# ESSENTIAL ARGINYL RESIDUES IN ASPARTATE AMINOTRANSFERASES

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Summary

The active sites of the aspartate aminotransferases from both pig heart supernate and beef kidney mitochondria contain functional arginyl residues. Inactivation with butanedione in the presence of borate buffer, its reversal by gel filtration and irreversible inactivation with phenylglyoxal provide strong evidence for a functional role of these residues. Quantitative studies with the pig heart enzyme indicate two arginyl residues per subunit are modified. Protection experiments suggest that these residues participate in binding substrate and are not involved in the interaction of apoenzyme with pyridoxal phosphate

## Introduction

It has become increasingly apparent that arginyl residues serve a general function as binding sites in a large number of enzymes, particularly those that act on substrates or cofactors carrying a negative charge. Thus, E. coli alkaline phosphatase (1), fructose 1,6-diphosphate aldolase (2), ribonuclease A (3) and a number of reverse transcriptases (4) all act on phosphorylated substrates and are inactivated by reagents that specifically modify arginyl residues. Similarly, such reagents inactivate several dehydrogenases (5-7), at least five kinases (8) and two synthetases (9), all requiring phosphate-containing coenzymes. In addition, arginyl residues participate in binding the substrate C-terminal carboxyl group of both

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carboxypeptidases A (10) and B (11). We have now extended these investigations to two aspartate aminotransferases, one from pig heart cytosol and another from beef kidney mitochondria. These enzymes catalyze reactions involving the dicarboxylic acids aspartate, α-ketoglutarate, oxaloacetate and glutamate. In addition, they employ pyridoxal phosphate as a cofactor. Braunstein (12) has proposed three different positively charged binding sites at the active center of the enzyme, one for each of the two carboxyl groups of the substrate and one for the phosphate of the coenzyme. Modification of these aspartate aminotransferases with butanedione and phenylglyoxal has revealed that arginyl residues are indeed critical for their activity. Protection experiments indicate that the substrates but not the coenzyme interact with these residues.

## Materials and Methods

Pig heart cytosol aspartate aminotransferase, prepared according to Jenkins et al. (13) was obtained from Boehringer-Mannheim Corp. as a suspension in ammonium sulfate. Beef kidney mitochondrial aspartate aminotransferase was prepared by the method of Scandurra and Cannella (14). The preparation of the apoenzymes has been described (14,15). Both enzymes were assayed as described previously (14,16) following the decrease in absorbance at 340nm at 25°. The assay solutions (3 ml total volume) were buffered with either 50mM potassium phosphate or sodium borate, pH 7.7, as indicated. Enzyme modifications with 10mM butanedione, Aldrich, were carried out at 25° in 50mM sodium borate, pH 8.0, or with 1.5mM phenylglyoxal, Aldrich, in 125mM sodium bicarbonate, pH 7.9. In some cases substrates or inhibitors were present during the modification reaction.

Arginine modification was determined using protein aliquots hydrolyzed in vacuo in 6 N HCl at 110° for 22 hours and analyzed on a Beckman 120C amino acid analyzer. The incorporation of $^{14}$C-phenylglyoxal (prepared from $^{14}$C-acetophenone, ICN, by the procedure of Riley and Gray (17)) was determined by liquid scintillation counting of aliquots diluted into Aquafluor and measured on a Packard Tricarb instrument.

## Results

The time course for the inactivation of pig heart aspartate aminotransferase by 10mM butanedione in 50mM borate buffer is shown in Fig. 1. Activity decreases to less than 5% of the control after 20 min. The presence of α-ketoglutarate, 1.75mM, is virtually without effect on the rate of inactivation. L-Glutamate, 70mM, affords partial protection, and no further effect is observed if both substrates are present simultaneously. Glutarate, 30mM, a competitive inhibitor of the enzyme, also affords approximately the

