CATALYTIC FUNCTION OF A HISTIDYL RESIDUE IN CYTOSOLIC ASPARTATE AMINOTRANSFERASE-CATALYZED REACTIONS

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Methylene blue-sensitized photooxidation of the cytosolic aspartate aminotransferase from pig heart yielded a preparation which was inactive in the transamination reaction with natural substrates (L-aspartate and α-ketoglutarate) but fully active in the α,β-elimination reaction with β-chloro-L-alanine as well as in the transamination reaction with L-alanine. The inactivation results from photooxidative destruction of a histidyl residue in accord with the result reported by Martinez-Carrion et al.[J.Biol.Chem. 242, 1426 (1967)]. These results indicates that the histidyl residue does not function as a base abstracting the α-hydrogen atom of these substrates but acts as a catalytic residue involved in the interaction with the distal carboxylate of a natural dicarboxylic substrate as proposed previously [Morino et al., J.Biol.Chem. 249, 6684 (1974)].

A common prerequisite step in the enzymatic transamination and α,β-elimination reactions is believed to be the labilization of the α-hydrogen atom of a substrate (1,2). A mechanistic consideration of the chemical process of affinity labeling of aspartate aminotransferase (EC 2.6.1.1) by β-chloro-L-alanine has led to the concept that the α-hydrogen-abstracting base would be provided by the ε-amino group of the lysyl residue which forms an aldimine bond with the coenzyme, pyridoxal 5'-phosphate (3,4). Furthermore, a stimulatory action of formate ion in the reactions of aspartate aminotransferase with L-alanine (5) and β-chloro-L-alanine (5,6) has driven us to speculate that formate ion binds to a discrete subsite and exerts a striking effect on the catalytic efficiency by inducing reactivity of the ε-amino group of the lysyl residue which is assumed to act as a base abstracting the α-hydrogen atom of the bound substrate. In the previous paper (6), we postulated a histidyl residue as the formate-binding site and presented a possible molecular mechanism of action of formate ion in accelerating the rate of reactions described above. If this hypothesis is a valid one, destruction of the histidyl residue should yield a preparation which is fully active but devoid of the formate-activation in both of transamination with L-alanine and α,β-elimination with

β-chloro-L-alanine. The presence of a critical histidyl residue in the active site of cytosolic aspartate aminotransferase was suggested from a dye-sensitized photoinactivation study (7).

In the present work, cytosolic aspartate aminotransferase was photooxidized in the presence of methylene blue. The resulting preparation was almost inactive in transamination with natural dicarboxylic substrates but retained full activities of the α,β-elimination reaction with β-chloro-L-alanine as well as of the transamination reaction with L-alanine.

MATERIALS AND METHODS

Cytosolic aspartate aminotransferase was isolated from pig heart by a modification of the procedure described for beef liver enzyme (8). Lactate dehydrogenase was purified from pig heart as described (9). β-Chloro-L-alanine was synthesized as described (10).

Assay for transamination between L-aspartate and α-ketoglutarate was performed as described previously (11). For the assay for the α,β-elimination reaction, the reaction mixture contained, in a total volume of 1.0 ml, 20 mM β-chloro-L-alanine, 0.1 mM NADH, 5 μg (approximately 2 units at 25°) lactate dehydrogenase and 0.1 M potassium pyrophosphate buffer (pH 7.5). The reaction was initiated by adding the aminotransferase and followed by recording the absorbance change at 340 nm. Incubations were at 25°.

For photooxidation, irradiation was performed by a 150-W tungsten lamp located at a distance of 5.5 cm from the bottom of a cylindrical cell (2.5 cm in diameter) thermostated at 19°. The cell contained, in a total volume of 5.0 ml, 15 mg cytosolic aspartate aminotransferase in the pyridoxal form, 10 μg methylene blue and 0.1 M potassium pyrophosphate buffer (pH 7.5). During illumination the solution was stirred by a magnetic stirrer mounted over the cell. At intervals, 5 μl-aliquots were withdrawn from the reaction mixture and assayed for the activities of transamination and α,β-elimination. The activity of α,β-elimination of β-chloro-L-alanine was determined also in the presence of 3 M potassium formate. Spectrophotometric measurement was performed in a Hitachi recording spectrophotometer model 124. Amino acid analysis was carried out in an automatic amino acid analyzer, Hitachi Perkin-Elmer KLA 3B, according to the procedure of Spackman et al.(12). Proteins were hydrolyzed in constant boiling HCl for 24 hours at 110° in sealed, evacuated tubes.

