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BOVINE PLASMA AMINE OXIDASE INTERACTIONS WITH CONCANAVALIN A IN SOLUTION AND WITH CONCANAVALIN A-SEPHAROSE

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Summary

The reaction of bovine plasma amine oxidase, a glycoprotein, with Concanavalin A in 0.1 M potassium phosphate buffer, pH 7.0 at 25°C were investigated by equilibrium and kinetic methods. A tentative mechanism for the reaction was derived. The Concanavalin A-enzyme interaction was used to show that the carbohydrate is not essential for activity and that the carbohydrate is covalently attached to the protein at a site distant from the active site. Concanavalin A-Sepharose 4B affinity chromatography of the enzyme was found to be useful for obtaining the pure enzyme. Chromatographic conditions which elute the enzyme are reported. The superiority of the Concanavalin A-Sepharose 4B over that of the substrate-affinity Sepharose affinity support is discussed. The phenylhydrazone derivative of the enzyme, which was inactive, was shown to bind to the concanavalin A-Sepharose column.

Introduction

Plasma amine oxidase (diamine:oxygen oxidoreductase (deaminating), EC 1.4.3.6) is a diamine oxidase [1] or a Cu-amine oxidase [2] which catalyzes the following reaction [3].

$$RCH_2NH_2 + O_2 + H_2O = RCHO + NH_3 + H_2O_2$$

The enzyme contains 2 gatoms of Cu^{2+} [2,4] and is a pink type II copper protein [5]. It also contains 1 mol of a carbonyl containing organic cofactor

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[6]. The molecular weight of the bovine enzyme has been reported to be 170 000 [7] and to be made up of two subunits, each with a molecular weight of 85 000 [7]. The formal mechanism for the oxidation of benzylamine at pH 7.0 has been reported [8,9]. The procedure for obtaining crystalline bovine plasma amine oxidase has been reported [10]. These preparations of the enzyme have been used to show that the enzyme contains essential histidine [11] and lysine residues [12]. The bovine enzyme has been shown to be a glycoprotein [13] which contained galactose, mannose, sialic acid, and *N*-acetylglucosamine. The glycoprotein nature of the bovine enzyme has prompted us to explore the uses of concanavalin A in bovine plasma amine oxidase research and the results of these explorations are described in this report.

Materials and Methods

Materials. Bovine plasma amine oxidase was isolated as previously described [2]. Where important, enzyme (specific activity 1000) which was purified by polyacrylamide gel electrophoresis was used, otherwise, the specific activity of the enzyme is indicated in the text. All of the chemicals used in this study were of reagent grade. Concanavalin A and Sepharose 4B were purchased from Pharmacia Fine Chemicals. α-Methyl-D-mannoside and α-methyl-D-glucoside were purchased from Sigma Chemical Co. Cyanogen bromide and ethylene glycol were the products of the Aldrich Chemical Co. and Eastman Organic Chemicals, respectively.

Enzyme assay and protein determination. The enzyme activity was measured spectrophotometrically [3], using a Cary Model 14 automatic recording spectrophotometry using a benzylamine concentration of 3.3 mM in 0.1 M potassium phosphate (pH 7.0) at 25°C. Specific activity was calculated as the number of units of enzyme per mg protein. One unit was defined as the amount of enzyme which catalyzed a change of 0.001 absorbance units per min. The protein concentration was determined spectrophotometrically at 280 nm by the use of an $E^{1\%}_{1cm}$ of 20.8 which was obtained from absorbance and dry weight determinations.

Kinetics. The preliminary kinetic studies indicated that the Concanavalin A-enzyme complex (molar ratio of 2 : 1) was being produced when the reaction was carried out in 1.0 ml of 0.1 M potassium phosphate buffer, pH 7.0 and at 25°C. Since this complex was insoluble, the rate of the reaction was followed by turbidity measurements at 600 nm. The rate of the reaction followed second-order kinetics, the equation of which is shown below [14]:

$$\ln \frac{(B)_0 - X}{(A)_0 - X} = \ln(B)_0/(A)_0 + ((B)_0 - (A)_0)\, kt \qquad (1)$$

In this equation, t stands for time, X is the amount of A or B which has reacted at time 5, while $(A)_0$ and $(B)_0$ represents the initial concentrations of bovine plasma amine oxidase and Concanavalin A, respectively, and k is the second-order rate constant. The concentration of X was determined from the measurement of turbidity at 600 nm and by correlating turbidity values with concentration of the product.

