Cofactor Investigation of Bovine Plasma Amine Oxidase

NaBH₄ Reduction of Enzyme-Substrate Mixture*

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MELVIN INAMASU AND KERRY T. YASUNOBU‡
From the Department of Biochemistry-Biophysics, University of Hawaii, Honolulu, Hawaii

WILFRIED A. KONIG
From the Chemisches Institut der Universität Tubingen, Tubingen, Germany

SUMMARY

It has been reported that treatment of amine-diamine oxidase mixtures with sodium borohydride resulted in the formation of a stable substrate-pyridoxal phosphate complex. When this procedure was applied to the bovine plasma amine oxidase in the presence of ¹⁴C-labeled substrate, the enzyme was inactivated and became radioactive. A pure radioactive product was isolated from acid hydrolyzates by ion exchange chromatography and preparative paper chromatography of the hydrolyzate. The compound was shown to be ϵ-N-benzyllysine by mass spectrometry. This adduct was apparently a reduced intermediate in the catalytic pathway of substrate oxidation by the enzyme. Formation of a substrate-pyridoxal phosphate complex could not be confirmed.

Investigations of diamine oxidases have led to the proposal that they are copper-pyridoxal phosphate enzymes (1–7). Evidence that the organic cofactor of the diamine oxidases is pyridoxal phosphate came from trapping experiments of the enzyme-substrate complex by NaBH₄, for example, Buffoni performing trapping experiments with the pig plasma amine oxidase. The product isolated was reported to be the reduced substrate-pyridoxal phosphate Schiff's base (6). Kumagai et al. (4) carried out similar experiments with the pig plasma amine oxidase and reported that they isolated pyridoxal-histamine complex from acid hydrolyzates of the enzyme. Adachi et al. (7) reported that they had isolated the pyridoxal-histamine complex from acid hydrolyzates of the enzyme. They isolated pyridoxylethylamine when ethylamine was the substrate in trapping experiments with the pig plasma amine oxidase. The product isolated was reported to be the reduced substrate-pyridoxal phosphate complex. When this procedure was applied to the bovine plasma amine oxidase and ¹⁴C-labeled benzylamine, the product isolated was shown to be ε-N-benzyllysine by mass spectrometry. This adduct was apparently a reduced intermediate in the catalytic pathway of substrate oxidation by the enzyme. Formation of a substrate-pyridoxal phosphate complex could not be confirmed.

EXPERIMENTAL PROCEDURES

Materials

Bovine Plasma Amine Oxidase—The enzyme was isolated by a modified procedure of Yamada and Yasanobu and had a specific activity of 300 to 560 (8). The source of most of the reagents used have been described in previous reports from this laboratory (1, 2, 8). Radiochemicals used in the present investigation were purchased from ICN and the specific activity of the benzylamine was 4.2 mCi per μmole. NaBH₄ was purchased from the Sigma Chemical Co.

Methods

Enzyme, Assay, Protein Determination, and Specific Activity—The enzyme was assayed by the method of Tabor et al. (9) in which the increase in absorbance at 250 nm is measured during the oxidation of benzylamine to benzaldehyde by the enzyme. One unit of enzyme is defined as the amount of enzyme causing an absorbance change of 0.001 per min at 25° at pH 7.2. The protein concentration was determined spectrophotometrically on the basis that the E₅₅₀ at 250 nm is 0.8. Specific activity is defined as the units per mg of protein.

Amino Acid Analyses—Samples were analyzed in the Beckman model 120 automatic amino acid analyzer according to the procedure of Spackman et al. (10). Ninhydrin-positive material was determined by the method of Moore and Stein (11).

Radioactivity Measurements—All radioactivity measurements were made in the Packard Tri-Carb liquid scintillation spectrometer, model 574. Corrections for background were made. The phosphor solution was prepared from 60 g of naphthalene, 0.2 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene, 4 g of 2,5-diphenyloxazole, 100 ml of methanol, 20 ml of ethylene glycol, and 834 ml of di-oxane.

Synthesis of Pyridoxyl-Benzylamine—About 1 mg of pyridoxal and 0.555 mg of benzylamine were mixed and reduced with 5 mg of NaBH₄. No purification of the product was attempted.