RESULTS

During photooxidation of cytosolic aspartate aminotransferase, the activity of the normal transamination reaction between L-aspartate and α-ketoglutarate decreased progressively via first order kinetics (Fig. 1, curve 1). In contrast, the activity of α,β-elimination reaction with β-chloro-L-alanine did not decrease but instead increased slightly with the progress of photooxidation (Fig. 1, curve 3). As reported previously (5), the rate of α,β-elimination reaction catalyzed by the native enzyme was markedly accelerated by the presence of formate ion. When measured in the presence of 3 M formate, the rate of α,β-elimination of β-chloro-L-alanine decreased in parallel with that of the normal transamination reaction (Fig. 1, curve 2). Activation of the α,β-elimination reaction by formate ion was negligible with an photooxidized

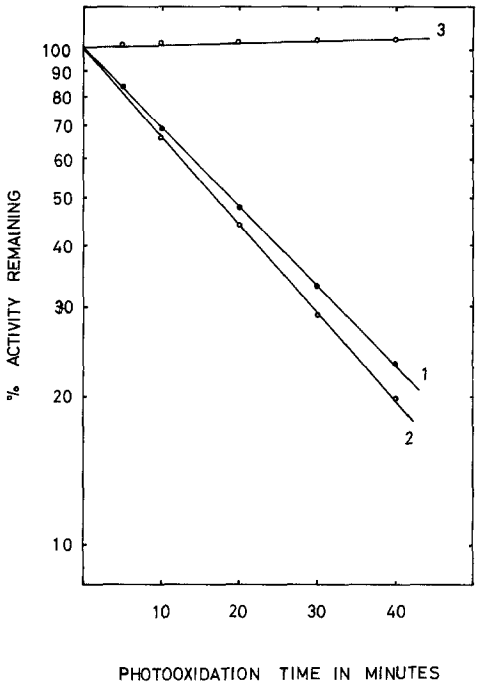

Fig. 1. Variations of the activity of transamination with natural substrates and that of α,β-elimination of β-chloro-L-alanine during photooxidation of cytosolic aspartate aminotransferase.

Photooxidation and enzyme assays were performed as described under " MATERIALS AND METHODS ".

Curve 1: the rate of transamination between L-aspartate and α-ketoglutarate.

Curve 2: the rate of α,β-elimination of β-chloro-L-alanine in the presence of 3 M potassium formate.

Curve 3: the rate of α,β-elimination of β-chloro-L-alanine in the absence of formate.

With the native enzyme, the rates were 160 μmoles/min/mg (normal transamination), 0.25 μmole /min/mg (α,β-elimination in the absence of formate), and 3.9 μmoles/min/mg (α,β-elimination in the presence of 3 M formate). These values were taken as 100 % in the respective reactions.

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preparation having only 10 % residual activity in the normal transamination reaction.

On amino acid analysis of photoinactivated preparations, destruction of 1.5 histidyl residues per monomeric unit of the enzyme was observed with a preparation retaining 30 % residual activity of the normal transamination, and 2.3 residues with a preparation showing 5 % residual activity. The inactive species exhibited an absorption band at 330 nm at pH 8.0 which shifted to 430 nm upon variation of pH to 5.0. These and other properties of photoinactivated preparations were in accord with those reported by Martinez-Carrion et al.(13).

L-Alanine is a poor substrate for aspartate aminotransferase. As previously described (5), the rate of conversion of the pyridoxal form of the native enzyme into the pyridoxamine form by L-alanine is markedly accelerated in the presence

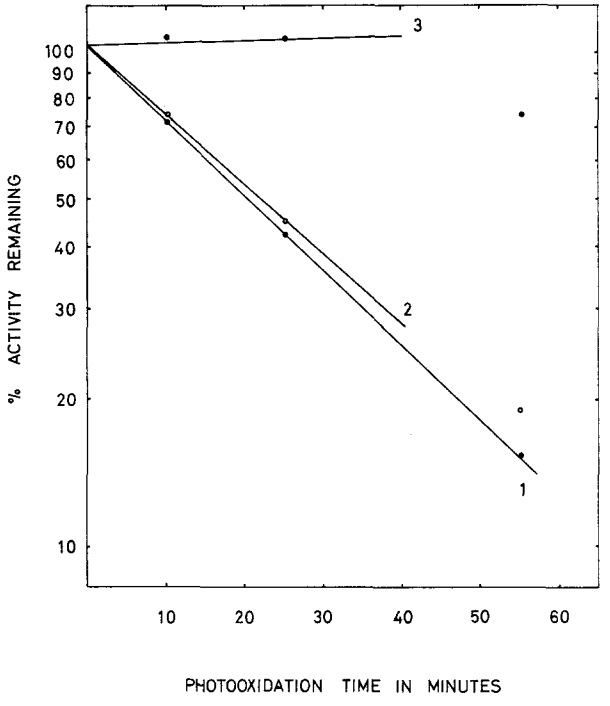

Fig. 2. Changes in the rate of transamination with L-alanine during photooxidation of cytosolic aspartate aminotransferase.

