A COMPARATIVE STUDY ON THE AFFINITY LABELLING OF ASPARTATE AMINOTRANSFERASE ISOZYMES BY β-BROMOPYRUVATE

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SUMMARY: An irreversible inactivation of pig heart muscle aspartate aminotransferase isozymes by β-bromopyruvate was studied. β-Bromopyruvate alone caused a slow inactivation of each isozyme. The addition of a small amount of L-cysteine sulfinate greatly accelerated the inactivation. This was particularly remarkable with the mitochondrial enzyme. From a comparison of the rate of the inactivation by the β-bromopyruvate: L-cysteine sulfinate system with that of the inactivation by β-bromo-L-alanine, it is quite likely that the enzyme is inactivated during a transamination reaction between the pyridoxamine form of the enzyme and β-bromopyruvate.

A comparison of the kinetic parameters indicates that the mitochondrial aspartate aminotransferase prefers 4-carbon dicarboxylic acids as the substrate or inhibitor whereas the supernatant enzyme prefers 5-carbon dicarboxylic acids (1,2,3). Thus it seems clear that there are subtle differences in the microenvironment of the substrate binding site in each isozyme.

As a probe to obtain the topological information of the active site, halo acids of various carbon chain length were examined for their reactivity with the mitochondrial and supernatant isozymes of aspartate aminotransferase in the pyridoxal form. Only β-bromopropionate was found to specifically inactivate the mitochondrial enzyme with the alkylation of a lysine residue per monomeric unit of the enzyme(4).

To get further insight into the fine structure of the active site, the reactivity of the pyridoxamine form with various halo acids was studied with the result that only the supernatant enzyme was inactivated by several halo acids(5).

In the course of these studies, β-bromopyruvate was found to convert the pyridoxamine form into the pyridoxal form of both isozymes. A slow inactivation

was observed during the prolonged incubation either of the pyridoxal or pyridoxamine form of these isozymes with β-bromopyruvate. However, a pronounced enhancement of the inactivation rate was found to occur in the presence of L-cysteine sulfinate.

The present communication describes briefly the comparative study on the mechanism of the inactivation of pig heart muscle aspartate aminotransferase isozymes by β-brompopyruvate in the presence of L-cysteine sulfinate.

MATERIALS AND METHODS: The aspartate aminotransferase isozymes were purified from pig heart muscles by an improved modification of the procedures described previously(6). Only the α-subform(7) of the supernatant aminotransferase was used in the present study. The mitochondrial enzyme used contained all subforms. β-Bromo-pyruvic acid(8), β-bromo-L-alanine(9) were synthesized by Dr. S. Imamoto, the Food Research Institute, Osaka, by the cited methods. Other chemicals were of commercial sources.

The enzyme assay was performed by following absorbance change due to the production of oxalacetate at 260 mμ. Spectral measurements were made with a Hitachi spectrophotometer Model 124 with a recorder.

RESULTS: A rather slow inactivation was observed during the incubation of both the supernatant and mitochondrial isozymes of aspartate aminotransferase with β-bromopyruvate(Fig. 1. A & B, curve 1). The addition of L-cysteine sulfinate markedly enhanced the inactivation rate(Fig. 1. A & B, curves 2,3), especially with the mitochondrial enzyme. Under the conditions described for Fig. 1., the lowest concentrations of L-cysteine sulfinate to attain a maximal inactivation rate seemed to be 2 mM and 0.2 mM with the supernatant enzyme and the mitochondrial enzyme, respectively.

In a separate experiment where the reaction of the pyridoxamine form of each isozyme with β-bromopyruvate was followed spectrophotometrically, a rapid conversion to its pyridoxal form was observed.

Hence, two explanations would be possible on the stimulating action of L-cysteine sulfinate in the inactivation by β-bromopyruvate. Firstly, L-cysteine

