Influences of Some Low Molecular Compounds on Enzymatic Activity and Isoelectric Point of Aspartate Aminotransferase from Rat Liver

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(Received April 2, 1993)

Summary The substances responsible for regulating cytosolic aspartate aminotransferase (AspATc) activity in the cytosolic fraction of rat liver were examined. AspATc was removed from the cytosolic fraction by passing the fraction through an affinity column to which anti-AspATc antiserum was conjugated. The unbound fraction from the column was found to decrease the activity of the purified AspATc. A fraction containing compounds of less than MW 1,000 was obtained by filtering the cytosolic fraction through a YM 2 membrane. This YM 2 filtrate decreased the activity of the purified enzyme; however, the enzymic activity was protected partially by the addition of 2-oxoglutarate or pyridoxal phosphate (PLP). The YM 2 filtrate also decreased the isoelectric points (pIs) of the purified enzyme. Influences of glucose and fructose on AspATc were examined, and fructose was found to decrease the enzymic activity and the pIs. Fructose was more effective on apoenzyme than holoenzyme, suggesting that fructose may bind to the Lys258 residue of AspATc which is the binding site of PLP. The effects of various amino acids including substrates on the enzymic activity were also examined. Some amino acids were found to decrease the enzymic activity to various extents, though the pIs were unaltered. These results suggest that under physiological conditions, AspATc activity is modified by various low molecular substances in various ways.

Key Words inactivation of AspATc, generation of AspATc subforms, isoelectric points of AspATc, glycation of AspATc

There are two isozymes of aspartate aminotransferase (EC 2.6.1.1) in animal cells, a cytosolic and a mitochondrial isozymes. Cytosolic aspartate aminotransferase (AspATc) has been separated by electrophoresis or chromatography into several subforms designated as α, β, γ, etc., in increasing order of negative charge (1, 2). The origin of these multiple forms is still unknown, although several possibilities have been proposed (3–9). Previously, we reported (10–12) that in
vitamin B6-deficient rat liver, the activity of AspATc was decreased and was not restored by the addition of PLP to the assay medium, but that the antigenic activity of the enzyme was not decreased. We also observed an increase in subforms of the enzyme with low isoelectric points (pIs) in vitamin B6-deficient rat liver, and furthermore we observed (13, 14) the similar phenomena as in vivo, when the cytosolic fraction of rat liver was incubated in vitro. This inactivation of AspATc during incubation in vitro was prevented by addition of both 2-oxoglutarate and PLP. Thus generation of variants during incubation seemed to be a reversible reaction. However, the variants present in vivo were not altered by any treatment, suggesting that some irreversible mechanism is involved in their generation.

In this paper we report studies on the factors responsible for inactivation of AspATc in purified enzyme system, and discuss the mechanism of their effect.

MATERIALS AND METHODS

Preparation and resolution of AspATc from rat liver. Male Wistar strain rats, weighing about 200 g, were fed a 70% casein diet for a week and then their livers were removed for purification of AspATc. The enzyme was purified essentially by the method of Huynh et al. (15). The final preparation of AspATc gave a single band on acrylamide gel electrophoresis with sodium dodecyl sulfate (SDS). Resolution of AspATc purified from rat liver was carried out as described previously (11). More than 80% of the resolution was attained without any inactivation. This preparation was used for the experiment as apoenzyme and the apoenzyme activity was expressed by subtracting holoenzyme activity from the total activity measured in the presence of PLP.

Preparation of cytosolic fraction. A liver cytosolic fraction was obtained from rats fed a 70% casein diet. For this, the liver was homogenized in a Teflon-glass homogenizer with 9 vol of 0.25 M sucrose containing 5 mM Tris-HCl and 0.1 mM EDTA (pH 7.4). The homogenate was centrifuged at 100,000 × g for 60 min and the resulting supernatant was used as the cytosolic fraction.

Preparation of affinity column conjugated anti-AspATc antibody. Anti-rat AspATc antiserum was raised in rabbits as described previously (12) and conjugated to Affigel-10 by the method recommended by the supplier of Affigel-10 (Bio-Rad Catalogue '89, p. 45). One hundred units of antibody could be conjugated to 2 ml of Affigel-10. The Affigel-10 column was used to remove AspATc from the cytosolic fraction of rat liver.

