Inactivation of cytosolic aspartate aminotransferase accompanying modification of Trp 48 by *N*-bromosuccinimide

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Reaction of *N*-bromosuccinimide with pig heart cytosolic aspartate aminotransferase led to loss of the enzymatic activity. Chemical analysis indicated the modification of two tryptophan residues. At a low ratio of *N*-bromosuccinimide to enzyme, oxidation of Trp 122 occurred without affecting the enzymatic activity. Increase in the ratio resulted in the oxidation of Trp 48 with a concomitant decrease in enzyme activity. The modified enzyme did not react with substrates and their analogs. Trp 48 is not within the active site but in the hinge region linking the large domain of the enzyme to the small domain that shows dynamic movement upon binding substrates. The present result suggests that oxidation of Trp 48 may impair the structural integrity of the interdomain interface.

Aspartate aminotransferase *Substrate induction* *Conformational change* N-*Bromosuccinimide* *Tryptophan residue* *Interdomain interface*

1. INTRODUCTION

Cytosolic aspartate aminotransferase (cAsp-AT) (EC 2.6.1.1) from pig heart has 9 tryptophan residues per monomeric unit of the enzyme [1]. Of these, Trp 140 is situated in front of the lower part of the pyridine ring of the bound coenzyme, pyridoxal-P [2]. The functional role of this active-site tryptophanyl residue is not known. However, its successful modification by nitrosodisulfonate led to a marked decrease in the enzyme activity of cAsp-AT [3]. All other tryptophan residues are located outside of the active site and their structural and functional roles, if any, are not known. This paper describes the reaction of NBS with cAsp-AT leading to a marked decrease in enzymic activity with concomitant oxidation of Trp 48 that is not within the active site of the enzyme but is located in a position important for the substrate-induced domain movement [2] of this enzyme.

Abbreviations: NBS, *N*-bromosuccinimide; pyridoxal-P, pyridoxal 5′-phosphate; HPLC, high-performance liquid chromatography

2. EXPERIMENTAL

The α-subform of cAsp-AT was isolated from pig heart as in [4,5]. The purified enzyme showed a specific activity of 250 units/mg protein. The enzyme concentration was calculated from the absorbance of 12.5 at 280 nm for a 1% solution. NBS was obtained from Wako, Osaka. Other reagents were of the highest grade commercially available.

A solution of 21.5 mM NBS in distilled water was prepared freshly prior to use. In typical experiments, appropriate volumes of the NBS solution were added to reaction mixtures (total volume 1.0 ml) containing 1 mg (21.5 nmol as monomeric unit) of cAsp-AT in 0.1 M potassium phosphate buffer (pH 5.0) to give final NBS concentrations of 0,1,2,3,4,5,6,7,8,10, and 15 molar excess over the monomeric unit of the enzyme. Incubation was performed for 10 min at 0°C. The reaction was ter-

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minated by adding $Na_2S_2O_3$ to a final concentration of 1 mM. The resulting samples were determined for enzyme activity and dialyzed overnight at 4°C against 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA.

The transamination activity was determined as described [5]. The rate of α,β-elimination reaction with 3-chloro-L-alanine was determined as in [6]. pH titration of the enzyme spectra was performed on solutions containing 1 mg/ml of the native or NBS-modified enzyme in 0.1 M imidazole-acetate buffer at various pH values and pK values were obtained from the plots of reciprocals of absorbance at 430 nm vs the ratio of base to acid in buffers [7].

For amino acid analysis, enzyme and peptide samples were hydrolyzed at 110°C for 22–24 h in 0.2 ml of 6 N HCl containing 0.05% 3-(2-aminoethyl)indole, 0.5% thioglycolic acid and 0.25% phenol in evacuated and sealed tubes [8]. Hydrolysates were analyzed for amino acid composition in a K101AS automated amino acid analyzer (Kyowa Seimitsu, Tokyo). Methionine sulfoxide was determined by the procedure using treatment with cyanogen bromide [9].

Peptide mapping of tryptic digests of enzyme samples was performed as follows. Native and NBS-modified preparations of cAsp-AT were treated with $NaBH_4$, carboxymethylated and digested with trypsin as in [10]. The tryptic digests (~1 mg) were taken in 200 μl of distilled water and chromatographed in a Hitachi model 638 HPLC system equipped with a Hitachi resin no. 5053 C18 (4 × 250 mm). The elution was performed with two successive gradients using 0.1% ammonium acetate/acetonitrile (19:1) (solvent A) and 0.1% ammonium acetate/acetonitrile (4:6) (solvent B); the first gradient from 0 to 40% solvent B, followed by the second gradient from 40 to 100% solvent B. Elution of peptides was monitored at both 210 and 280 nm.