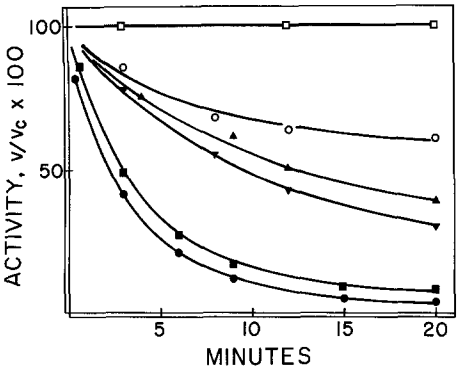


Figure 1. Inactivation of pig heart aspartate aminotransferase with butanedione. Enzyme, 0.85 mg/ml, in either 50mM borate (●) or 50mM phosphate (○) was incubated with 10mM butanedione and aliquots removed at the indicated times for assay. Protection experiments, carried out in the same way in borate buffer, contained α-ketoglutarate, 1.75mM (■); glutarate, 30mM (▼); L-glutamate, 70mM (▲); or α-ketoglutarate, 1.75mM, and L-glutamate, 70mM (◢). The control (□) was incubated in borate buffer without butanedione

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same degree of protection as L-glutamate. The degree of inactivation is markedly reduced if the modification reaction is carried out in 50mM phosphate buffer rather than in borate. Borate alone has no effect on the activity of aspartate aminotransferase.

The inactivation due to butanedione is reversible. Gel filtration of a modified enzyme sample having 15% activity through a Bio-Gel P-4 column equilibrated with phosphate buffer, pH 8, fully restores native activity after 80 min. If gel filtration is performed in borate buffer, no return of activity occurs over the same time period.

Similar effects of butanedione on enzymatic activity have been observed with the aspartate aminotransferase isolated from beef kidney mitochondria. Approximately 70% of the activity of the enzyme is lost after 20 min reaction with 10mM butanedione. The presence of α-ketoglutarate protects slightly, L-glutamate is more effective and a combination of the two is intermediate in preventing the loss of activity.

Inactivation of the pig heart enzyme correlates with the modification of

two arginyl residues per enzyme subunit (Table I). Amino acid analysis of a sample treated with butanedione for 35 min indicates 24.3 arginyl residues per subunit compared to 26.1 for the unmodified control. Neither histidine, lysine nor any other amino acid is modified. Gel filtration to remove borate and incubation in phosphate buffer for an hour restores activity to 100% and increases the arginyl content to 26.6 residues per subunit.

TABLE I

Activity and Basic Amino Acid Composition of Butanedione-Modified Pig Heart Aspartate Aminotransferase

| Enzyme | V/Vc x 100 | Residues/Subunit Lys | His | Arg | Arg Modified per subunit |
|---|---|---|---|---|---|
| Control | 100 | 19.7 | 7.7 | 26.1 | --- |
| +Butanedione[a] | 8 | 19.5 | 7.9 | 24.3 | 1.8 |
| +Gel filtration[b] | 89 | 19.5 | 7.7 | 26.6 | --- |
| +Phenylglyoxal[c] | 15 | | | | 1.9 |

[a]The native enzyme, 1.04 mg/ml, was modified with 10mM butanedione in 50mM borate, pH 8.0, 25°, for 30 min. One aliquot was assayed for activity and another, 400 µl, was precipitated with HCl, washed and hydrolyzed for amino acid analysis.

[b]A third aliquot, 500 µl, was gel filtered through a 0.7 x 17 cm column of Bio-gel P-4 equilibrated and developed with 50mM potassium phosphate, pH 8.0. The eluent fraction containing enzyme was incubated at 20° for 60', assayed and processed for amino acid analysis. There were no differences between samples in the neutral and acid amino acids.

[c]Native enzyme, 0.4 mg/ml, was modified with 1.5mM $^{14}$C-phenylglyoxal in 125mM bicarbonate, pH 7.9, 25°, for 30 min. One aliquot was assayed for activity and the rest analyzed for radioactivity after gel filtration through Bio-gel P-4.

Phenylglyoxal in bicarbonate buffer also inactivates both the beef kidney mitochondrial and the pig heart supernatant enzymes. As in previous instances (2,5,8) activity is not restored by dialysis or gel filtration. In the case of the pig heart enzyme, activity at various times of reaction correlates with the incorporation of $^{14}C$-labeled reagent. Assuming a stoichiometry of two moles of phenylglyoxal per mole of arginine (17), extrapolation indicates complete inactivation on modification of two arginyl residues per subunit. These results are consistent with those obtained using butanedione and further suggest that residues other than arginine are not modified.