The experimental procedure for the measurement of the rate of alanine-transamination is essentially identical with that described previously (5). The conversion of the pyridoxal form of the enzyme to the pyridoxamine form was monitored by following the decrease in absorbance at 370 nm. This wavelength was chosen since the pyridoxamine form enzyme did not absorb at this region.

The reaction mixture for photooxidation was as described under " MATERIALS AND METHODS ". At intervals, 0.5 ml-aliquots were mixed with 0.5 ml of 0.1 M potassium phosphate buffer (pH 7.5). When indicated, the buffer contained 2 M potassium formate (final concentration: 1 M). The reaction was initiated by adding 40 µl of 1.0 M L-alanine and the decrease in absorbance at 370 nm was recorded at 25°. The absorbance at 370 nm represents the concentration of the pyridoxal form present. The pseudo first order rate constant for the conversion of the pyridoxal form to the pyridoxamine form was calculated from plots of log $[100(A_o - A_t)/ A_o]$ versus time, where A_o denotes the initial value of absorbance at 370 nm; A_t, that at time, t.

Curve 1: the rate of transamination between L-aspartate and α-ketoglutarate.

Curve 2: the rate of transamination with L-alanine in the presence of 1.0 M potassium formate.

Curve 3: the rate of transamination with L-alanine in the absence of formate

With the native enzyme, the rates were 160 µmoles/min/mg (normal transamination), 0.08/min (transamination with L-alanine in the absence of formate), and 2.7/min (transamination with L-alanine in 1.0 M formate). These values were taken as 100 % in the respective reactions.

of formate. Fig. 2 shows that the rate of the transamination with L-alanine remains virtually unaltered after photooxidation. In addition, the presence of 1 M formate did not appreciably affect the rate of the reaction of the photoinactivated enzyme with L-alanine. During photooxidation, the rate of transamination with L-alanine in the presence of 1 M formate decreased in parallel with that of the normal transamination reaction between L-aspartate and α-ketoglutarate. These results are consistent with the observation described above for the reaction with β-chloro-L-alanine and provide additional evidence that a histidyl residue is not an α-proton-abstracting base but is closely related with the binding site of formate ion.

DISCUSSION

Aspartate aminotransferase catalyzes transamination of L-alanine and α,β-elimination of β-chloro-L-alanine. It is believed that, in common with the transamination reaction with natural substrates, these reactions proceed via enzymatic abstraction of the α-hydrogen atom of the bound substrate. The present investigation has clearly demonstrated that photooxidative destruction of a histidyl residue in the cytosolic aspartate aminotransferase yielded a preparation which is devoid of the normal transamination activity but fully active in the α,β-elimination reaction with β-chloro-L-alanine as well as in the transamination reaction with L-alanine. This finding is in contradiction to the concept (14) that the histidyl residue is involved in the abstraction of the α-hydrogen atom of the bound substrates and rather favors the idea that the proton-abstracting base in the active site is provided by the ε-amino group of the lysyl residue which originally forms an aldimine linkage with the coenzyme (3).

A possible formation of a charge relay system between formate ion, a hypothetical imidazolyl group and the ε-amino group of the lysyl residue within the

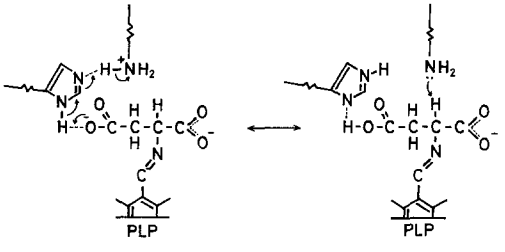

Fig. 3. Interactions of a substrate with catalytic residues within the active site of aspartate aminotransferase.

active site has been considered to explain the stimulatory action of formate in the aspartate aminotransferase-catalyzed reactions with L-alanine and β-chloro-L-alanine (6). Cheng and Martinez-Carrion (15) postulated that a histidyl residue is an anion binding site. The present result is consistent with this and, furthermore, enables us to conclude that the histidyl residue susceptible to photooxidation constitutes the binding site for formate ion. In the transamination reaction with natural dicarboxylic substrates (L-aspartate or L-glutamate), the distal carboxyl group of these substrate would bind to the same histidyl residue as formate anion binds. Thus the high catalytic efficiency observed in the normal transamination reaction could result from the interaction of the distal carboxylate of a substrate with the histidyl residue which enhances the reactivity of the lysyl ε-amino group as a base to abstract the α-hydrogen atom of the substrate (Fig. 3). This mechanism may provide a molecular basis of the apparently strict substrate specificity of aspartate aminotransferase.

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