Quantitative precipitin reactions. The reactions were carried out in analogous manner to the precipitin reaction used in immunology to investigate antigen-antibody reactions [15]. The total volume for each sample was 1.2 ml and the solvent was 0.1 M potassium phosphate buffer, pH 7.0. The enzyme concentration was kept constant at 0.2 mg and from 0.065 to 0.194 mg of the lectin were mixed vigorously in separate tubes and were allowed to stand at 4°C for 36 h. At this time, the samples were centrifuged at 27 000 × *g* in the Sorvall RC 2B centrifuge for 20 min. Aliquots of the supernatants were measured at 280 nm to determine the protein contents and enzyme activities. The amount of protein in the precipitates were determined by dissolving the washed precipitates in 1.0 N NaOH and by taking 280 nm absorbances of the samples.

Preparation of Concanavalin A-Sepharose. Concanavalin A-Sepharose was prepared by the cyanogen bromide coupling method described by Cuatrecasas and Anfinsen [16]. Any remaining active groups were blocked by suspending the gels in 1 M ethanolamine at pH 8.0 and by stirring gently for 2 h [17].

Determination of the activity of the lectin-enzyme precipitate. The Concanavalin A-enzyme precipitate was obtained by incubating a 3-fold molar excess of concanavalin A (0.38 mg) and enzyme (0.2 mg) in 0.1 M potassium phosphate buffer, pH 7.0 at 4°C for 36 h. The precipitated enzyme was washed twice with 1.0 ml of buffer and was centrifuged at 27 000 × *g* for 30 min. To the washed precipitate, 3.0 ml of 0.1 M potassium phosphate buffer, pH 7.0 which contained 1.0 ml of 0.1 M benzylamine was added. Such mixtures were allowed to proceed for 1, 2, 5, and 10 min with constant stirring. Immediately after the reaction, the samples were filtered and the absorbance at 250 nm were measured.

Results

Precipitin reaction of Concanavalin A and homogeneous plasma amine oxidase

The conditions under which maximum precipitation between Concanavalin A and polysaccharide have been worked out by So and Goldstein [18]. In this study, various mixtures of Concanavalin A to plasma amine oxidase were reacted at constant enzyme concentrations at 4°C for 36 h in 0.1 M potassium phosphate buffer, pH 7.0. After centrifugation, the supernatants were collected and the supernatants were analyzed for enzyme activities and for protein contents. The precipitates were dissolved in 1.0 N NaOH and the absorbances of the samples were read at 280 nm. The results obtained from these experiments are shown in Fig. 1. At a 1 : 1 molar ratio of Concanavalin A to enzyme, the complex formed was mainly soluble and active since the amount of protein which precipitated was small. About 80% of the amine oxidase existed as the insoluble Concanavalin A-enzyme complex when the molar ratio was 2 : 1. At molar ratios greater than 2, the activity in the supernatant approached zero and the amount of protein in the supernatant continued to increase due to the presence of excess of lectin.

Kinetics of Concanavalin A-amine oxidase complex formation

The rate of the insoluble Concanavalin A-enzyme complex formation was determined at 25°C in 1.0 ml of 0.1 M potassium phosphate buffer, pH 7.0