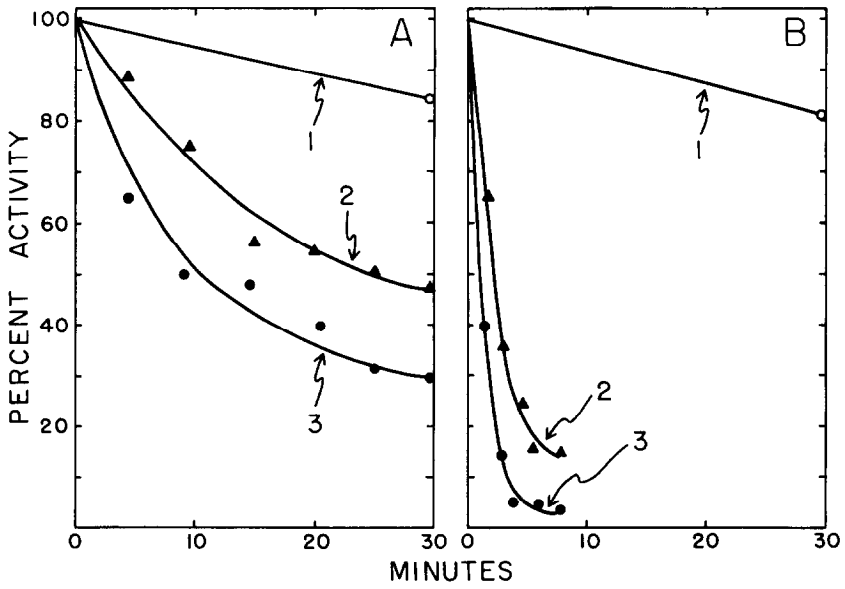

Fig. 1. Effect of L-cysteine sulfinate on the inactivation of aspartate aminotransferases by β-bromopyruvate

A. The supernatant enzyme (0.3 mg) was incubated with 40 mM β-bromopyruvate (curve 1), 20 mM β-bromopyruvate plus 2.0 mM L-cysteine sulfinate (curve 2), or 40 mM β-bromopyruvate plus 2.0 mM L-cysteine sulfinate (curve 3), at 30° in 0.5 ml of 0.1 M sodium cacodylate buffer, pH 7.0, containing 0.8 mM ethylenediamine tetraacetate.

B. The mitochondrial enzyme (0.38 mg) was incubated with 40 mM β-bromopyruvate (curve 1), 20 mM β-bromopyruvate plus 0.2 mM L-cysteine sulfinate (curve 2), or 40 mM β-bromopyruvate plus 0.2 mM L-cysteine sulfinate (curve 3), at 30° in 0.5 ml of the same buffer as described above.

In both of the experiments A and B, an aliquot (5 µl) was removed at each of the indicated times for the determination of aspartate aminotransferase activity. The assay mixture contained 20 mM L-aspartate, 10 mM α-ketoglutarate and 0.2 M Tris-HCl buffer, pH 8.1. The increase in the optical density at 260 mµ was recorded at 25° in a Hitachi spectrophotometer Model 124.

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sulfinate serves to maintain the level of the pyridoxamine form of the enzyme by continuously converting the pyridoxal form formed by transamination with β-bromopyruvate back to the pyridoxamine form. The pyridoxamine form thus kept at a steady high level in the incubation mixture reacts efficiently with β-bromopyruvate; or, alternatively, the accumulation of β-bromo-L-alanine formed from β-bromopyruvate by transamination with L-cysteine sulfinate results in the inactivation of pyridoxal form of the enzyme during a β-elimination reaction as observed in the reaction of the supernatant aspartate aminotransferase with L-serine-O-sulfate(10) and also in the reaction of L-aspartate β-decarboxylase with β-chloroalanine(11).

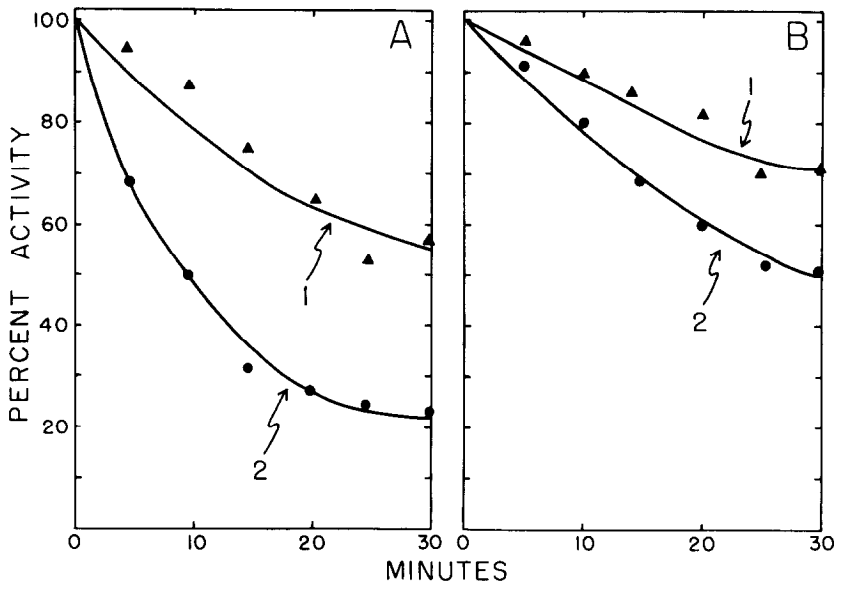


Fig. 2. Inactivation of aspartate aminotransferases by β-bromo-L-alanine
A. The same as in Fig. 1, A., except that β-bromopyruvate or β-bromopyruvate plus L-cysteine sulfinate was replaced by 20 mM (curve 1) or 40 mM β-bromo-L-alanine (curve 2).
B. The same as in Fig. 2, B., except that β-bromopyruvate or β-bromopyruvate plus L-cysteine sulfinate was replaced by 20 mM (curve 1) or 40 mM β-bromo-L-alanine (curve 2).

