INACTIVATION OF ASPARTATE AMINOTRANSFERASE DURING TRANSAMINATION WITH CHLOROPYRUVATE. EVIDENCE AGAINST MODIFICATION OF CYS_{390} IN CYTOSOLIC ISOENZYME

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SUMMARY: Both the cytosolic and mitochondrial isoenzyme of aspartate aminotransferase from pig heart were inactivated during transamination with chloropyruvate. Inactivation occurred with L-alanine as the amino group donor in the presence of potassium formate. When L-glutamate or L-aspartate was employed as the amino group donor in the transamination reaction with chloropyruvate, no inactivation occurred. This is in contrast to the case of inactivation by bromopyruvate (Okamoto, M. & Morino, Y.(1973) J. Biol. Chem. 248, 82-90) where these natural dicarboxylic amino acid substrates were effective in the transamination reaction leading to syncatalytic inactivation (Birchmeier, W. & Christen,P.(1974) J. Biol. Chem. 249, 6311-6315). The Cys_{390} in the cytosolic isoenzyme which was modified in the syncatalytic inactivation was not modified under the present condition for inactivation with either chloropyruvate or bromopyruvate.

Both the cytosolic and mitochondrial aspartate aminotransferases (EC 2.6.1.1) from pig heart were shown to be irreversibly inactivated during reaction with β-chloro-L-alanine (1) or bromopyruvate (2,3 & 4). Inactivation by β-chloro-L-alanine resulted from covalent modification of an essential lysyl residue which is involved in aldimine formation with the coenzyme in both isoenzymes (5,6). Inactivation by bromopyruvate was shown to result from the modification of a cysteinyl residue in both isoenzymes (3). With the cytosolic isoenzyme, the modified residue was identified to be Cys_{390} and bromopyruvate was claimed to act as a syncatalytic modifier rather than as an affinity label (4).

Chloropyruvate, less reactive as an alkylating reagent than bromopyruvate, was tested for the capability to label the active site as an affinity label rather than as a syncatalytic modifier. Indeed, chloropyruvate was found to inactivate both isoenzymes of aspartate aminotransferase during transamination with L-alanine in the presence of potassium formate whereas incubation of the enzymes with chloropyruvate in the presence of natural dicarboxylic amino acid substrates did not lead to the inactivation of both isoenzymes.

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Abbreviations used are: Cys, cysteinyl residue; EDTA, ethylenediaminetetraacetate.

EXPERIMENTAL PROCEDURES

Materials: Both the cytosolic and mitochondrial aspartate aminotransferases were purified from pig herat by a modification of the procedure described for beef liver isoenzymes (7). Chloropyruvic acid was synthesized by the method described (8) and recrystallized from hot chloroform. Sodium iodo[1-^{14}C]acetate (57 mCi per mmol) was obtained from the Radiochemical Center, Amersham.

Methods: Assay for the normal transamination between L-aspartate and α-ketoglutarate was performed as described previously (3). Progress of inactivation was monitored by determination of transaminase activity on aliquots withdrawn at various time intervals from the reaction mixtures prepared for control and inactivation as described for the individual experiments. For the analysis of sulfhydryl groups, the cytosolic isoenzyme (50 mg) was incubated at 37° C with 100 mM chloropyruvate in the presence of 0.3 M L-alanine, 3 M potassium formate, 1 mM dithiothreitol and 0.2 M sodium cacodylate buffer (pH 6.5) in a final volume of 3 ml. As a control experiment, the reaction mixture identical to that described above except for the omission of L-alanine was prepared and incubated at 37°C. After incubation for 3 hours the remaining activities were 4 % and 94 % in the presence and absence of L-alanine, respectively. Then both preparations were dialyzed for 24 hours against 2 liters of 0.1 M sodium acetate buffer (pH 6.0). The enzyme activity was checked and found to be the same as described above. The samples were reduced by $NaBH_4$ and dialyzed against 10 mM sodium acetate buffer (pH 5.5) for 6 hours and lyophilized. The dry samples were dissolved in 2 ml 6 M guanidine-HCl containing 0.1 M Tris-HCl buffer (pH 8.0), 1 mM EDTA and 1 mM dithiothreitol and incubated for an hour at 37°C. To these solutions was added a sufficient amount of sodium iodo[1-^{14}C]acetate (2.2 x 10^5 dpm per μmol) to make a final concentration of 10 mM and the mixtures were incubated for 10 minutes at 37°C. The samples were dialyzed against distilled water and heavy precipitates formed were collected by centrifugation. Precipitates were dissolved in 3 ml of 70 % formic acid and 50 molar excess of cyanogen bromide was added. The mixtures were kept at room temperature (15 to 20°C) for 24 hours and then diluted 10 times with distilled water and lyophilized. Dried samples were dissolved in 5 ml of 6 M urea in 5 % formic acid and applied to a Sephadex G-75 column (3 x 130 cm) equilibrated with the same solvent. Peptides were eluted with 6 M urea in 5 % formic acid. Fractions of 5 ml were collected at a flow rate of 15 ml per hour. The addition of urea gave better resolution and abolished the anomalous elution of the peptide with 75 residues before the one with 212 residues (4,9). Bray's solution (10) was used as the scintillation medium. Radioactivity was determined in a Packard scintillation spectrometer model 3385. Absorption spectra were obtained with a Hitachi recording spectrophotometer model 124 and circular dichroism spectra with a Jouan Dichrograph II.