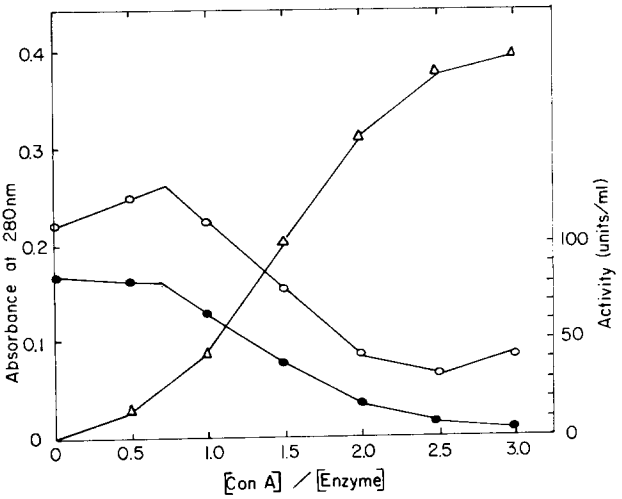

Fig. 1. Precipitin reaction between plasma amine oxidase and Concanavalin A. Various molar ratios of Concanavalin A to enzyme were incubated as described in the methods section at 4°C for 36 h. The absorbance at 280 nm (○———○), and activity (●———●) of the supernatant as well as the protein in the precipitate (△———△) were measured.

from turbidity measurements at 600 nm. The enzyme concentration was kept constant at 0.3 μM and Concanavalin A was varied so that the lectin/enzyme ratio varied from 1.0 to 4.0. When the data was plotted according to the second-order reaction, a series of parallel lines were obtained. From the slopes of the lines, k_{obs} was calculated and a plot of k_{obs} versus the lectin/enzyme ratio is shown in Fig. 2. For Concanavalin A to enzyme molar ratios between 0—1, the rate of insoluble complex formation was slow; between 1—2, k_{obs} increased indicating fast insoluble complex formation; and beyond a ratio of 2,

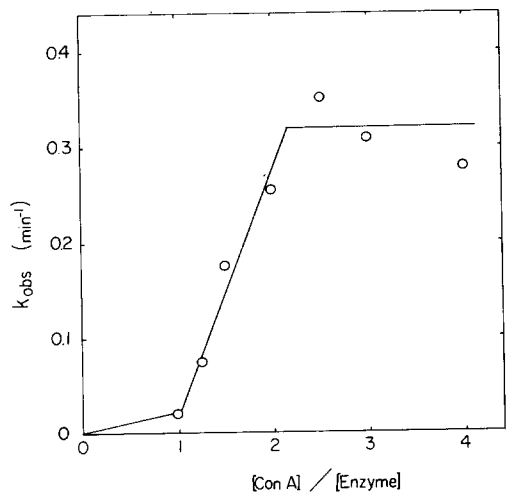

Fig. 2. Dependence on Concanavalin A concentration of second-order rate constant for plasma amine oxidase and Concanavalin A insoluble complex formation. The second-order rate constants, k_{obs} were obtained from Fig. 2.

it appeared that the k_{obs} was independent of the Concanavalin A concentration.

Determination of activity of the lectin-enzyme precipitate

The Concanavalin A-enzyme precipitate was obtained by the precipitin reaction. After incubating 0.2 mg of the enzyme with 0.38 mg of Concanavalin A (lectin/enzyme rate of 3.0) at 4°C for 36 h, in 1.2 ml of 0.1 M potassium phosphate buffer, pH 7.0, it was collected by centrifugation and washed with the buffer. The supernatant contained no enzyme activity. Aliquots were added to the substrate, benzylamine, and the reactions were allowed to proceed for various time intervals. Immediately after each reaction, the sample were filtered and the absorbances at 250 nm were determined. The enzyme was as active as the uncomplexed enzyme.

Interactions and elutions of partially purified enzyme from Concanavalin A-Sepharose columns

When 2.15 mg of partially purified enzyme (spec. act. 475 units/mg) was applied to a Concanavalin A-Sepharose column (1.5 × 8 cm) preequilibrated with 0.1 M potassium phosphate buffer, pH 7.0, the enzyme was absorbed to the column while the impurities passed through the column as shown in Fig. 3. The enzyme was eluted from the column by a buffer containing 0.1 M α-methyl-D-mannoside (Fig. 3). The specific activity of enzyme eluted was doubled and yields of enzyme varied from 50—70%. The enzyme was also eluted by 0.1 M sodium borate buffer, pH 9.0 (Fig. 4A), while no enzyme was eluted with 0.1 M sodium carbonate buffer, pH 9.5.