Preparation of YM 2 filtrate. The liver of a rat fed a 70% casein diet for a week was homogenized in a Waring blender with 2 vol of water. The homogenate was centrifuged at 100,000 × g for 60 min and the resulting supernatant was passed through a YM 2 filter (Amicon), which cuts off substances with molecular weights of over 1,000.

Chemicals. All reagents used were of the highest grade available and were purchased from Wako Pure Chemicals, Boehringer Mannheim or Sigma Chemicals.
Co. pI markers were obtained from Bio-Rad Laboratory.

*Incubation of AspATc.* Usually the cytosolic fraction of rat liver or purified enzyme solution in 0.1 M potassium phosphate buffer (pH 7.4) was incubated at 37°C for various periods and then remaining activity in a suitable volume of the incubation mixture was measured.

*Isoelectric focusing.* Acrylamide gels (5%) of 1 mm thickness containing 2% Ampholine were prepared on glass plates and a current was applied in a horizontal apparatus (Atto Co. Ltd.) with 1 M NaOH as catholyte and 1 M H₃PO₄ as anolyte. Gels were chilled to 10°C and pretreated at 400 V for 1 h. Samples were then loaded onto the gels with a plastic applicator and focused to equilibrium (overnight, 400 V). The gels were washed with 5% salicylic acid-10% TCA solution for overnight to remove ampholyte and then stained for protein with 0.25% Coomassie Brilliant Blue G in 10% acetic acid-30% methanol solution.

*Analysis of amino acids.* Apo-AspATc samples incubated with D-glucose or D-fructose were reduced with 10 mM sodium borohydride (20°C, for 1 h), extensively dialyzed against water and hydrolyzed in 6 N HCl at 110°C for 24 h. Amino acids were automatically analyzed in an LKB Alpha Plus 4151 amino acid analyzer. Amino acids contained in the YM 2 filtrate were also analyzed as well.

*Other methods.* AspATc activity was measured by the modification of the method of Karmen (16) described previously (12). One unit of enzyme activity was defined as the activity producing 1 μmol product in a minute at pH 7.4. D-Glucose-6-phosphate and D-fructose-6-phosphate contained in the YM 2 filtrate were determined by a sequential enzymatic method as described before (17). Glucose was determined by means of glucose oxidase using Wako B kit. Pentose and ketose were also determined as orcinol and resorcinol reactive substances, respectively (18, 19).

**RESULTS**

*Inactivation of AspATc by low molecular compounds in rat liver cytosol*

AspATc was removed from the cytosolic fraction of liver by passing the fraction through an affinity column (Affigel-10) to which anti-AspATc antiserum had been conjugated. When the purified AspATc was incubated with the unbound fraction from the column, the AspATc activity was decreased, suggesting that the cytosolic fraction contained some factors that inactivated AspATc (Table 1). Passage of the cytosolic fraction through a Sephadex G-25 column to remove low molecular substances partially reduced its effect on inactivating AspATc activity (data not shown), suggesting that some low molecular compounds present in the cytosolic fraction were responsible for its effect on AspATc activity. Therefore, we prepared a fraction containing compounds of below MW 1,000 by filtering the cytosolic fraction through a YM 2 membrane. The activity of purified AspATc was decreased by incubation with the YM 2 filtrate (Table 2). However, the extent of decrease in the enzymic activity was not proportional to the amount of the filtrate.

Vol. 39, No. 4, 1993
Table 1. Inactivation of purified AspATc by the cytosolic fraction and influences of 2-oxoglutarate and PLP.

| Addition                               | Activity remaining |
|----------------------------------------|--------------------|
|                                        | units/ml | %    |
| None (not incubated)                   | 6.24     | 100  |
| None                                   | 2.63     | 42.8 |
| 2 mM 2-oxoglutarate                    | 4.39     | 71.4 |
| 0.1 mM PLP                             | 4.00     | 65.0 |
| 2 mM 2-oxoglutarate, 0.1 mM PLP        | 5.41     | 88.0 |

AspATc was removed from the cytosolic fraction by passing the fraction through a column conjugated anti-AspATc antiserum. AspATc purified from rat liver was incubated with the unbound fraction at 37°C for 20 h and then remaining activity was determined. The incubation mixture contained about 50 μg of purified AspATc in 1 ml. Values are means for two determinations in duplicate experiments. Other conditions are described in the text.

