Stereospecificity of Sodium Borohydride Reduction of Schiff Bases at the Active Site of Aspartate Aminotransferase*

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Sodium borohydride treatment of holoaAspartate aminotransferase results in the reduction of the Schiff's base formed between pyridoxal phosphate and Lys 258. Treatment of the reduced enzyme with papain followed by acid hydrolysis liberates e-N-[3H]pyridoxyl lysine which is degraded to [3H]pyridoxamine diHCl and stereocohemically analyzed with apoaspartate aminotransferase. Sodium borohydride treatment of active site carbamylated aspartate aminotransferase reconstituted with pyridoxyl phosphate and sodium aspartate results in the trapping of an enzyme-substrate complex through the reduction of the Schiff's base formed between pyridoxal phosphate and aspartate. Active site bound N-[3H]pyridoxyl aspartate is liberated by treatment with papain and degraded to [3H]pyridoxamine diHCl for stereochemical analysis. Borohydride reduction of the holoenzyme occurs from the re face of the pyridoxal phosphate Lys 258 Schiff's base. Similarly, reduction of active site carbamylated enzyme-substrate complex occurs from the re face of the pyridoxal phosphate-aspartate Schiff's base. These results indicate that when active site carbamylated enzyme binds substrate to pyridoxal phosphate it does so stereospecifically and without changing the face of the Schiff base that is available for reduction as compared to native enzyme.

Stereospecific studies of aspartate aminotransferase as well as other pyridoxal phosphate-dependent enzymes have led to a detailed understanding of the role played by pyridoxal phosphate in the mechanism of catalysis by those enzymes. Dunathan (1) suggested that one of the primary catalytic functions of pyridoxal phosphate enzymes is to orient the cofactor-substrate complex in a reactive conformation. Such an orientation has the breaking and forming bonds orthogonal to the plane of the conjugated π system so as to stabilize the developing charge. Also, the cofactor-substrate complex would be rapidly bound by these enzymes on a single face, thereby forcing all the breaking and making of bonds to occur on the opposite side. In aspartate aminotransferase, the Cα-H bond is broken and the C-4'-H bond formed through a cis process occurring on the si face of the cofactor-substrate complex (Scheme 1).

Recent studies (2-4) have shown that in at least two other pyridoxal phosphate enzymes, reduction of enzyme-bound Schiff's base intermediates is an effective means of determining the exposed "solvent side" of cofactor-substrate complexes. The emerging mechanism is one where holoenzymes, in the absence of substrate, show a preference for hydride attack from the re site but when substrate is present, attack appears to occur from the si face (Scheme 1). Conformational change in the enzyme or a rotation of the pyridoxal phosphate upon substrate binding must be postulated in order to account for these results (5, 6). A problem with this approach is that transformation to products occurs extremely fast and the reduced Schiff's base intermediates can only be isolated by the dilution technique (7), and necessarily with very low radiochemical specific activities.

We have shown that after carbamylation of the active site Lys 258 (8), aspartate aminotransferase is still able to undergo the half-transamination reaction with conversion of active site bound pyridoxal phosphate to pyridoxamine phosphate. Subsequent work (9) showed that stereospecific deuterium addition in D2O solvent occurs from the si side resulting in a deuterium atom being introduced at the pro-S hydrogen of the pyridoxamine methylene group (Scheme 1). These results agreed with similar observations using the nonphosphorylated pyridoxal coenzyme (10) and constitute a demonstration of the retention of stereochemistry in both half-transamination processes, those which utilize phosphorylated coenzyme under single turnover conditions and those in hr, utilizing the nonphosphorylated coenzyme. They also illustrate that free Lys 258 is not required to maintain overall stereospecificity in the half-transamination process.

This present work describes our study of the sodium borohydride reduction of both holoaAspartate aminotransferase and carbamylated aspartate aminotransferase trapped in an enzyme-substrate complex in order to determine the solvent "exposed face" of the cofactor Schiff's base with both the active site Lys 258 and when forming an enzyme-substrate complex.

**EXPERIMENTAL PROCEDURES**

*Materials—Commercically available chemicals were of reagent grade or highest purity available and used without further purification. Papain and alkaline phosphatase were purchased from Sigma Chemical Co. Nonradioactive N-pyrodoxy l-Aspartate and e-N-pyrodoxy l-lysine were prepared by reported procedures (4, 11, 12). Sodium borohydride (282 to 320 mCi/mmol) was purchased from New England Nuclear Co.

