# SELECTIVE MODIFICATION OF TYROSINE AND CYSTEINE RESIDUES IN ASPARTATE AMINOTRANSFERASE FROM PIG HEART CYTOSOL

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SUMMARY. In the region of the active site of aspartate aminotransferase two amino acid residues - one Tyr and one Cys - are accessible to selective modification by appropriate reagents. Modification of each of the two residues singly results in certain changes of the enzyme's physico-chemical properties, but does not abolish its ability to catalyse the transamination reaction. Complete inactivation, associated with irreversible amination of the protein-bound pyridoxal-P to pyridoxamine-P, is observed only on modification of both residues.

As reported in several recent papers (1,2,3,4), treatment with tetranitromethane $[C(NO_2)_4]$ under mild conditions is apt to inactivate cytoplasmic aspartate : 2-oxoglutarate aminotransferase (Asp-aminotransferase, EC 2.6.1.1) from pig heart. The extent of inactivation and the number of tyrosine and cysteine residues modified depend on experimental conditions, such as pH, concentration of $C(NO_2)_4$, duration of incubation, presence of substrates or inhibitors.

The aim of the present work was to identify the residues whose modification is responsible for inactivation of the enzyme, and to study the functional role of these residues.

## MATERIALS AND METHODS

Pig-heart Asp-aminotransferase$_{cyt}$ was purified as described previously (5). The enzyme preparations had the following absorbancy ratios (at pH 5.0): $E_{280}/E_{430} = 10.5 \pm 0.5$; $E_{430}/E_{380} = 3.1 \pm 0.2$. Specific activity, assayed according to (6), ranged from 37 000 to 40 000 optical mU per mg. Protein concentrations were estimated by measurement of optical density at 280 nm ($E_{280}^{0.1\,\%} = 1.44$).

Tetranitromethane treatment of the enzyme was performed at pH 8.2 - 8.4 in 0.05 M Tris-HCl buffer. To the buffered protein solution (1.0 - 4.5 mg/ml), methanolic $C(NO_2)_4$ solution was added portionwise in absence or in presence of the substrate pair ($7 \times 10^{-2}$ M L-glutamate + $1.75 \times 10^{-3}$ M 2-oxoglutarate). Initial

concentration of $C(NO_2)_4$ in the methanolic solution was measured by adding aliquots of the solution to 0.1 M aqueous β-mercaptoethanol solution and measuring the extinction at 350 nm due to formation of nitroform ($E_{350}$ = 14 400) (7). The reaction was terminated by addition of concentrated, freshly prepared aqueous mercaptoethanol solution (to a final concentration of 0.1 - 0.15 M). Protein and low-molecular reaction products were separated on a column of Sephadex G-25 equilibrated with 0.05 M Tris-HCl (pH 8.2 - - 8.5).

Alkylation of the enzyme with N-ethyl-maleimide was carried out with a 400 - 450 fold molar excess of reagent (per enzyme subunit of m.w. 45 000) in 0.05 M Tris-HCl buffer, pH 7.5, in the presence of glutamate and 2-oxoglutarate (in concentrations stated above). The protein was then separated from low-molecular components by passage through a column of Sephadex G-25 equilibrated with 0.05 M Tris-HCl, pH 8.2.

Samples of modified enzyme were characterized by measurements of specific activity, Tyr($NO_2$) content ($E_{430}$ = 4100 at pH 8.2) (7), and number of HS groups. The thiol groups were estimated by spectrophotometric titration with 5,5'-dithio-bis(2-nitrobenzoate) (DTNB, purchased from Calbiochem) according to Ellman (8). Concentrations of modified protein were estimated by measurement of optical density at 280 nm, using the same extinction coefficient as for the native enzyme (1).

Tryptic proteolysis of transaminase was carried out in 0.2 M $NH_4$-bicarbonate buffer (pH 8.6) at 37° for 20 - 22 h, after denaturation of the protein samples at pH 3.0 (5 min. in the boiling water-bath). After proteolysis, the lyophilized digests were subjected to peptide mapping on paper, using electrophoresis (pyridine-acetate buffer, pH 3.4; 35 V/cm; 2.5 h) in the first direction and descending chromatography (solvent system - butanol: acetic acid:pyridine:water = 15:3:10:12; 16 h) in the second. Tyr ($NO_2$)-containing peptides were isolated preparatively as previously described (4).

Absorption spectra were recorded on "Specord" (GDR) or "Hitachi" (Japan) spectrophotometers.

## RESULTS AND DISCUSSION

The properties of Asp-aminotransferase modified by treatment with tetranitromethane may differ markedly, depending on the presence or absence of substrates during the incubation with modifying reagent. As seen on Fig. 1 (A and B), nitration of 1.0 - 1.3 tyrosine residues in absence of substrates results in 50 - 60 % loss of activity, while the - considerably more rapid - nitration of approximately the same number of residues in presence of the substrate pair is associated with 95 % inactivation. Irreversible amination of the cofactor to protein-bound pyridoxamine-P (see ref. 2) occurs only on inactivation of the enzyme by $C(NO_2)_4$ treatment in presence of substrates.