Table 2. Inactivation of purified AspATc by the YM 2 filtrate of the cytosolic fraction.

| YM 2 filtrate (ml) | 2-Oxoglutarate (2 mM) | Activity remaining (%) |
|-------------------|-----------------------|------------------------|
| 0                 | 100                   |
| 0.2               | 15.7 ± 1.9            |
| 0.4               | 32.4 ± 1.9            |
| 0                 | 101.0 ± 0.4           |
| 0.2               | 63.4 ± 7.7            |
| 0.4               | 80.5 ± 7.4            |

The cytosolic fraction of rat liver was filtered through a YM 2 membrane and about 50 μg of purified AspATc was incubated with the indicated amounts of the filtrate in total 1 ml. Values are means for 5 determinations ± SD. Other experimental conditions are given in the text.

added. The YM 2 filtrate possibly contains various low molecular compounds including not only inactivators but also protectors and/or activators of AspATc activity. The decrease in enzymic activity was prevented by the addition of 2-oxoglutarate to the incubation system, as in the crude system.

Characterization of the inactivators of AspATc

Some properties of the factors inactivating AspATc were examined. Heat-treatment of the YM 2 filtrate for 2 min at various temperatures of up to 95°C had little effect on its inactivation of the enzymic activity. Additions of a sulfhydryl reagent or EDTA to the incubation system also had no significant effect on inactivation of the enzymic activity (data not shown).
INACTIVATION OF AspATc

Influences of YM 2 filtrate on the IEF pattern of purified AspATc

The isoelectric focusing (IEF) patterns of AspATc incubated with YM 2 filtrate, PLP and 2-oxoglutarate are shown in Fig. 1. Enzyme itself gave several bands and addition of PLP to the purified enzyme showed a higher pI value (holoenzyme). When the enzyme focused with YM 2 filtrate, the IEF patterns were markedly changed, showing that bands with higher anodic mobility increased regardless of the presence or absence of PLP. Addition of 2-oxoglutarate to these systems increased bands with lower anodic mobility.

Influences of various amino acids on the activity and IEF patterns of AspATc

Aspartate and glutamate, which are substrate of AspATc, were the most effective for inactivating the enzyme (more than 90% inhibition). Alanine, citrulline, histidine, leucine, methionine, phenylalanine, and tryptophan were moderately effective (70–90% inhibition), and glycine, ornithine and serine were slightly effective (40–60% inhibition). The other amino acids tested did not affect the enzymic activity. After inactivation in the presence of all these amino acids except glycine and ornithine, the activity could be restored almost completely (> 80%) by brief incubation in the presence of PLP. These amino acids may contribute to produce pyridoxamine (PMP) form and then apo-form by releasing PMP. In contrast the enzymic activity inactivated by the addition of ornithine or glycine could not be restored in the presence of PLP (data not shown). As shown in Fig. 2, these amino acids did not alter the IEF pattern of AspATc.
Fig. 2. Effects of glycine and ornithine on isoelectric focusing pattern of AspATc. Purified enzyme was incubated with 0.5 mM glycine or ornithine. Conditions are as in Fig. 1. Additions are as follows: 1, none; 2, glycine; 3, ornithine; 4, YM 2; 5 and 6, none (not incubated); 7, YM 2+PLP; 8, ornithine+PLP; 9, glycine+PLP; 10, PLP.

Influences of carbohydrate on the activity and IEF patterns of AspATc

Besides amino acids some sugars must be contained in the YM 2 filtrate and so influences of glucose and fructose on the enzymic activity were examined. When AspATc was incubated in the presence of glucose or fructose, the enzymic activity was decreased with increasing of the incubation periods. As shown in Fig. 3, fructose was a more effective inactivator of AspATc than glucose, especially on the apoenzyme. Figure 4 shows the influences of these sugars on the IEF patterns of apo-AspATc. Apoenzyme itself showed pl values compared with the holoenzyme and IEF pattern was diffusive, indicating that conformation of the apoenzyme is unstable because of lack of PLP. The IEF pattern was slightly altered by the addition of glucose (lane 2) and largely altered by fructose (lane 3). When a short incubation with PLP was carried out, the pattern of AspATc without or with glucose was recovered to very similar one to that of holo-AspATc, whereas the pattern of AspATc incubated with fructose was substantially unaltered by the addition of PLP.