NaBH₄ Reduction of Enzyme-¹⁴C-Labeled Benzylamine Mixtures—A 100-fold excess (0.5 ml of a 0.1 M solution) of benzylamine was added to 0.5 ml of a 0.1 M phosphate, pH 7.2, solution of enzyme which contained 5.6 mg of enzyme (specific activity of 553). After 30 s of reaction, 4 mg of NaBH₄ in 0.1 ml of 0.1 N NaOH were added. At various times, 0.15 ml aliquots of this mixture were applied to Sephadex G-25F columns (1 X 10 cm) to separate protein from reagents. The protein was assayed immediately for activity. A control in which 0.1 ml of NaOH solution was added to the enzyme without NaBH₄, was assayed at the end of the reaction time. Larger quantities of the derivatized enzyme were prepared with...
\(^{14}C\)-labeled benzylamine as follows. About 273 mg of enzyme and 53.5 mg of benzylamine in 35 ml of 0.1 m phosphate buffer, pH 7.2, were incubated for 30 s. About 25 mg of NaBH\(_4\) were added and the reaction was allowed to proceed for 5 to 6 hours. The enzyme then was precipitated by the addition of 12.6 g of solid (NH\(_4\))\(_2\)SO\(_4\) (50% saturation), the solution was centrifuged, and the precipitate was collected. The precipitate was dissolved in 3 ml of water and applied to a column (2 x 26 cm) of Sephadex G-25 F pre-equilibrated and eluted with 0.02 m NH\(_4\)HCO\(_3\) solution. Aliquots of the eluted enzyme (0.02 ml) were assayed for \(^{14}C\) and protein content and the labeled protein portions were pooled. Aliquots containing 20 mg of labeled protein were transferred to test tubes and dried in a desiccator and constant boiling HCl was added. The tubes were evacuated and sealed and the sample was hydrolyzed for 24 hours at 110\(^\circ\)C. The hydrolyzates were pooled, dried, and filtered through glass wool. The solution was applied to a column (2 x 80 cm) of Dowex 1 equilibrated with N-ethylmorpholine-\(N\)-picoline-pyridine buffer, pH 8.0 (12). The column was eluted stepwise with 200 ml of pH 8.0 buffer followed by 400 ml of pH 8.8 buffer at a flow rate of 18 ml per hour. Four-milliliter fractions were collected and assayed for radioactivity and ninhydrin-positive material. The radioactive fractions were pooled and dried. They then were applied to a column (2 x 15 cm) of Dowex 50-H\(^{+}\) (made from HCl and water). The column was washed successively with 30 ml of H\(_2\)O, 51 ml of 2 N NH\(_4\)OH, 33 ml of 4 N NH\(_4\)HCO\(_3\), and 50 ml of 8 N NH\(_4\)OH. The fractions again were assayed for radioactivity and ninhydrin-positive substances. The radioactive eluates from the Dowex 50 chromatography were pooled. Aliquots were applied to sheets of water-washed Whatman No. 3MM paper. The solvent system used for chromatography was 1-butanol-acetic acid-H\(_2\)O (4:1:2). After spraying the guide strips on both sides of the chromatogram, the paper was cut and the desired compounds were eluted with water.

**Synthesis of e-N-Benzyllysine**—The compound was synthesized by addition of solid NaBH\(_4\) to a 1-m solution of N-\(\alpha\)-acetyl-\(\tau\)-lysine methyl ester (15 mg) and 1 ml of benzaldehyde-saturated water solution. The mixture was taken to dryness and then hydrolyzed with 5.7 N HCl for 24 hours. The dried hydrolyzate was applied to a column of Dowex 50 (H\(^{+}\) form, 2 x 10 cm, see previous paragraph) and eluted with 2 N NH\(_4\)OH. The product was localized by spotting aliquots of the eluate on paper and by spraying the paper with ninhydrin. The e-N-benzyllysine-containing fractions were pooled and dried.

**Mass Spectral Analyses**—The LKB 9000 mass spectrometer with gas chromatographic inlet was used. The gas chromatographic column (3-foot glass column packed with 3% OV 17 on Chromosorb WAW, 100 to 120 mesh, 140\(^\circ\)C temperature, and temperature rise of 5\(^\circ\) per min) detected the number of compounds present in the sample. The mass spectrometer was run with an ion source temperature of 250\(^\circ\), ionizing voltage of 70 e.v. and an accelerating voltage of 3.5 kv. About 100 \(\mu\)g of sample was heated with 1 ml of CH\(_4\)COOH and 1 ml of HCl (1.25 \(N\)) at 100\(^\circ\) for 1 hour and, after removing the solvent, trifluoroacetylated with a mixture of 200 \(\mu\)l of trifluoroacetic acid anhydride (1 volume) and CH\(_2\)Cl\(_2\) (4 volumes) for 1 hour at room temperature. For each run in the mass spectrometer, about 2 \(\mu\)g of derivative in CH\(_2\)Cl\(_2\) were analyzed.