In order to examine the possibility that arginyl residues might be involved in the interaction of apoenzyme with pyridoxal phosphate, coenzyme binding was measured by spectrophotometry. The native and phenylglyoxal modified enzymes were converted to the apo form as previously described (16). A sample of apoenzyme was then treated with phenylglyoxal and all three samples -- apo(native), apo(phenylglyoxal-modified), and phenylglyoxal-modified modified(apo) -- were incubated with pyridoxal phosphate. Activity and coenzyme binding measurements were made subsequent to gel filtration to remove free coenzyme (Table II). Under the reconstitution conditions employed, approximately 1.2 moles of pyridoxal phosphate are bound per mole of unmodified coenzyme and 55% of native activity is recovered. Modification with phenylglyoxal, either before or after removal of coenzyme, does not affect the amount of pyridoxal phosphate which can recombine with the modified apoenzyme. In both cases about 1.1 moles of coenzyme are bound per mole of enzyme, the same as with the unmodified enzyme. However, in neither case is there any restoration of native activity.

## Discussion

A number of amino acid residues have been implicated in the mechanism of action of aspartate aminotransferase (12). One lysyl residue per subunit serves to form a Schiff base with pyridoxal phosphate and may function as a proton donor-acceptor during transaldimination. One histidyl residue per

TABLE II

Activity and Coenzyme Binding of Phenylglyoxal-modified Pig Heart Aspartate Aminotransferase

| Enzyme | Relative Activity, $V/V_c \times 100$ | | Mole PLP / Mole Enzyme |
|---|---|---|---|
| | Before Resolution[a] | After Reconstitution[b] | |
| Control | 100 | 55 | 1.2 |
| +Butanedione[c] | 3 | 2 | 1.1 |
| Apo+Butanedione | - | 2 | 1.1 |

[a]Apoenzyme and coenzyme were resolved as in (15)

[b]Enzymes were reconstituted by incubation with a 3-fold molar excess of pyridoxal 5'-phosphate (PLP) for 1 hr, 20°.

[c]Modification as in Fig. 1.

subunit may also be involved in proton transfer. Modification of one cysteinyl residue leads to 95% inactivation, its rate of modification being increased markedly by the presence of substrates (19). Similarly, one tyrosyl residue undergoes syncatalytic modification (16) and nitration of one tyrosine in the apoenzyme abolishes coenzyme binding (20). The participation of a tyrosyl residue in stabilizing the coenzyme conformation is an important feature of the dynamic stereochemical mechanism proposed by Karpeisky and coworkers (21).

The data shown here indicate that arginyl residues are critical to the mechanism of action of two different aspartate aminotransferases. The specificity of the reagents employed, the dependence of butanedione inactivation or the presence of borate, and the effect of gel filtration are all consistent with arginine modification (5,10). Moreover, amino acid analysis demonstrates that only arginyl residues are modified. The protection experiments suggest that these residues participate in substrate binding.

Arginine has previously been suggested as a possible cationic site for binding the phosphate group of the coenzyme as well as the carboxyl groups of the substrates (12). The present results would seem to rule out an interaction between arginine and the coenzyme but confirm that substrate binding requires at least one arginyl residue per subunit. It is not possible, on the basis of data available thus far, to determine whether it is the α- or ω-carboxyl group of the substrate, or both, which interacts with arginine, but the latter may be more likely since the α-carboxyl group has been thought to associate with the imidazole group of a histidine (12).

The finding that arginyl residues are critical for substrate binding in the two enzymes examined here provides an important means to extend the functional comparisons among transaminases from various sources. We are currently studying those enzymes which are specific for monocarboxylic amino acids for their response to arginine modification and also mitochondrial and supernatant enzymes from the same tissue and species. The present study emphasizes the ever more apparent incidence of functional arginyl residues at the active site of enzymes acting on anionic substrates.

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