Sites of probable glycation of AspATc

AspATc incubated in the presence of glucose or fructose was analyzed for amino acid composition. As shown in Table 3, 20–25% of lysine in AspATc was decreased by incubation with fructose. Numbers of lysyl residue modified with fructose were calculated to be 5–6 residues per subunit. In addition to lysine, serine and glycine were also decreased by about 20%. Glucose was ineffective on modification of AspATc under the condition examined.

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Table 3. Amino acid composition of AspATc incubated with or without sugar.

| Amino acid    | Without addition | With glucose | With fructose |
|---------------|------------------|--------------|---------------|
| Aspartic acid | 1.00             | 1.00         | 1.00          |
| Threonine     | 0.61             | 0.60         | 0.53          |
| Serine        | 0.72             | 0.79         | 0.58          |
| Glutamic acid | 1.14             | 1.21         | 1.25          |
| Glycine       | 0.93             | 1.18         | 0.76          |
| Alanine       | 0.90             | 0.98         | 0.82          |
| Valine        | 0.79             | 0.77         | 0.75          |
| Isoleucine    | 0.45             | 0.49         | 0.45          |
| Leucine       | 0.97             | 1.02         | 1.00          |
| Tyrosine      | 0.38             | 0.38         | 0.37          |
| Phenylalanine | 0.67             | 0.66         | 0.64          |
| Lysine        | 0.61             | 0.59         | 0.47          |
| Histidine     | 0.20             | 0.25         | 0.18          |
| Arginine      | 0.67             | 0.63         | 0.59          |
| Proline       | 0.45             | 0.46         | 0.43          |

1 and 2 contain asparagine and glutamine, respectively. Values are expressed as molar ratios to aspartic acid. Cysteine and tryptophan were not determined under the condition used. The experiment was repeated and the values are means for 2 determinations in the typical one. The experimental conditions are described in the text.

Table 4. Amino acid composition of the YM 2 filtrate prepared from liver of rat fed 70% casein diet.

| Amino acid       | mM   | Amino acid | mM   |
|------------------|------|------------|------|
| PHO-serine       | 0.06 | Valine     | 2.57 |
| Taurine          | 2.62 | Cystine    | 0.03 |
| P-ETH-amine      | 0.21 | Methionine | 0.64 |
| Aspartic acid    | 1.11 | Isoleucine | 1.21 |
| Threonine        | 1.60 | Leucine    | 2.25 |
| Serine           | 2.04 | Tyrosine   | 0.72 |
| Asparagine       | 1.17 | $\beta$-Alanine | 0.55 |
| Glutamic acid    | 4.75 | Phenylalanine | 0.59 |
| Glutamine        | 14.63 | $\gamma$-ABA | 0.04 |
| Sarcosine        | 16.52 | Ornithine  | 0.80 |
| Glycine          | 2.42 | Lysine     | 1.63 |
| Alanine          | 7.31 | Histidine  | 0.63 |
| Citrulline       | 0.15 | Proline    | 2.47 |

1DL-o-Phosphoserine. 2o-Phosphoethanolamine. 3$\gamma$-Aminobutyric acid.
Fig. 3. Effects of fructose and glucose on AspATc activity. 130μg of AspATc was incubated with 50mM fructose or glucose in the final volume of 600μl at 37°C, an aliquot was taken at the time indicated and the remaining activities were determined. Values were expressed as % activity of control (without addition) which was essentially unchanged during the incubation. ○, apoenzyme with fructose; △, apoenzyme with glucose; ●, holoenzyme with fructose; ▲, holoenzyme with glucose. Other experimental conditions are described in the text.

Fig. 4. Effects of fructose and glucose on isoelectric focusing pattern of AspATc. Apoenzyme was incubated at 37°C for 100h (lanes 1–3) and then the incubation was continued in the presence of 10⁻⁴M PLP for further 2h (lanes 5–7). Additions are follows: 1 and 5, none; 2 and 6, 50mM glucose; 3 and 7, 50mM fructose; 4, pI markers (phycocyanin, 4.65; β-lactoglobulin B, 5.10; bovine carbonic anhydrase, 6.00; human carbonic anhydrase, 6.50; equine myoglobin, 7.00; human hemoglobin A, 7.10; human hemoglobin C, 7.50; lentil lectin, 8.20, 8.30, 8.60; cytochrome C, 9.60).