RESULTS

Inactivation of aspartate aminotransferase by chloropyruvate

Incubation of both isoenzymes with chloropyruvate alone did not lead to the inactivation of these enzymes. In contrast to the case with bromopyruvate (3,4), inactivation with chloropyruvate was strongly dependent on the nature of the amino acid cosubstrates. L-Alanine was found to be the most effective whereas the natural amino acid substrate, L-glutamate or L-aspartate, was much less effective (Fig. 1). When L-alanine was used as an amino acid substrate, the presence of formate was required for efficient inactivation. The rate of inactivation increased with the increase in the concentration of formate. This stimulatory action of formate is analogous to that found in the reaction of aspartate aminotransferase with L-alanine (1) or β-chloro-L-alanine (1,11).

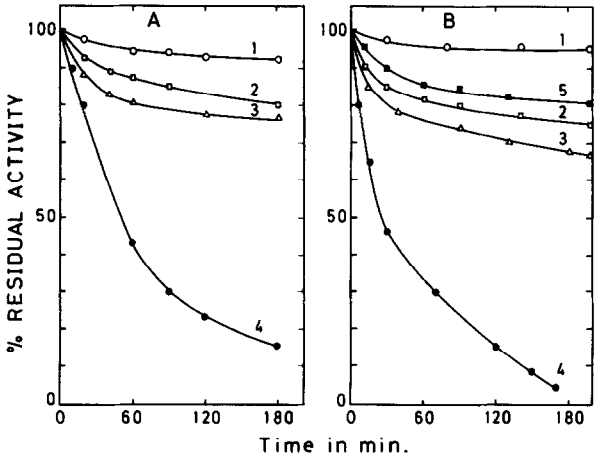

Fig. 1. Effect of amino acid substrates and formate ion on the inactivation of aspartate aminotransferase isoenzymes by chloropyruvate.

Each reaction mixture contained, in a total volume of 1.0 ml, 2.5 mg mitochondrial isoenzyme (A) or 1.5 mg cytosolic isoenzyme (B), 150 mM (A) or 100 mM (B) chloropyruvate, and 0.2 M Tris-HCl buffer (pH 7.5). Incubations were at 25°C. Curve 1, in the absence of any amino acid substrate. Following additions were made in the experiments represented by curves 2-5. Curve 2, 100 mM L-glutamate; curve 3, 40 mM L-cysteine sulfinate; curve 4, 0.3 M L-alanine and 3 M potassium formate; curve 5, 100 mM L-aspartate. Activity for normal transamination was determined on aliquots withdrawn from each incubation mixture at the indicated times. In both A and B, addition of 0.3 M L-alanine alone gave results essentially identical to those indicated by curves 1. Specific activities of the cytosolic and mitochondrial isoenzymes were 210 and 190 μmoles per min per mg protein at 25°C, respectively, and these values were taken as 100 %.

The rather puzzling ineffectiveness of the natural dicarboxylic amino acid substrates could be attributed to the fact that α-ketoglutarate or oxalacetate produced from the corresponding amino acid during enzymatic transamination competes with chloropyruvate and protects the enzyme against inactivation. As can be seen from Table I, the presence of keto acid substrates exhibited strong protection against inactivation by chloropyruvate. This finding suggests that chloropyruvate must bind to the active site prior to inactivation. Moreover, it explains why the natural amino acids were ineffective as cosubstrates during the inactivation reaction.