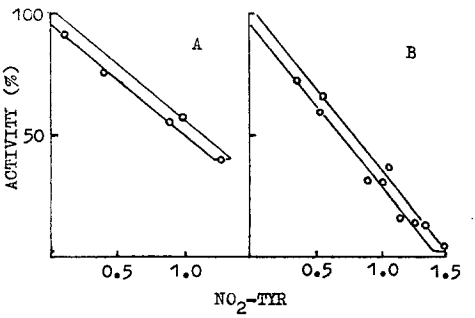


Fig. 1.

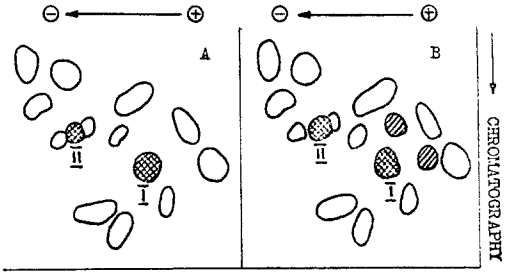


Fig. 2.

Fig.1. Extent of inactivation of Asp-aminotransferase on treatment with $C(NO_2)_4$ in absence (A) and in presence (B) of substrate pair, plotted against amount of $Tyr(NO_2)$ appearing in the protein.

Fig.2. Areas of peptide maps of nitrated aminotransferase preparations in the region containing $Tyr(NO_2)$-peptides. $Tyr(NO_2)$ was revealed on exposure to ammonia vapors. The peptide maps were developed with 0.2% solution of ninhydrin in acetone; $Tyr(NO_2)$-containing peptides are hatched, double hatching marks peptides showing most intense yellow color on exposure to ammonia. A. Samples of enzyme modified with $C(NO_2)_4$ in presence of the substrate pair and containing 0.8-1.0 residue of nitrotyrosine per protein subunit. B. Samples of Asp-aminotransferase treated with $C(NO_2)_4$ in presence or absence of the substrates and containing 1.3-1.5 $Tyr(NO_2)$ residues per subunit.

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On peptide maps of tryptic digests of Asp-aminotransferase modified by $C(NO_2)_4$ in the presence of substrates and containing 0.8 - 1.0 $Tyr(NO_2)$ residue per protein subunit (Fig. 2, A), two $Tyr(NO_2)$-containing peptides are revealed on exposure of the map to ammonia vapors. We demonstrated earlier (4) that peptides I and II contain the major part of fragment TA 26-1 of the enzyme's peptide chain (9). In this chain, Tyr-40 is the residue undergoing "syncatalytic" nitration (2) in the presence of substrates. If more drastic conditions are used for $C(NO_2)_4$ treatment, so that 1.3 - 1.5 $Tyr(NO_2)$ residues per protein subunit are produced, four yellow spots are usually seen on the peptide map in ammonia vapor (Fig. 2, B), two of them in the positions of peptides containing the functionally important Tyr-40. On peptide maps of tryptic digests from nitrated aminotransferase prepared by prolonged ex-

TABLE I

HS GROUP CONTENT OF ASP-AMINOTRANSFERASE TREATED WITH TETRANITROMETHANE IN ABSENCE AND IN PRESENCE OF SUBSTRATES[a]

| Protein sample | $Tyr(NO_2)$[b] | HS[c] prior to SDS addition[d] | After SDS addition[d] | Number of HS groups modified |
|---|---|---|---|---|
| Holoenzyme (aldimine form) | - | 1.4-1.5 | 3.9-4.1 | - |
| Enzyme treated by $C(NO_2)_4$ without the substrates | 1.1-1.3 | 0.1-0.2 | 2.5-2.6 | 1.4-1.5 |
| Enzyme treated by $C(NO_2)_4$ in presence of the substrates | 1.3-1.5 | 0.1-0.2 | 1.5-1.6 | 2.4-2.5 |

[a] 60-fold molar excess of tetranitromethane (per subunit). [b] $Tyr(NO_2)$ residues per subunit. [c] Amount of HS groups per subunit titratable according to Ellman (8). [d] SDS, sodium dodecylsulfate.

posure to $C(NO_2)_4$ in absence of the substrates, four spots are mostly detectable, corresponding to those of the $Tyr(NO_2)$-peptides of "syncatalytically" nitrated aminotransferase. Under sufficiently mild conditions of nitration, when the $Tyr(NO_2)$ content does not exceed 1.3 residues per subunit, the spots containing the functionally important Tyr-40 residue are clearly predominant on the peptide maps. Thus, in either case - i.e., on $C(NO_2)_4$ treatment in presence or in absence of the substrates - this tyrosine residue undergoes preferential (most rapid) nitration.