To test the possible inactivation by a β-elimination reaction, the reaction of both isozymes in the pyridoxal form with β-bromo-L-alanine, presumably a transamination product, was examined. As shown in Fig. 2, a significant inactivation of each isozyme was observed upon the incubation with β-bromo-L-alanine, with concomitant formation of pyruvate (probably due to a β-elimination reaction). The inactivation of the mitochondrial enzyme by β-bromoalanine proceeded at a rate much slower than that by β-bromopyruvate plus cysteine sulfinate and never exceeded 50% under the present experimental condition.

Compared with the mitochondrial enzyme, the inactivation of the supernatant enzyme by β-bromoalanine was faster and the rate was comparable to that of its inactivation by β-bromopyruvate plus cysteine sulfinate.

With both of the enzymes, a very slow conversion of the pyridoxal form to the pyridoxamine form was observed upon the addition of β-bromoalanine as judged

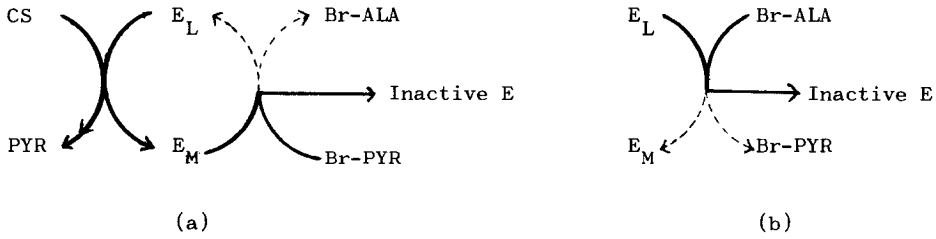
from their spectral changes. However, α-ketoglutarate added to prevent a possible conversion of the enzyme to pyridoxamine form was totally without effect on the inactivation process at a concentration range from 0.5 mM to 10 mM.

DISCUSSION: The results described herein indicates that, especially in the case of the mitochondrial enzyme, the inactivation by β-bromopyruvate in the presence of L-cysteine sulfinate occurred during a transamination reaction between the pyridoxamine form of the enzyme and β-bromopyruvate, and there seems to be little, if any, contribution of a β-elimination reaction in the inactivation process. Indeed, the stimulating effect of L-cysteine sulfinate reached a maximum at a concentration as low as 0.2 mM(Fig. 1.B). And this amount of L-cysteine sulfinate would have been a minimum necessary for maintaining the enzyme in the pyridoxamine form during the time required for its inactivation by β-bromopyruvate.

In the case of the supernatant enzyme, there was no significant difference between the rate of inactivation of the pyridoxal form by β-bromoalanine and that of the pyridoxamine form by β-bromopyruvate. Therefore it is difficult to assess the relative importance of a transamination reaction or a β-elimination reaction in contributing to the inactivation of the supernatant enzyme by β-bromopyruvate.

The relation of the rate of the inactivation with that of transamination or β-elimination reaction will be referred here briefly. With either of the supernatant or mitochondrial enzyme, the rate of inactivation by β-bromoalanine was less than 0.1 per cent of that of the β-elimination of the same substrate under the conditions described for Fig. 2. The rate of inactivation by β-bromopyruvate in the presence of L-cysteine sulfinate was 1 per cent of that of the transamination of β-bromopyruvate with the pyridoxamine form of the supernatant enzyme whereas the inactivation rate was 7 per cent of that of the transamination of β-bromopyruvate with the pyridoxamine form of the mitochondrial enzyme. Details will be described elsewhere.

Although it is yet premature to offer a substantial explanation for the observed inactivation, a tentative scheme(Scheme 1) seems to conform to the present experimental data.



Scheme I. Schematic representation of inactivation mechanism
(a) Inactivation during transamination with β-bromopyruvate
(b) Inactivation during β-elimination reaction with β-bromo-L-alanine
CS=L-cysteine sulfinate PYR=pyruvate Br-ALA=β-bromo-L-alanine
Br-PYR=β-bromopyruvate E_L=pyridoxal form E_M=pyridoxamine form
Inactive E=inactivated enzyme (pyridoxal or pyridoxamine form)

Multiple intermediary steps have been postulated for the reaction sequence of the enzymatic transamination(12,13). Kinetic, spectral and chemical analyses of the inactivation process as well as the products obtained by the two sets of inactivation procedures are very important and are now in progress to elucidate the structure of key intermediates directly involved in the inactivation.

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