Contents of amino acids and carbohydrate in the YM 2 filtrate

The YM 2 filtrate was analyzed for amino acids and carbohydrates, and the data are shown in Tables 4 and 5, respectively.

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Table 5. Carbohydrate contents in the YM 2 filtrate prepared from liver of rat fed 70% casein diet.

| Carbohydrate            | mM     |
|-------------------------|--------|
| Glucose-6-phosphate     | 0.81 ± 0.00 |
| Fructose-6-phosphate    | 0.18 ± 0.02 |
| Glucose                 | 8.10 ± 0.19 |
| Pentose\(^1\)           | 4.39 ± 0.05 |
| Ketose\(^2\)            | 0.47 ± 0.00 |

\(^1\) Estimated as ribose. \(^2\) Estimated as fructose.

**DISCUSSION**

In the study on factors responsible for inactivation of AspATc in vitro, we found that some low molecular substances in rat liver cytosol are very effective as inactivators. We also found that the inactivation of the enzyme could be partially protected by the addition of 2-oxoglutarate or PLP (Table 1). We prepared the YM 2 filtrate which contains compounds of below MW 1,000 from rat liver. The addition of the YM 2 filtrate to AspATc was effective in decreasing the enzymic activity, but the extent of the decrease was not proportional to the amounts of the filtrate added (Table 2). Such phenomenon can be understood if we consider that the filtrate may contain various substances, including inhibitors, protectors and activators of the enzyme.

Then amino acids, which must be contained in the YM 2 filtrate, were examined and some of the amino acids, including aspartate, glutamate, alanine, citrulline, histidine, leucine, methionine, phenylalanine, tryptophan, glycine, ornithine, and threonine, were effective to inactivate the enzyme, but the inactivated enzyme was reactivated by the addition of PLP except for glycine and ornithine. This means that those amino acids contributed to form pyridoxamine form of the enzyme, which eventually converted to the apoenzyme, because PMP is known to have a lower affinity than PLP for the enzyme protein. Glycine and ornithine were moderately effective to inactivate AspATc and the activities were not restored by addition of PLP. Since glycine and ornithine are known not to bind the active site of AspATc, the mechanism of the inactivation remains to be studied. These amino acids did not alter the IEF pattern of AspATc (Fig. 2).

Next we examined the effects of glucose and fructose on AspATc activity as some sugars must be contained in the YM 2 filtrate. It was found that fructose was effective for the inactivation of AspATc but also for the alteration of the IEF patterns (Figs. 3 and 4), whereas glucose which is contained in the filtrate at a high level had little effect on both the enzyme activity and the IEF pattern under the condition examined. Recently, glycation (non-enzymatic glucosylation) of several proteins and the decrease in functional activities of the glycated proteins have been
reported in *in vivo* and *in vitro* systems (18, 19). Fructose was more reactive to the apoenzyme than the holoenzyme. This may show that the lysyl residue which is the binding site of PLP, is the most active site for modification of AspATc with fructose, although some other lysyl residues were also modified (Table 3). Fructose itself does not seem to be contained in the cytosolic fraction of rat liver at a level high enough for binding to AspATc. Various substances with aldehyde or ketone residue such as intermediary metabolites of glucose must be contained at various levels. It would be possible to consider that such various compounds attack some lysyl residues of AspATc and that these modified AspATc cause variants on the pI values.

There are some reports indicating that AspATc from pig heart contains sugars such as glucose, mannose and sialic acid, etc. (6, 20), although the physiological meaning for these sugars has not been elucidated. We previously observed increases in AspATc variants with low pI values and low enzymic activities in the liver cytosol from vitamin B6-deficient rat (10, 11). When rats were subjected to vitamin B6 deficiency, apoenzyme must be increased and so various compounds with aldehyde or ketone residue would be easily bound to Lysine 258 of AspATc which is a binding site of PLP. This might be a mechanism of increasing in inactive AspATc molecules with lower pI values in the liver from vitamin B6-deficient rat. Various low molecular compounds which may cause alterations of AspATc activity and of pI values, would be contained in cytosol of rat liver. Thus in the physiological conditions, AspATc may be regulated by such various low molecular compounds in various ways.

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