*Instrumentation—Nuclear magnetic resonance spectra were measured on Bruker WP-80 spectrometer. Ultraviolet spectra and kinetic measurements were obtained on Cary 15 and Cary 210 spectrophotometers. Scintillation counting was done in Triton X-100 based scintillation solutions on a Beckman LS100C instrument. Counting efficiency was determined by addition of a known quantity of [3H]toluene standard (New England Nuclear). Electrophoresis was obtained with a Savant Instruments High Voltage Electrophoresis apparatus.

*Chromatography—High voltage paper electrophoresis employed Whatman No. 1 paper using a pyridine/acetic acid/water (1:5:94)
buffer (pH 4) and 3000 V DC for 30 min. Precoated Silica Gel G plates from Analtech Inc. (0.25 mm thickness) were used for thin layer chromatography. The following solvent systems were used: ethanol/water (7:3 or 4:1) and ethanol/concentrated NH₄OH (85:15). Visualization methods included UV, ninhydrin spray, iodine vapor, and Gibb's reagent (13).

Isolation and Carbamylation of Aspartate Aminotransferase—Supernatant aspartate aminotransferase (EC 2.6.1.1) was isolated from porcine heart and apoenzyme prepared as previously described (14). Carbamylation and reconstitution with pyridoxal phosphate were carried out by treatment of the apoenzyme as previously reported (8).

Reduction of Holoaspartate Aminotransferase with Sodium Borohydride—In a typical holoenzyme reduction, the holoaspartate aminotransferase (100 mg) dissolved in 0.1 N sodium phosphate buffer at pH 7.5 (7 ml) was added directly to 25 mCi of sodium borohydride and the reaction monitored by UV absorbance at 430 nm until reduction was complete. The reaction mixture was then dialyzed against two changes (1 liter) of distilled water and lyophilized. The residue was hydrolyzed with papain (3 pmol) in pyridine acetate buffer (3 ml), pH 5.5, and 0.01 M 2-mercaptoethanol at 37°C overnight. The clear hydrolysate was fractionated on a Sephadex G-25 column (2 x 40 cm). The column was eluted with 0.1 M sodium borate, pH 8, and 5-ml fractions were collected. Tubes 8 to 11 were combined and dialyzed against water (2 liters) and lyophilized. The residue was hydrolyzed with papain (3 pmol) in pyridine acetate buffer (3 ml), 0.01 M 2-mercaptoethanol at 37°C overnight. The clear hydrolysate was filtered through an Amicon UM-05 filter. The filtrate was dissolved in 10 ml Tris buffer (6 ml) pH 8.1, and treated with alkaline phosphatase (10 mg) at 37°C in darkness for 18 h. The alkaline phosphatase was precipitated by addition of 100% trichloroacetic acid (1 ml), and the mixture centrifuged at 15,000 rpm for 1 h. The supernatant liquid was evaporated to dryness in vacuo at 40°C. The residue was dissolved in distilled water (2 ml) and N²[^1]H]pyridoxyl L-lysine isolated by chromatography on AG 50 H⁺ resin (2 x 40 cm) with a linear gradient of 2 to 5 N HCl (350 ml each) (Fig. 1). No impurities or significant changes in specific radioactivity could be observed by paper electrophoresis or thin layer chromatography.