The spectral analysis of chloropyruvate-inactivated preparations of both isoenzymes revealed considerable gross change in comparison with the control preparations treated with chloropyruvate alone. Circular dichroic spectra of the inactivated species (Fig. 2, bottom, curve 2) were distinctly different from those of the control preparations, suggesting that upon inactivation a profound change had occurred in the active site chromophore or its vicinity.

Either of chloropyruvate or bromopyruvate could react simply as an alkylating reagent and inactivate the enzyme through alkylation of nucleophilic side chains,

Table I

Protection of aspartate aminotransferase isoenzymes against chloropyruvate-mediated inactivation by competitive keto acid substrates.

In a final volume of 1.0 ml each, 1.5 mg either isoenzyme were incubated with 50 mM chloropyruvate in the presence of 3 M potassium formate and 0.1 M potassium cacodylate buffer (pH 6.5) at 25°C. When indicated, concentrations of L-alanine, α-ketoglutarate and pyruvate were at 0.3 M, 50 mM and 50 mM, respectively. Specific activities of the cytosolic and mitochondrial isoenzymes were 210 and 190 μmoles per min. per mg protein at 25°C, respectively, and these values were taken as 100 %.

Isoenzyme	Incubation Time (minutes)	Remaining Activity (per cent)			
		Chloropyruvate + L-Alanine	Chloropyruvate + L-Alanine + Pyruvate	Chloropyruvate + L-Alanine + α-Keto-glutarate	Chloropyruvate Alone
Cytosolic	10	73 %	85 %	99 %	97 %
	60	34	69	98	92
	120	13	60	97	88
Mitochondrial	10	87	95	97	97
	60	53	78	87	90
	120	36	68	82	86

in addition to acting as a keto acid substrate. Cys_{390} of the cytosolic aspartate aminotransferase is known to be modified syncatalytically in the presence of a substrate pair (9) and it had been implicated as the modified residue during bromopyruvate-mediated inactivation (4). Since the primary structure of the cytosolic isoenzyme is known (12, 13), each of the five cysteinyl residues can easily be labeled through carboxymethylation with radioactive iodoacetate and consequently identified by chromatographic separation of cyanogen bromide-cleaved peptide fragments on a Sephadex column. This procedure was employed to examine whether or not Cys_{390} is involved in inactivation with chloropyruvate. Such analysis showed that approximately equal amount of radioactivity was distributed into each of three fractions (Fig. 3). Fraction I should contain Cys_{45}, Cys_{82} Cys_{191}. Of these three sulfhydryls, the fully exposed Cys_{45} and Cys_{82} were blocked by chloropyruvate. Hence the radioactivity in Fraction I represents that derived from the Cys_{191}-containing peptide. Fraction II contained pyridoxyl peptide which absorbed at 325 nm at pH 7.0 and hence should contain the Cys_{252}-peptide fragment. Radioactivity in Fraction III results from the Cys_{390}-containing

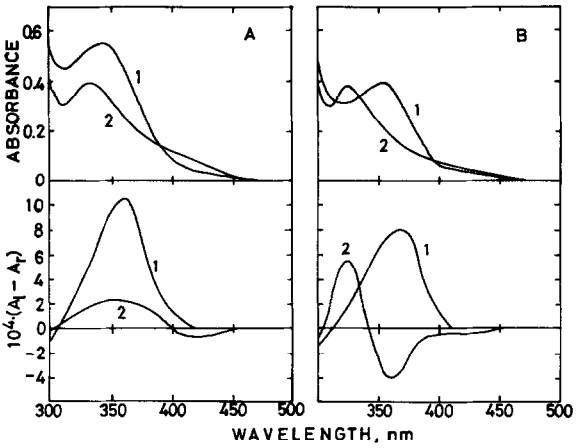

Fig. 2. Absorption and circular dichroic spectra of aspartate aminotransferases treated with chloropyruvate in the presence and absence of L-alanine.

A. mitochondrial enzyme; B. cytosolic enzyme. Upper, absorption spectra; lower, circular dichroism spectra. Each reaction mixture contained, in a total volume of 1.0 ml, 3.0 mg mitochondrial isoenzyme or 2.8 mg cytosolic isoenzyme, 150 mM chloropyruvate, 3 M potassium formate and 0.1 M Tris-HCl buffer (pH 7.5). Curves 1, in the absence of L-alanine; curves 2, in the presence of 0.3 M L-alanine. Incubations were at 33°C for 4 hours. These spectra were taken after dialysis overnight against 0.1 M potassium pyrophosphate buffer (pH 7.5). Spectra were corrected for dilution due to dialysis. Remaining activities checked after dialysis were 97 % (curve 1) and 28 % (curve 2) in A., 96 % (curve 1) and 4 % in B.