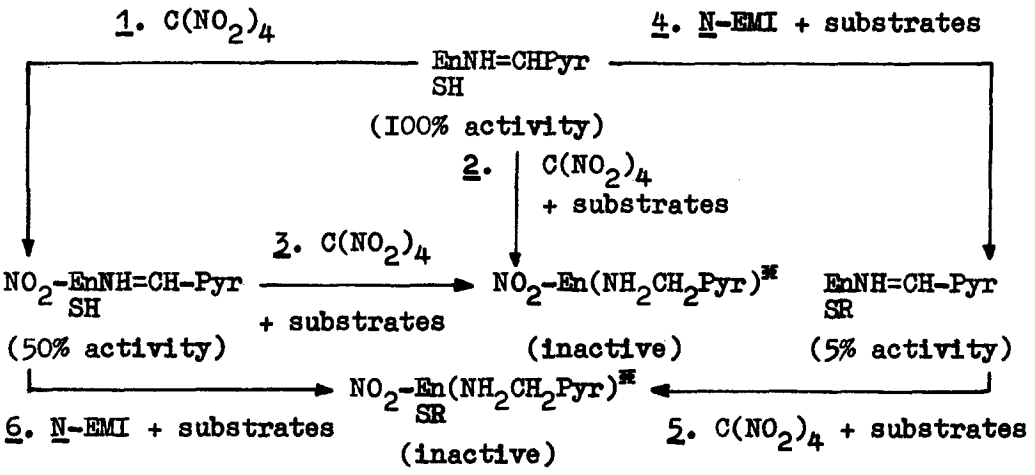
We further studied in detail the modification of HS groups on treatment of the enzyme by tetranitromethane with substrates present or absent. From the data of Table I it is evident that when the enzyme reacts with $C(NO_2)_4$ in presence of the substrates, one thiol group more disappears than in the case of $C(NO_2)_4$ treatment without substrates. The additional HS group oxidized in presence of the substrates is a semi-buried thiol group (it is

titratable with DTNB only in the presence of denaturing agents, e.g., sodium dodecylsulfate). The cysteine residue that becomes susceptible to oxidation by tetranitromethane in presence of the substrate pair is presumably situated in proximity of the active site; it is apparently identical to the functionally important cysteine residue slowly reacting with pMB (10) and undergoing alkylation by N-ethylmaleimide in presence of a substrate pair, i.e., under "syncatalytic" conditions (11). Blocking of the HS group of this residue by any of the reagents mentioned above results in marked lowering of the enzyme's activity. Since no complete inactivation is observed in these cases, and the capacity for interconversion of the aldimine and amino forms of enzyme-bond cofactor is retained, the HS group in point does not participate directly in the catalytic act, although it is presumably located in the region of the active site.

In the peptide chain of Asp-aminotransferase (9) the functionally important cysteine residue is Cys-390 (Yu.Torchinsky, E.Severin, and associates, FEBS Letters, in the press), i.e., the one most close to the C-terminus.

It was obviously of importance to study the changes in physico-chemical properties of Asp-aminotransferase caused by modification singly of either the tyrosine or the cysteine residue under consideration. To this effect a series of experiments were carried out involving sequential modification of these two residues of the protein. Scheme 1 is a concise graphic presentation of the results obtained in these experiments. It shows that complete loss of catalytic activity of Asp-aminotransferase was associated with irreversible amination of the enzyme-bound cofactor and required that the important residues - Tyr-40 and Cys-390 - be both modified (reactions 2, 3, 5, 6). If only Tyr-40 is nitrated and the Cys-390 residue remains intact (reaction 1), the enzyme retains its ability to catalyse the transamination reaction. Subsequent oxidation of this cysteine residue with $C(NO_2)_4$ in presence of the substrates (reaction 3) abolishes catalytic activity. The inference that inactivation of Asp-aminotransferase by tetranitromethane in presence of the substrate is a result of concomitant modification of both Tyr-40 and the important thiol group is supported by experiments on modification of the enzyme with N--ethyl-maleimide and $C(NO_2)_4$ in sequence (reactions 4, 5 and 6).

Scheme I

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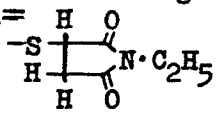
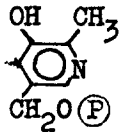
Modified enzyme preparations marked by an asterisc (*) have identical spectral properties.
($NH_2CH_2Pyr$), cofactor in the amino form, non-convertible to the aldimine form, EnNH=CH-Pyr; SH - functionally important thiol group of Asp-aminotransferase; SX--important thiol group of the enzyme oxidized by tetranitromethane; SR=



Pyr=



If aminotransferase alkylated with N-ethylmaleimide (reaction 4) is treated thereafter with $C(NO_2)_4$ in the presence of substrates (reaction 5), catalytic activity is abolished and the bound cofactor is converted to its amino form, which cannot be reconcerted into the pyridoxal-P aldimine. The same result is obtained when the modifications are carried out in inverted order, i.e., first by $C(NO_2)_4$ without substrates (reaction 1) and then in their presence with N-ethylmaleimide (reaction 6).

Essentially similar conclusions concerning the role of simultaneous modification of the two residues have been reached by P.Christen (personal communication).

We were able to demonstrate, moreover, that inactivation of Asp-aminotransferase on modification of the two important groups located in the region of the active center is not attended by significant distortion of the substrate-binding site. The enzyme thus modified retains its capacity to bind substrates or their in-

hibitory analogues, and 2-oxoglutarate is capable to form a Schiff base (ketimine) with the protein-bound pyridoxamine-P of the modified enzyme (unpublished results, 1972).

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