Abeles [11], there is increasing evidence for base catalysis in the bovine serum amine oxidase catalyzed conversion of the substrate-amine to product imine [12,13].



Scheme 1. Mechanism explaining the experimental observations of C-14 incorporation without tritium incorporation. The ^{14}C-labeled substrate (*) forms a Schiff base with cofactor, **1**, which undergoes reduction by [^{3}H]$NaCNBH_3$. Although the enzyme will initially incorporate tritium, **2**, subsequent enolization will generate the more stable form of the cofactor, **3** (adapted from [1]).

In the earlier study of bovine serum amine oxidase [1], no tritium was found to be incorporated into recovered benzylamine, contrary to results reported by Suva and Abeles [11]. The latter authors demonstrated the presence of [1(S)-^{3}H]benzylamine following [^{3}H]$NaBH_4$ reduction of plasma amine oxidase at pH 8.0, but used a different substrate, pH and reductant from Hartmann and Klinman [1]. With benzylamine as substrate for bovine serum amine oxidase, pre-steady-state and steady-state isotope effects indicate that the major enzyme form is the enzyme substrate complex [14], such that [^{3}H]$NaCNBH_3$ reduction could lead to tritiated cofactor but would not be expected to label substrate. For methylamine oxidase no effort was made to determine whether tritium had been incorporated into substrate. As discussed previously [1], the stable incorporation of C-14 into enzyme implies that the secondary amine (**3**) either oxidizes more slowly than the primary amine generated under catalytic conditions or is resistant to oxidation.

3.3. *Retention of radiolabel following dialysis*

In our previous study of bovine serum amine oxidase, the retention of counts in radiolabeled enzyme was found to be dependent on the method of treatment. Whereas incubation of labeled enzyme for 2.5 h followed by rechromatography led to full retention of counts, extensive dialysis or denaturation led to the release of ~50% of the radiolabel from the protein.

Although this result was originally attributed to half of the sites reactivity [1], it has now been shown that earlier preparations of bovine serum amine oxidase were only half active [15]. To assay for retention of label for methylamine oxidase, enzyme was dialyzed with the use of a Centricon apparatus which permits close to full recovery of radioactivity in the supernatant

Table 1

Effect of dialysis on the retention of radiolabel in reductively trapped methylamine oxidase

Time of inactivation (min)	Supernatant (cpm)	Protein (cpm)	% cpm in protein
20	886	2842	76
60	1177	4384	79
90	1037	3877	79
120	1496	6437	81

and protein. As summarized in table 1, such treatment led to the loss of ~20% of radiolabel. Since phenylhydrazine titration with methylamine oxidase exhibited a similar end point (of 0.8 mol of reactive phenylhydrazine sites per mol of enzyme active sites), we suggest that methylamine oxidase of specific activity 0.850 U/mg corresponds to enzyme which is ~80% active.

3.4. *Implications for structure of the organic cofactor in copper amine oxidases*

In 1984, Lobenstein-Verbeek et al. [3], and Ameyama et al. [4] independently reported results which implicated PQQ as the organic cofactor in bovine serum amine oxidase. Spectral data in support of the presence of PQQ in both serum amine oxidases and lysyl oxidase were subsequently published [16–18]. It should be emphasized, however, that an unambiguous structural proof of PQQ as the covalently bound cofactor in the copper amine oxidases has not yet been advanced. The latter point is particularly important in light of the recent X-ray studies of Hol et al. [19] on methylamine dehydrogenase. Despite the prior evidence for PQQ in this protein [20], X-ray diffraction patterns indicate an active site dicarbonyl which lacks the pyridine ring present in authentic PQQ [19].

In concluding, we note that while the reductive trapping data presented herein and in Hartmann and Klinman [1] cannot distinguish covalently bound PQQ from alternative structures, they do provide strong chemical evidence for a dicarbonyl-containing cofactor in the pro- and eukaryotic copper amine oxidases.

Acknowledgements: This work was supported by a grant from the National Institutes of Health (GM 39296).

REFERENCES

[1] Hartmann, C. and Klinman, J.P. (1987) J. Biol. Chem. 262, 962–965.
[2] Hartmann, C. and Klinman, J.P. (1988) Biofactors 1, 41–49.
[3] Lobenstein-Verbeek, C.L., Jongejan, J.A., Frank, J. and Duine, J.A. (1984) FEBS Lett. 170, 305–309.
[4] Ameyama, M., Hayashi, U., Matsushita, K., Shinagawa, E. and Adachi, O. (1984) Agric. Biol. Chem. 48, 561–565.
[5] Duine, J.A., Frank, J. and Jongejan, J.A. (1987) Adv. Enzymol. 59, 170–212.
[6] Duine, J.A. and Jongejan, J.A. (1989) Annu. Rev. Biochem. 58, 403–426.
[7] Van Iersel, J., Van der Meer, R.A. and Duine, J.A. (1986) Eur. J. Biochem. 161, 415–419.
[8] Neumann, R.H., Hevey, R. and Abeles, R.H. (1975) J. Biol. Chem. 250, 6362–6367.
[9] Omitted.
[10] Jentoft, N. and Dearborn, D.G. (1979) J. Biol. Chem. 254, 4359–4365.
[11] Suva, R.H. and Abeles, R.H. (1978) Biochemistry 17, 3538–3545.
[12] Farnum, M.F., Palcic, M.M. and Klinman, J.P. (1986) Biochemistry 25, 1898–1904.
[13] Farnum, M.F. and Klinman, J.P. (1986) Biochemistry 25, 6028–6036.
[14] Palcic, M.M. and Klinman, J.P. (1983) Biochemistry 22, 5957–5966.
[15] Klinman, J.P., Hartmann, C. and Janes, S.M. (1989) in: 1st International Congress on PQQ and Quinoproteins (Duine, J.A. and Jongejan, J.A. eds) p.297.
[16] Knowles, P.F., Pandeya, K.B., Rius, F.S., Spencer, C.M., Moog, R.S., McGuirl, M.A. and Dooley, D.M. (1987) Biochem. J. 241, 603–608.
[17] Moog, R.S., McGuirl, M.A., Cote, C.E. and Dooley, D.M. (1986) Proc. Natl. Acad. Sci. USA 83, 8435–8439.
[18] Williamson, P.R. and Kagan, H.M. (1987) J. Biol. Chem. 262, 8196–8201.
[19] Vellieux, F.M.D., Huitema, F., Groendijk, H., Kalk, K.H., Frank, J., Jongejan, J.A., Duine, J.A., Petratos, K., Drenth, J. and Hol, W.G.J. (1989) EMBO J. 8, 2171–2178.
[20] Van der Meer, R.A., Jongejan, J.A. and Duine, J.A. (1987) FEBS Lett. 221, 299–304.