In order to see if hydrophobic interactions are involved in the binding of

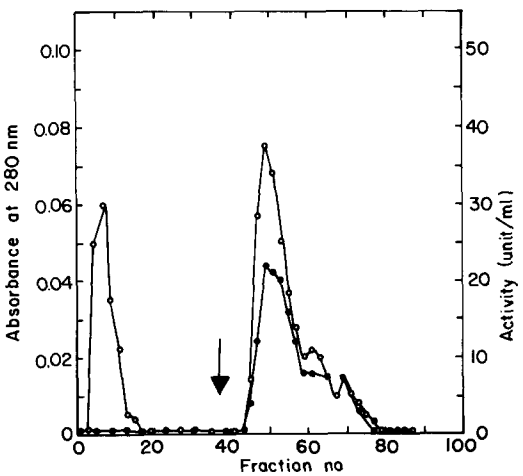

Fig. 3. Concanavalin A-Sepharose affinity chromatography of partially purified plasma amine oxidase. The enzyme (2.15 mg, 475 units/mg) was applied onto a concanavalin A-Sepharose column (1.5 × 8 cm) which had previously been equilibrated with 0.1 M potassium phosphate buffer, pH 7.0. The column was first washed with the equilibration buffer. At the point indicated by the arrows, elution was done with the equilibration buffer containing 0.1 M α-methyl-D-mannoside. Fractions (2.0 ml) were collected, and absorbance at 280 nm (○———○) and enzyme activity (●———●) were measured.

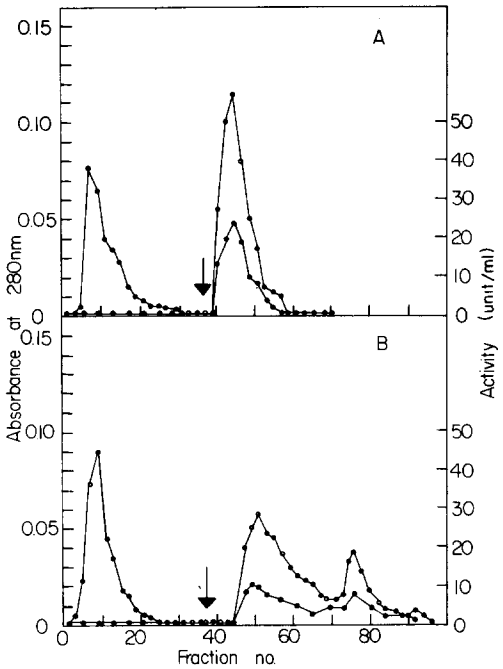

Fig. 4. Elution of plasma amine oxidase by borate buffer and ethylene glycol from Concanavalin A-Sepharose column. The enzyme (2.15 mg, 475 units/mg) was applied onto a Concanavalin A-Sepharose column (1.5 × 8 cm) which had previously been equilibrated with 0.1 M potassium phosphate buffer, pH 7.0. The column was first washed with the equilibration buffer. At the points indicated by the arrows, elution was performed with 0.1 M sodium borate buffer, pH 9.0 (A) and equilibration buffer containing 50% (v/v) ethylene glycol (B). Fractions (2.0 ml) were collected, and absorbance at 280 nm (○———○) and enzyme activity (●———●) were measured.

enzyme to the affinity column, elution was attempted with 50% (v/v) ethylene glycol, a polarity reducing reagent. As shown in Fig. 4B, the enzyme was eluted in two distinct peaks (45.8% of activity and 18% of protein).

Chromatography of the enzyme-phenylhydrazine complex on Concanavalin A-Sepharose

Cu-amine oxidases have been shown to form a phenylhydrazine complex in which one mole of phenylhydrazine is incorporated irreversibly into the enzyme with inactivation of the enzyme activity [6]. When the enzyme-phenylhydrazine complex was passed through the lectin-Sepharose column, it was absorbed by the affinity column and it was eluted by 0.1 M α-methyl-D-glucoside.