Reduction of Carbamylated Aspartate Aminotransferase with Sodium Borohydride—Carbamylated apoaspartate aminotransferase (200 mg) reconstituted with pyridoxal phosphate was incubated with 50 mM L-aspartate in 0.1 M sodium borate buffer (5 ml), pH 8, at 37°C until Schiff's base formation was complete (maximal absorbance at 440 nm). The incubation mixture was then added to 25 mCi of sodium borohydride and the reduction monitored by the decrease at 440 nm and concomitant increase at 330 nm (Fig. 2). After 2 h, 10 mg of inactive sodium borohydride were added to complete the reduction. The reduced mixture was then passed through a Sephadex G-25 column (2 x 40 cm) eluted with 0.1 M sodium borate, pH 8, and 5-ml fractions were collected. Tubes 8 to 11 were combined and dialyzed against water (2 liters) and lyophilized. The residue was hydrolyzed with papain (3 pmol) in pyridine acetate buffer (3 ml), 0.01 M 2-mercaptoethanol (pH 5.5) at 37°C overnight. The clear hydrolysate was filtered through an Amicon UM-05 filter. The filtrate was dissolved in 10 ml Tris buffer (6 ml) pH 8.1, and treated with alkaline phosphatase (10 mg) at 37°C in darkness for 18 h. The alkaline phosphatase was precipitated by addition of 100% trichloroacetic acid (1 ml), and the mixture centrifuged at 15,000 rpm for 1 h. The supernatant liquid was evaporated to dryness in vacuo at 40°C. The residue was dissolved in distilled water (2 ml) and N²[^1]H]pyridoxyl L-aspartate isolated by ion exchange chromatography on AG 50 H⁺. Linear gradient elution with 2 to 5 N HCl (400 ml each) was performed and 3-ml fractions were collected. Fractions 60 to 80 were collected (Fig.

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1 The experiments reported herein have been repeated at least twice.
Stereospecificity of Transaminase Enzyme-Substrate Complexes

8647

PL-CIS

YS

F2

\( \frac{4}{4} \)

-4.0

FIG. 1. Separation of e-N[\(^3\)H]pyridoxyl L-lysine produced from acid hydrolysis of reduced holoaspartate aminotransferase. Solid line, cpm; broken line, absorbance at 294 nm. Inset, separation of a prepared mixture of (A) N-pyridoxyl-L-aspartate, (B) e-N-pyridoxyl lysine, (C) pyridoxal HCl, (D) pyridoxamine diHCl by high voltage paper electrophoresis. PL-LYS, e-N-pyridoxyl lysine.

PL-ASP, N-pyridoxyl L-aspartate.

FIG. 2. Spectral changes of carbamylated aspartate aminotransferase upon the addition of pyridoxal phosphate, sodium aspartate, and sodium borohydride. 1 carbamylated apoenzyme and pyridoxal phosphate; 2, same as 1 after addition of 50 mM sodium aspartate; 3, same as 2 after reduction with sodium borohydride.

3) and evaporated to dryness and no impurities or changes in specific radioactivity were detectable by electrophoresis or thin layer chromatography.

Stereochemical Analysis of [\(^3\)H]Pyridoxyl Derivatives—Oxidative degradation of the [\(^3\)H]pyridoxyl derivatives employed a modification of literature procedures (2-4). The derivative was dissolved in distilled water (2 ml). Argon was bubbled through and the solution was cooled to 4°C and treated with 1 N NaOH (0.62 ml) and 5% sodium hypochlorite (0.46 ml). After 10 min, the reaction mixture was added dropwise to boiling water (15 ml) through which a stream of N\(_2\) was being passed, and heating was continued for 15 min. The solution was cooled rapidly to 0°C, neutralized with 0.1 N HCl, and evaporated to dryness in vacuo. The residue in distilled H\(_2\)O (1 ml) was chromatographed on Amberlite CG 50 H\(^+\) (1.5 x 20 cm). Distilled water elution (60 x 5 ml) was followed by a linear gradient between water and 0.1 N HCl (250 ml each) (Figs. 4 and 5). [\(^3\)H]Pyridoxalamine diHCl was eluted in fractions 73 and 74 (5-ml fractions). Final purification was obtained by preparative electrophoresis. The stereochemistry of the tritium at C-4' of the pyridoxamine was determined by incubation with aspartate aminotransferase apoenzyme. The apoenzyme (3 mg) was incubated with [\(^3\)H]pyridoxamine (0.84 \(\mu\)mol) and 1 mM \(\alpha\)-ketoglutarate (0.3 ml) in a total of 3 ml of 0.1 M Tris buffer, pH 8, until pyridoxal formation was complete (407 nm). The reaction was usually complete in 4 h. The incubation mixture was freeze-dried and the water was trapped.

The [\(^3\)H]pyridoxal formed was isolated from the residue by ion exchange chromatography on Amberlite CG 50 H\(^+\) (1.5 x 20 cm). Distilled water elution (60 x 5 ml) was followed by a linear gradient between water and 0.1 N HCl (250 ml each). Final purification was obtained by paper electrophoresis.