**RESULTS**

**Preliminary Studies**—The conditions suitable for the so-called trapping of the enzyme-substrate complex with NaBH\(_4\) were examined. The inhibition of the beef plasma amine oxidase by sodium borohydride was found to be dependent upon the pH of the reaction mixture. However, no effect of NaBH\(_4\) in the absence of benzylamine was observed in the range from pH 6 to 9. At increasingly higher pH values beyond 9, activity losses were noted relative to the control sample. At pH 10.5, approximately 50% activity remained following incubation of the enzyme with sodium borohydride alone. Therefore, the reactions of enzyme, benzylamine, and borohydride were conducted at pH 7.2 where pH effects upon the enzyme were not significant.

**Rate of Enzyme Inactivation in Trapping Experiments**—As shown in Fig. 1, the enzyme lost 82\% of its initial activity after 3 hours while a control sample of enzyme treated in an identical manner except for the addition of NaBH\(_4\) lost only 8\% of its activity.

In a separate experiment, the absorption of the reaction mixture without NaBH\(_4\) was measured after 5 hours to estimate the amount of benzaldehyde formed. This quantity of benzaldehyde then was substituted for the amine substrate and incubated with another enzyme sample. Sodium borohydride was added and the reaction was allowed to proceed for 5 hours. The enzyme, after passage through a column of Sephadex G-25 F, retained 99\% of its initial enzyme activity.

**Moles of \(^{14}C\)-Labeled Benzylamine Incorporated in Enzyme**—The moles of substrate (or product) incorporated into the enzyme using \(^{14}C\)-labeled benzylamine was determined. The results, summarized in Table I, show that 1 to 2 moles of radioactive compound were incorporated per mole of enzyme.

**Hydrolysis of \(^{14}C\)-Labeled Enzyme**—The purified labeled enzyme was hydrolyzed with 5.7 N HCl and an aliquot was applied to paper for chromatographic analysis. The developed chromatogram showed a discrete radioactive compound with an RF of 0.83. The recovery of radioactivity at this stage was about 90\% of the initial radioactivity.

**Further Purification of Radioactive Compound**—The filtered acid hydrolysate of the enzyme then was applied to a column of Dowex 1 and eluted as described under "Experimental Procedures."

![Fig. 1](http://www.jbc.org/) Kinetics of the inactivation of plasma amine oxidase during the reaction of the enzyme-substrate mixture with NaBH\(_4\). The reaction conditions are described under "Experimental Procedures." Curves show the activity in the absence (A) and the presence (B) of NaBH\(_4\) in the reaction mixture.

**TABLE I**

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<th>Incorporation of (^{14}C) into enzyme with (^{14}C)-labeled benzylamine (substrate) and NaBH(_4) reduction of enzyme</th>
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\* Specified activity of benzylamine used.

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FIG. 2. Absorption spectrum of the radioactive compound and pyridoxyl-benzylamine. a, radioactive compound; b, pyridoxyl-benzylamine; and c, synthetic ε-N-benzyllysine.

procedure.” About 80 to 85% recovery of the applied radioactivity was obtained in this step.

The pooled radioactive fraction was further purified by Dowex 50-H+ column chromatography. The column separated the lysine from the astigmine, the label remaining with the lysine fraction. Paper chromatography of the pooled radioactive fraction showed the presence of two ninhydrin-positive substances. The major component was lysine and the minor component contained the radioactivity.

In the final purification step, preparative paper chromatography led to the isolation of a pure radioactive compound. The yield of radioactivity in this step was about 70%. Rechromatography of the eluted compound showed a single spot when the chromatogram was sprayed with ninhydrin and the radioactivity was associated with this compound.

Chemical Properties of Radioactive Product—The isolated compound was ninhydrin-positive, eluted at the lysine position in the automatic amino acid analyzer, and had an $R_P$ of 0.53 in the solvent system 1-butanol-HOAc-H2O (4:1:2).

One possible structure for the compound was pyridoxyl-benzylamine. Therefore, this compound was synthesized and the absorption spectra of the radioactive compound were isolated from bovine plasma amine oxidase and authentic pyridoxyl-benzylamine were determined (Fig. 2). The spectra of these two compounds are quite different, which rules out the possibility that the reduced Schiff’s base of pyridoxal and benzylamine had been isolated. Another possible structure, ε-N-benzyllysine, also was synthesized as described above. The mass spectra of these two compounds are very similar (Fig. 2).

