The Stereochemistry of Pyridoxal Phosphate Enzymes

THE ABSOLUTE STEREOCHEMISTRY OF COFACTOR C'4 PROTONATION IN THE TRANSAMINATION OF HOLOSERINE HYDROXYMETHYLASE BY D-ALANINE*

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SUMMARY

Serine hydroxymethylase catalyzes the reversible cleavage of several β-hydroxy-α-amino acids to glycine and the appropriate aldehyde. With D-alanine as substrate, the α-hydrogen is labilized and a slow transamination to pyruvate and the pyridoxamine phosphate enzyme occurs. The absolute stereochemistry of this transamination has been determined by using pyridoxamine pyruvate transaminase to analyze the pyridoxamine phosphate product of the serine hydroxymethylase transamination.

These two enzymes were found to protonate the cofactor C'4 from the same face. Thus, all five pyridoxal phosphate enzymes so far examined show the same absolute stereochemistry of cofactor protonation, adding the proton from the si face of C'4, so that the labile proton occupies the pro-S position of the pyridoxamine methylene group.

Enzymatic transamination involves the reversible protonation of the bound pyridoxal phosphate cofactor C'4 carbon during the key step of the reaction. The absolute stereochemistry of this protonation has been determined for three different transaminases, glutamate aspartate transaminase (1-3), pyridoxamine pyruvate transaminase (4), and for the pyridoxal phosphate-dependent L-glutamate decarboxylase, which undergoes transamination and inactivation under certain conditions (5). In all cases, the cofactor C'4 carbon is protonated from the si face of the planar intermediate yielding pyridoxamine or pyridoxamine phosphate with the enzymerelated proton in the pro-S configuration (6).

Each of these enzymes acts on an L-amino acid to cleave either the Cα−H or Cα−COOH bond. It was of particular interest to determine if the class of pyridoxal phosphate enzyme which cleaves the Cα−R bond of an L-amino acid substrate also protonates the C'4 from the si face. Serine hydroxymethylase, shown by Schirch to be the same as threonine aldolase (7), cleaves the Cα−R bond of several β-hydroxy-α-amino acids and undergoes slow transamination with D- but not L-alanine as expected from stereochemical arguments (8, 9).

MATERIALS AND METHODS

Serine hydroxymethylase from lamb liver was purified and assayed according to the method of Ulevitch (10). A standard assay solution contained 0.1 mM DL-threo-β-phenylserine, in 0.05 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, pH 7.5, 0.001 M EDTA, 0.025 M Na2SO4 buffer, pH 7.5 (Buffer A), at 25°C. The volume was 1.0 ml. The reaction was initiated by the addition of 0.01 ml of a serine hydroxymethylase solution. D-enantiomer formation was observed at 279 nm (ε = 1410). The enzyme used had a specific activity of 2.3 μmoles of benzaldehyde per min per mg of enzyme under the above conditions and appeared pure on acrylamide disc gel electrophoresis (10).

All radioactivity counting was done on a Packard Tri-Carb scintillation spectrometer model 3230 with a scintillation fluid mixture containing 330 ml of Triton X-100 (Parkard), 28 ml of Liquifluor (New England Nuclear), and 642 ml of toluene (11). The counting efficiency was 29%.

Pyridoxal phosphate specifically labeled at C'4 was prepared by reduction of the pyridoxal phosphate imine formed at pH 12 in 11 M ammonia with NaB3H4 (63 μCi per μeq of H−) (New England Nuclear), the pyridoxal phosphate being in 2-fold excess over the available hydride. The purified [H]pyridoxamine phosphate was reoxidized by passage through a column (2.4 × 1.5 cm) of active MnO2 prepared by the method of Mancera (12), and the [H]pyridoxal phosphate isolated by placing the oxidation mixture on a Dowex 1-acetate column equilibrated with 0.1 M ammonium acetate, pH 4.5. Unreacted pyridoxamine phosphate is not retained by the column under these conditions. The column was then washed with 0.1 M acetic acid to remove any other trace impurities and the [H]pyridoxal phosphate eluted with 0.1 M chloroacetic acid. The over-all yield of purified pyridoxal phosphate was 25%. Its specific activity of 53 μCi per μmol remained unchanged through thin layer chromatography in two solvent systems (13), although some minor non-radioactive impurities were present. The high specific activity of [H]pyridoxal phosphate relative to NaB3H4 is an indication of the existence of a large isotope effect in the MnO2 oxidation, as well as a selective loss of [H]pyridoxal phosphate by overoxidation.