3. RESULTS AND DISCUSSION

3.1. *Inactivation of cAsp-AT by NBS*

The inactivation showed a critical dependency on the ratio of NBS to enzyme (fig.1). Increase in the molar ratio up to 4 decreased the enzyme activity only by about 10%. Further increase in NBS resulted in progressive inactivation of the enzyme. Thus, the ratio of 4 appears to be a critical ratio at which the inactivation starts. At a ratio of 8, the enzyme showed about 30% remaining activity for the transamination of natural dicarboxylic substrates as well as for the α,β-elimination reaction with 3-chloro-L-alanine. Further increase in the ratio led to the formation of aggregates of modified enzyme. The inactivation profile for either the apoenzyme or the pyridoxamine form of cAsp-AT did not differ from that for the pyridoxal form of the enzyme. Substrates such as glutamate (40 mM), aspartate (40 mM) or 2-oxoglutarate (20 mM) did not protect either form of the enzyme from inactivation by NBS. By contrast, glutarate (20 mM) or 2-methylaspartate (20 mM) increased the critical ratio of NBS to enzyme to 6 or 7, respectively, but the protection was not complete. These data are partly shown in fig.2.

3.2. *Identification of modified amino acid residues*

Modified preparations of cAsp-AT with 30% residual activity were hydrolyzed as described in section 2. Comparison with the native enzyme showed that two tryptophan residues per monomeric unit of the enzyme were oxidized by NBS and that other residues were not changed (table 1). The extent of inactivation as a function of the number of remaining tryptophan residues indicated that the enzyme activity was not appreciably affected during oxidation of the first

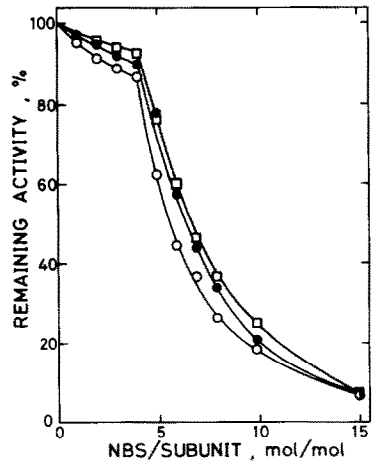

Fig.1. Inactivation of cAsp-AT as a function of NBS to enzyme ratio. (□) Pyridoxal form, (○) pyridoxamine form, (●) apoenzyme. See text for details.

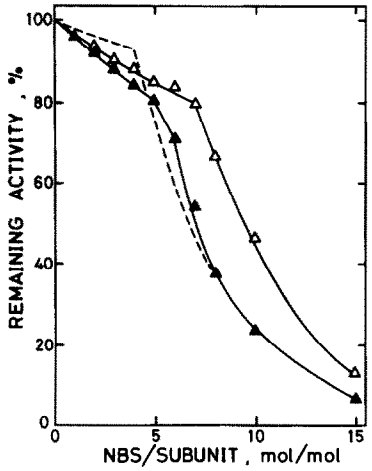

Fig.2. Effect of substrate or substrate analog on the inactivation of cAsp-AT by NBS. (– – –) Pyridoxal form alone (data from fig.1), (▲) in the presence of 40 mM L-glutamate, (△) in the presence of 20 mM 2-methylaspartate. See text for details.

tryptophan residue and the inactivation occurred progressively with the oxidation of an additional tryptophan residue (fig.3). Nonspecific oxidation of additional tryptophan residues appeared to occur above the NBS to enzyme ratio of 6–7. A similar result was obtained with either the apoenzyme or the pyridoxamine form of cAsp-AT.

Table 1

Comparison of amino acid residues between the native and NBS-modified preparation of cAsp-AT

Amino acid residues	Amounts found (mol/mol subunit)	
	Native enzyme	Modified enzyme
Trp (9)	9.03	6.80
Lys (19)	18.95	18.96
His (8)	8.00	7.90
Arg (26)	25.69	25.50
Tyr (12)	12.10	11.90
Phe (23)	22.98	22.94
Methionine sulfoxide	0	0
Cysteic acid	0	0

Data for the sample with 30% remaining activity are shown. Numbers of residues derived from the sequence are shown in parentheses

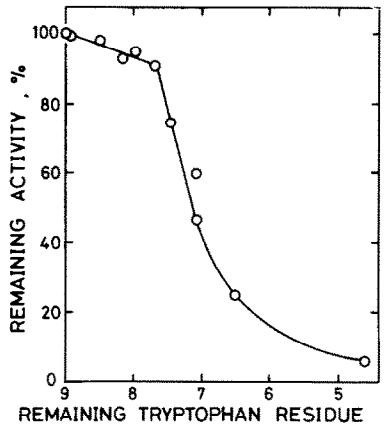

Fig.3. Extent of inactivation of cAsp-AT as a function of the number of remaining tryptophan residues. Samples obtained in the experiments described for fig.1 were analyzed for tryptophan contents.

To locate these two tryptophan residues in the known sequence of cAsp-AT [1], peptide-mapping patterns of tryptic digests were compared between the native enzyme, and modified enzyme samples with 93 and 49% remaining activity (fig.4). Peak 1 was absent in the chromatogram of tryptic digests from the enzyme with 93% remaining activity (fig.4C). With the sample from the enzyme with 49% remaining activity, peak 1 was also missing and peak 2 decreased in intensity by about 50% (fig.4D). This result indicates that the tryptophan residue of a peptide in peak 2 was closely related with the enzyme activity while that in peak 1 was not. Determination of amino acid compositions of these two peptides isolated from the native enzyme revealed that the peptide in peak 1 contained Trp 122 and that in peak 2 contained Trp 48 (table 2). The peptide in peak 3 contained Trp 140. It is noted that this active-site residue [2] was not oxidized by NBS (fig.4).