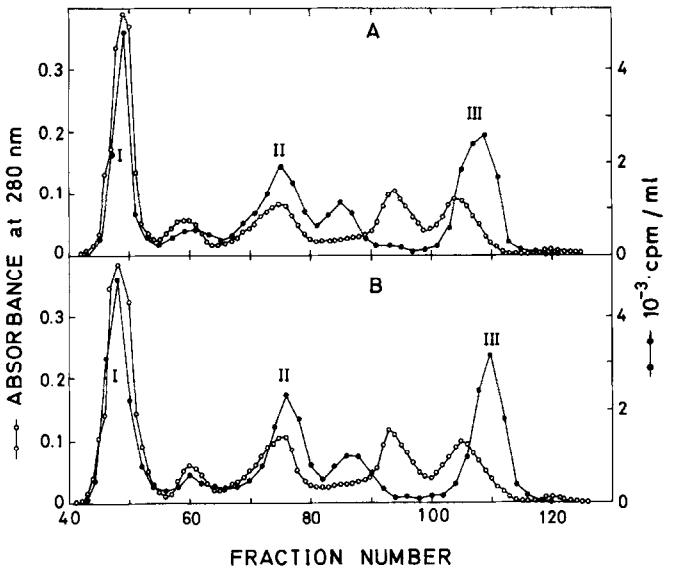

Fig. 3. Fractionation of cyanogen bromide-cleaved peptides on a Sephadex G-75 column in 6 M urea containing 5 % formic acid.

Experimental details were described under Methods. A. inactivated cytosolic enzyme (remaining activity, 4 %); B. control preparation (remaining activity, 94 %).

peptide. Thus all five cysteinyl residues present in the cytosolic enzyme can be identified by examining the distribution of radioactivity on the chromatogram of cyanogen bromide-peptides from a preparation which has been treated with ^{14}C-iodoacetate. As can be seen from Fig. 3, no difference in the distribution pattern of radioactivity was observed between the preparation treated with chloropyruvate alone and that inactivated by chloropyruvate in the presence of L-alanine and potassium formate. This finding indicated that any cysteinyl residue was not modified either in the presence or absence of L-alanine and hence the inactivation resulted from modification of residue(s) other than cysteinyl residues.

DISCUSSION

When the data for curves 4 in Fig. 1 were plotted in a first order fashion, the resulting plot did not yield a typical straight line. This probably results from the fact that during the reaction pyruvate accumulates and exerts a protective action against inactivation by competing with chloropyruvate. Indeed, the keto acid substrates, pyruvate and α-ketoglutarate, when added to the reaction mixture for inactivation, protected the enzymes against inactivation. Furthermore, chloropyruvate did not inactivate the enzymes in the presence of L-glutamate which was an efficient cosubstrate in the syncatalytic inactivation with bromopyruvate (4). All these findings are in favor of the idea that chloropyruvate acts as an active site-directed reagent under the present experimental condition, i.e., in the presence of L-alanine and potassium formate.

In the present study, search for a modified cysteinyl residue was performed in an indirect way by identification of unmodified cysteinyl residues as carboxymethylcysteine resulting from treating an inactivated enzyme with ^{14}C-iodoacetate Such analysis on the preparation of cytosolic isoenzyme inactivated by bromopyruvate in the presence of L-alanine and potassium formate revealed that the cyanogen bromide-cleaved peptides was indistinguishable from that of the control preparation (unpublished observation). This finding indicates that, as with the case of inactivation by chloropyruvate, the Cys_{390} was not modified in the inactivation with bromopyruvate. A parallel experiment on the preparation inactivated by bromopyruvate in the presence of L-glutamate (a condition for syncatalytic inactivation (4)) revealed that radioactivity in Fraction III (Fig. 3) was insignificant and hence the Cys_{390} was modified in this case. This result is in accord with the data reported (4). Thus there seems to be two different modes of action of bromopyruvate (as a syncatalytic modifier or as an affinity label) in inactivating the cytosolic isoenzyme, depending upon the nature of the amino acid cosubstrate and reaction conditions.

The present result has shown that inactivation of the cytosolic isoenzyme by chloropyruvate does not result from modification of the Cys_{390} but from modification of some residue(s) within the active site. Direct identification of the structure of the modified site in the cytosolic and mitochondrial isoenzyme is currently under way with the use of radioactive chloropyruvate.

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