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RESULTS AND DISCUSSION

[1H]Pyridoxal phosphate specifically labeled at C₄ was incorporated into serine hydroxymethylase as follows. A Sephadex G-25 column (1.3 cm X 80 cm) was equilibrated with Buffer A. An 0.3-ml aliquot of this buffer containing 0.1 µmole of [1H]pyridoxal phosphate was applied to the column followed by 7 ml of Buffer A and 10 ml of 2 µl cysteine in Buffer A at pH 7.5. Finally, 0.6 ml of serine hydroxymethylase (12.3 mg per ml) was applied to the column and the enzyme eluted in the same buffer. In this manner, pyridoxal phosphate is removed from the enzyme by thiocyanate formation (14). The apoenzyme then enters the [1H]pyridoxal phosphate zone reforming holoenzyme. The tritiated holoenzyme is then eluted well ahead of the low molecular weight compounds.

Holoenzyme prepared in this manner (2.5 mg; specific radioactivity 0.7 X 10⁶ cpm per mg) was allowed to react at room temperature with 0.05 µl p-alanine in 0.7 ml of Buffer A, pH 7.5, for 216 hours. Carrier pyridoxine pyrophosphate (0.25 amoles) was then added and the solution made 0.13 M in HCl. The protein was removed by ultrafiltration. After adjusting the pH to 7.5 with sodium hydroxide, the solution was applied to a Dowex 1-acetate column (1.3 X 6 cm) equilibrated with 0.1 M NH₄, pH 10.6. The excess alanine was eluted with 0.1 M ammonium acetate, pH 7.1. [1H]Pyridoxamine phosphate was then eluted with 0.1 M ammonium acetate, pH 5.5. Pyridoxal phosphate remains bound to the column under these conditions. All of the radioactivity originally present in the holoenzyme was eluted with [1H]pyridoxamine phosphate. The sample was then evaporated to dryness, dissolved in 0.5 ml of 0.1 M Tris buffer, pH 8.7, and the pH adjusted to 7.5.

Incubation of this [1H]pyridoxamine phosphate with 1 unit of alkaline phosphatase (Escherichia coli, Sigma) for 16 hours at 35° led to complete hydrolysis of the 5'-phosphate. The [1H]pyridoxamine was purified by passing it through a Dowex 1-acetate column at pH 7.0. It was then incubated with 0.15 mg of pure alkaline phosphatase (Escherichia coli, Sigma) for 16 hours at 35°, and the pH adjusted to 7.0. Finally, 0.6 ml of serine hydroxymethylase (12.3 mg per ml) was applied to the column and the enzyme eluted in the same buffer, containing 5 X 10⁻⁴ M pyruvate. When transamination to pyridoxal was complete (A₃₉₃:A₃₈₀ = 3.4 for pyridoxal (15), the solution was freeze dried and the water and solid residues counted.

When [1H]pyridoxamine and pyruvate are incubated under the conditions of the enzymatic transamination, nonenzymatic exchange of H of less than 1% is observed.

A sample of racemic [1H]pyridoxamine phosphate from the original NaBH₄ reduction was also hydrolyzed to [1H]pyridoxamine, transaminated to pyridoxal with pyridoxamine pyruvate transaminase, the solution freeze dried, and the water and solid residues counted.

Table I shows that the [1H]pyridoxamine derived from serine hydroxymethylase transamination retains the bulk of its H-label on reverse transamination to pyridoxal by pyridoxamine pyruvate transaminase. Only half of the tritium in chemically synthesized, racemic [1H]pyridoxamine remains in the [1H]pyridoxamine product of transamination by the pyridoxamine pyruvate transaminase. Since pyridoxamine pyruvate transaminase is known to labelize the pro-S proton of pyridoxamine (3), it follows that a solvent H occupied this position in the [1H]pyridoxamine from serine hydroxymethylase transamination. Thus, solvent H was added to the si face of the C₄ carbon during that transamination.

The loss of at most 12% of the H originally in [1H]pyridoxal phosphate reflects the presence of some racemic [1H]pyridoxamine in the material transaminated by pyridoxamine pyruvate transaminase. Whether this racemic material results from a lack of complete stereospecificity in the reaction of serine hydroxymethylase with the abnormal substrate, p-alanine, or from some nonenzymatic process which occurs during transamination and analysis has not been determined.

All five of the pyridoxal phosphate-dependent enzymes so far examined protonate the cofactor from the si face of the C₄ carbon. If it is assumed that the proton is always added to the "exposed" or solvent-facing side of the planar cofactor, there would seem to be a remarkable regularity in the geometry of cofactor binding to the various apoenzymes. The present case is particularly interesting since the transamination proceeds with a p-alanine acid. Thus, a change in the amino acid substrate configuration is not accompanied by a change in the exposed face of the bound cofactor.

REFERENCES

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