Mass Spectra Analysis of Radioactive Compound—The pure radioactive compound which had been O-methylated and trifluoroacetylated was analyzed in the mass spectrometer. The product gave only one peak on gas chromatography. The mass spectrum of this radioactive derivative is shown in Fig. 3. The molecular ion of the O-methylated trifluoroacetic acid-derivative compound had a m/e ratio of 346. The most abundant ion occurred at a m/e ratio of 91 units. The major peaks are consistent with the expected fragmentation pattern of the reduced benzaldehyde-lysine complex. Thus, the compound formed upon the addition of NaBH₄ to a plasma amine oxidase-benzylamine mixture is ε-N-benzyllysine and not pyridoxyl-benzylamine.

DISCUSSION

The diamine oxidases are widely distributed in nature, being found in bacteria, fungi, plants, and animals (13). Mammalian diamine oxidases are found in the plasma and kidney. Both types have been isolated in a highly purified crystalline form, making it possible to investigate the prosthetic groups of the enzyme (2, 4). Identification of the organic cofactor is difficult since it cannot be freed from the enzyme by conventional procedures such as acid or base treatment of the enzyme at 4°C (1).

There have been reports that the cofactor is pyridoxal phosphate. Several have reported the isolation of the substrate-pyridoxal adduct from acid hydrolyzates of the NaBH₄-reduced enzyme-substrate mixtures (4, 6, 7) using ³⁵C-labeled substrates to provide a convenient and sensitive label of the product.

This trapping technique was applied here to bovine plasma amine oxidase. Aerobic rather than anaerobic conditions (14) were chosen since they appeared to provide optimal conditions for trapping the cofactor (enzyme)-substrate complex.

Sufficient amounts of enzyme and ³⁵C-labeled substrate were used to make possible the isolation of a pure radioactive compound from the NaBH₄ trapping experiment. The spectrum, $R_P$ and chromatographic behavior of the product all indicated that the product was not the expected pyridoxal-benzylamine. The mass spectrum of the radioactive compound proves that the radioactive product isolated from the enzyme was ε-N-benzyllysine.

The question arises whether the production of ε-N-benzyllysine in these experiments is peculiar to the bovine plasma amine oxidase or whether it was also formed in previously reported experiments with pig plasma amine oxidase (6), kidney histaminase (4), and the A. niger amine oxidase (7). All of these enzymes have been reported to contain pyridoxal phosphate as a cofactor. Consider first A. niger amine oxidase. The re-
ported mechanism for the oxidation of substrate by this enzyme (15) is almost identical with that reported for the bovine plasma amine oxidase (14). Thus, trapping the substrate on the enzyme with NaBH₄ should yield the same product with both amine oxidases. We have examined the experimental evidence of Adachi et al. (7) and find that the electrophoretic mobility and the fluorescence data of the isolated material from the trapping experiments do not agree with the properties of the synthetic ε-N-ethyl-pyridoxal amine. Likewise, when the electrophoretogram of the product obtained from kidney histaminase and the synthetic N-histaminy1-pyridoxal amine are compared, they have similar electrophoretic mobilities but again their mobilities are not identical (4). Thus, the claim of these studies that the cofactor of the enzyme is pyridoxal phosphate cannot be accepted at the present time.

Two mechanisms may be proposed for the formation of ε-N-benzyllysine. One is that the benzaldehyde reaction product subsequently forms a Schiff’s base with a reactive lysine ε-NH₂ group of the enzyme. This seems unlikely based on the present finding that the addition of NaBH₄ to enzyme-benzaldehyde mixtures do not inhibit the enzyme. The amount of benzaldehyde used was equivalent to the amount of the aldehyde produced in the absence of NaBH₄. A more plausible mechanism is trapping of an enzyme-substrate intermediate formed prior to the release of benzaldehyde.

Sodium borohydride reduction of the substrate-d-amino acid oxidase mixture also results in the trapping of the keto acid product which forms a Schiff’s base with the ε-NH₂ group of a lysine residue in the enzyme (16). Massey et al. (17) subsequently confirmed this finding but reported that the activity of d-amino acid oxidase is not affected. Bovine plasma amine oxidase, however, is inactivated.

REFERENCES

Cofactor Investigation of Bovine Plasma Amine Oxidase: NaBH4 REDUCTION OF ENZYME-SUBSTRATE MIXTURE
Melvin Inamasu, Kerry T. Yasunobu and Wilfried A. Konig


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