Cofactor Investigation of Bovine Plasma Amine Oxidase

NaBH₄ REDUCTION OF ENZYME-SUBSTRATE MIXTURE*

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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 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 are copper-pyridoxal phosphate enzymes (1–7).

Materials

Bovine Plasma Amine Oxidase—The enzyme was isolated by a modified procedure of Yamada and Yasunobu 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 µmol. 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 benzoaldehyde 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°C at pH 7.2. The protein concentration was determined spectrophotometrically on the basis that the E₁ cm at 280 nm is 9.8. 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 0.535 mg of benzylamine were mixed and reduced with 5 µg of NaBH₄. No purification of the product was attempted.

NaBH₄ Reduction of Enzyme-¹⁴C-Labeled Benzylamine Mixtures—A 1000-fold excess (0.5 µl of a 0.1 M solution) of benzylamine was added to 0.5 µl 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 µg of NaBH₄ in 0.1 ml of 0.1 N NaOH were added. At various times, 0.15 µl aliquots of this mixture were applied to Sephadex G-25F columns (1 X 10 cm) to separate protein from reactants. The protein was assayed immediately for activity. A control in which 0.1 µl 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...
**RESULTS**

**Preliminary Studies**—The conditions suitable for the so-called trapping of the enzyme-substrate complex with NaBH₄ 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 NaBH₄ 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 NaBH₄ lost only 8% of its activity.

In a separate experiment, the absorbance of the reaction mixture without NaBH₄ 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.

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**Table I**

| Experiment | Enzyme Cpm/μmole | U Cpm/μmole | Moles U/C mole enzyme |
|------------|------------------|-------------|-----------------------|
| 1          | 0.18             | 180         | 380                   | 0.5                    |
| 2          | 0.27             | 16          | 14                    | 1.1                    |
| 3          | 7.76             | 940         | 14                    | 0.7                    |
| 4          | 9.90             | 400         | 180                   | 2.2                    |

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

The pooled radioactive fraction was further purified by Dowex 50-H+ column chromatography. The column separated the lysine from the arginine, 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.

Chromatography of the eluted compound showed that the product was ninhydrin-positive, eluted at the lysine position in the automatic amino acid analyzer, and had an $R_f$ of 0.53 in the solvent system 1-butanol-HOAc-H$_2$O (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 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-derivatized 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 NaBH$_4$ 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 NaBH$_4$-reduced enzyme-substrate mixtures (4, 6, 7) using C-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 C-labeled substrate were used to make possible the isolation of a pure radioactive compound from the NaBH$_4$ trapping experiment. The spectrum, $R_f$ 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-histaminyl-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-ε-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 ε-amino acid oxidase is not affected. Bovine plasma amine oxidase, however, is inactivated.

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