Discussion

Bovine plasma amine oxidase is a glycoprotein which contains 7—8% carbohydrate which included mannose, galactose, sialic acid, and glucosamine [13]. Concanavalin A has a reported molecular weight of 110 000 at pH 7.0 and exists as a tetramer [19]. Each of its subunits has the capacity to bind one mole of glucose or mannose or glycoproteins which contain these sugars. The

report on the kinetics of the turbidimetric reaction has been published [20]. The recent study on the kinetics of finding of Concanavalin A with chromogenic mono- and disaccharides, as detected by stopped-flow spectroscopy, showed the mono- and biphasic natures of the reactions, respectively [21]. In the present investigation the kinetics and stoichiometry of the reaction were measured by quantitative precipitin and turbidity measurements, the latter technique which indicated the reaction followed second order kinetics. The reaction appeared to proceed as follows as pH 7.0 and at 25°C:

Concanavalin A + Enzyme = Concanavalin A-enzyme soluble (1)

Concanavalin A + Concanavalin A-enzyme soluble = (Concanavalin A)$_2$-enzyme insoluble (2)

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2 Concanavalin A + Enzyme = (Concanavalin A)$_2$-enzyme insoluble (3)

Reaction 1 occurred chiefly when the lectin/enzyme ratio was below 1. At greater ratios of lectin/enzyme, the (Concanavalin A)$_2$-enzyme complex which is insoluble is chiefly present. The rate constant for Reaction 3 was about 0.3 min^{-1}. It is interesting to note that the insoluble complex would be predicted to have the composition (Concanavalin A)$_2$-enzyme which indicated that there are two Concanavalin A combining sites in the enzyme and despite the presence of 4 carbohydrate binding sites in Concanavalin A, only one of these sites is being utilized in complex formation with the enzyme. A priori, it would appear that complexes with the composition (Concanavalin A)$_n$-enzyme where n is greater than 2 should form but due to the insolubility of (Concanavalin A)$_2$-enzyme complex, the (Concanavalin A)$_n$-enzyme aggregates are not formed. However, the kinetics of the reaction must be checked by other physicochemical techniques before the proposed mechanism can be accepted as established.

It was important to determine if the (Concanavalin A)$_2$-enzyme complex was active or not. When the activity was checked with the substrate benzylamine, it was determined that the complex was fully active. Since the Concanavalin A tetramer has a molecular weight of 110 000 it is a fairly large molecule. In the (lectin)$_2$-enzyme complex, if Concanavalin A reacted with the carbohydrate which is near the active center, the enzyme activity would most likely be inhibited. Therefore, it would appear that the carbohydrate, i.e. mannose in the enzyme is located at some distant from the active center. Furthermore, the carbohydrate in the enzyme appears to be unessential for activity since the lectin-Concanavalin A-enzyme complex is fully active.

Besides the use of Concanavalin A as a probe of the carbohydrate attachment site in the enzyme, obviously it can be used to purify the enzyme when it is coupled to Sepharose and used as an affinity column. A procedure for obtaining pure preparations of the bovine plasma amine oxidase has been published by our laboratory [10]. In this method, the final step of enzyme purification entails repeated crystallization of the enzyme using ammonium sulfate. This is a time-consuming and a low yield method. In the present investigation, when the enzyme (after the hydroxyapatite chromatography

step) was purified by Concanavalin A-Sepharose chromatography, the specific activity of the enzyme was nearly doubled and final purification could be obtained by passing the eluate through a Biogel A 1.5 M column [2]. The enzyme absorbed to the Concanavalin A-Sepharose column was eluted by α-methyl-D-mannoside and by 0.1 M borate buffer, pH 9.0 but not by 0.1 M sodium carbonate pH 9.5. Although ethylene glycol, a polarity reducing, hydrophobic bond breaker, was only partially effective in eluting the enzyme from the affinity column, it would appear that the enzyme binds to the column both by hydrophobic interactions and carbohydrate recognition. Several papers dealing with the effect of ethylene glycol [22,23] and borate [24] on carbohydrate-Concanavalin A interactions have been reported. In our hands, the substrate-affinity chromatography procedure reported by Toraya et al. [25] yields altered enzyme which was quite unstable and showed variable specific activity. Moreover, the diamino-butane-Sepharose solid support could be used only once while the Concanavalin A-Sepharose could be used repeatedly.

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