RESULTS

Sodium Borohydride Reduction of Holoaspartate Aminotransferase—Reduction of the Schiff's base formed between pyridoxal phosphate and Lys 258 results in the disappearance of the characteristic absorbance at 430 nm and the appearance of an increase at 330 nm in the UV spectrum. Proteolytic hydrolysis with papain followed by mild acid hydrolysis of the reduced enzyme cleaved the peptide and phosphate linkages to give e-N-[\(^3\)H]pyridoxyl L-lysine in a yield of 1.8 \(\mu\)mol and a specific radioactivity of 3.74 x 10\(^7\) dpm/\(\mu\)mol. This material was identical in ion exchange elution profile (Fig. 1), electrophoretic mobility, UV spectrum (15), and thin layer chromatography with synthetic e-N-pyridoxyl lysine (4). This was then oxidatively degraded with sodium

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partate Aminotransferase-Apoaspartate aminotransferase (17). Reduction of the enzyme-substrate complex formed between the reconstituted carbamylated apo-transaminase and L-aspartate occurs primarily from the re face since 90% of the tritium of the derived pyridoxamine was retained in the lyophilized residue and not in the water (Table II). Further, the [3H]pyridoxal HCl isolated from the lyophilized residue was identical in ion exchange elution profile, electrophoretic mobility, and UV spectrum with reference pyridoxal HCl and had the same specific radioactivity as the [3H]pyridoxamine diHCl.

### DISCUSSION

In both the native and active site carbamylated aspartate aminotransferase, reduction with sodium borohydride can occur from either the si or re face of the Schiff's base intermediate (Scheme I). The [3H]pyridoxyl lysine or [3H]pyridoxyl aspartate so produced when oxidatively degraded with sodium hypochlorite yields pyridoxamine stereospecifically labeled at either the pro-S or pro-R hydrogen of the methylene carbon (C-4'). Since aspartate aminotransferase removes the pro-S hydrogen of pyridoxamine during transamination, loss of tritium in forming pyridoxal (Scheme I) represents attack by hydride upon the si face of the cofactor-substrate complex. Alternately, retention of tritium by the pyridoxal (Scheme IB) represent hydride attack from the re face of the cofactor-substrate complex.

Our results show that holoaspartate aminotransferase Schiff's base displays a preference for hydride attack from the re side, since the tritium was retained by the pyridoxal (Table II). This result was expected; however, the degree of stereospecificity (90%) is much higher than that found by other researchers in the field (2-4).

We have used active site carbamylated aspartate aminotransferase for reduction with sodium borohydride of the enzyme-substrate complex because reduction of native aspartate aminotransferase enzyme-substrate complex fails to give appreciable yields of trapped intermediates. Enzyme carbamylated at active site Lys 258 binds pyridoxal phosphate, but not as a Schiff's base. When substrate is added an enzyme-substrate complex is formed with the same UV and fluorescence (quenched) characteristics as the native enzyme (8).
this case, the complex can be reduced by borohydride because conversion to a pyridoxamine complex is so slow. In this case of enzyme-substrate trapping, our results show the same preference for hydride attack from the re side. This result was unexpected because, primarily on theoretical grounds, it has been hypothesized (1) that the cofactor-substrate complex is rigidly bound by the enzyme on a single face, thereby forcing all the making and breaking of bonds to occur on the opposite side. In both native and active site carbamylated aspartate aminotransferase, the Cα-H bond can be broken and the new C4'-H bond formed (Scheme I) through a cis process occurring on the si face of the cofactor-substrate complex. It is also held that reduction with externally added reagents would occur from the si face as well (1). This belief has been strengthened by the reports that reduction of carbamylated aspartate aminotransferase, binding of substrate occurs such a manner that the re face of the complex is available for hydride reduction, just as with the native enzyme. In light of the fact that active site carbamylated aspartate aminotransferase undergoes incorporation of H in the half-transamination reaction converting pyridoxal phosphate to pyridoxamine phosphate in D2O with identical stereospecificity as that occurring in native enzyme (from the si face of the complex) (9), we are led to conclude that in our system there is no change of sidedness in the Schiff's base exposure to external agents when comparing enzyme-lysine-pyridoxal phosphate and enzyme-aspartate-pyridoxal phosphate complexes. Also, our system has two sides, one where all the enzymatic processes take place (si face) and the other where external agents can react (re face).

REFERENCES