3.3. *Molecular properties of cAsp-AT modified by NBS*

A modified enzyme with 43% remaining activity showed no sign of peptide bond cleavage on SDS-polyacrylamide gel electrophoresis. As described above, preparations which were modified to an extent lower than 30–40% remaining activity showed a tendency to form aggregates. For this reason, spectral studies were performed on modified enzyme samples with 40–48% remaining activity.

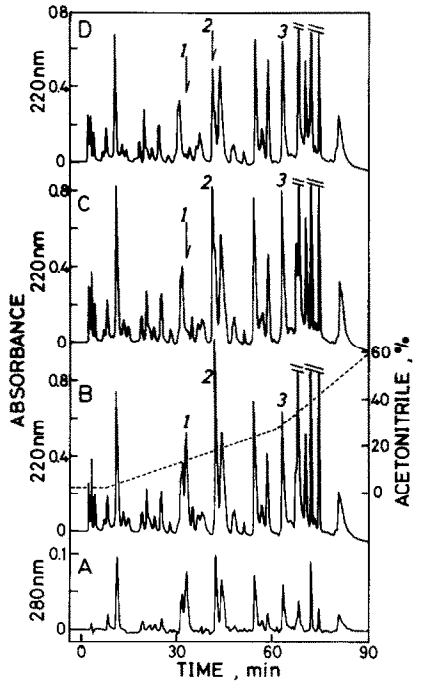

Fig.4. Comparison of HPLC tryptic peptide mapping between the native and NBS-modified preparations of cAsp-AT. (A) From the native enzyme; monitored at 280 nm. (B) From the native enzyme; monitored at 220 nm. (C) From NBS-modified enzyme with 93% remaining activity. (D) From NBS-modified enzyme with 49% remaining activity. (– – –) Gradient of acetonitrile.

The pyridoxal form of cAsp-AT exhibits two absorption bands at 362 and 430 nm which are attributed to the bound pyridoxal-P and known to act as a pH indicator. The protolytic pK value for the internal aldimine nitrogen in the native enzyme is 6.3 at an ionic strength of 0.1 [8]. The modified enzyme with 87% remaining activity showed a pK value of 6.3 while that with 48% remaining activity showed a higher value of 7.23. This seems to indicate that oxidation of Trp 48 might affect the ionic environment of the internal aldimine bond of the bound coenzyme. There was no sign of dissociation of pyridoxal-P by oxidation of Trp 48. Substrate analogs such as succinate, glutarate, *erythro*-3-hydroxyaspartate and 2-methylaspartate are known to form complexes with the pyridoxal form of cAsp-AT, respectively exhibiting characteristic absorption spectra in the visible region [11]. When the pyridoxal form of cAsp-AT

Table 2

Amino acid composition of tryptophan-containing peptides from peaks 1 and 2 in chromatogram B in fig. 4

Amino acids	Peptide in peak 1		Peptide in peak 2	
	Molar ratio	(Integer)	Molar ratio	(Integer)
S-Carboxymethyl-cysteine	–[a]		1.14	(1)
Aspartic acid	2.86	(3)	2.11	(1)
Threonine	0.81	(1)	0.89	(1)
Glutamic acid	–		1.14	(1)
Proline	–		2.15	(2)
Glycine	1.00	(1)	–	
Valine	–		3.13	(3)
Leucine	–		1.00	
Tyrosine	0.93	(1)	–	
Lysine	0.99	(1)	–	
Arginine	–		1.05	(1)
Tryptophan	0.60	(1)	0.67	(1)
Assignment[b]	WYNGTNNK (residues 122–129)		TDDCQPWVLPVVR (residues 42–54)	

[a]Not detectable or less than 0.05
[b]Based on the known amino acid sequence of cAsp-AT [1]

modified by NBS to various extents was spectrally monitored for the ability to form these complexes, it was found that the extent of formation of these complexes was quantitatively accounted for by the fraction of catalytically active species remaining in the modified preparations. Thus, oxidation of Trp 48 seems to result in loss of the binding capacity for substrates and substrate analogs.

Crystallographic studies of cAsp-AT [2] and the mitochondrial isoenzyme [12] indicated that the substrate specificity for dicarboxylates is determined by two arginyl residues at position 292 and 386 and that binding of dicarboxylic substrates induces a dynamic movement of the small domain (residues 14–47 and 350–412) toward the active-site pocket provided by the large domain (residues 70–300). In cAsp-AT, Trp 48 is located in the region that connects the small domain to the large domain. The present result suggests that the conversion of the side chain of this tryptophan residue into oxyindole exerts a profound influence on the structural integrity of the interdomain region and thus impairs the proper movement of the small do-

main which is necessary for productive binding of substrates and subsequent catalysis.

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