REACTIVITY OF THE CYSTEINE AND TYROSINE RESIDUES OF ASPARTATE TRANSAMINASE FROM CHICKEN HEART CYTOSOL

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SUMMARY

Aspartate transaminase (EC 2.6.1.1) from chicken heart cytosol contains 4 thiol groups per subunit. Two of them are fully buried. One exposed SH group is readily modified by iodoacetamide, N-ethylmaleimide, tetranitromethane, 5,5'-dithiobis(2-nitrobenzoate), 4,4'-dipyridyl disulfide and p-mercuribenzoate. A further SH group is semi-buried: while inaccessible for alkylating reagents and disulfides, it can be blocked by p-mercuribenzoate at pH about 5 (but not at pH 8). Treatment of the enzyme with tetranitromethane in the absence of substrates leads to nitration of maximally 0.8 tyrosine residue per subunit; in the presence of amino and keto substrate 1.65 eq of nitrotyrosine is formed, with a moderate decrease of enzymic activity.

During the last 15 years, chemical modification of transaminases was studied mainly using preparations of aspartate transaminase from pig heart cytosol (1). This enzyme is a dimeric protein possessing 5 thiol groups per subunit, viz., two exposed SH groups, two fully buried and one semi-buried SH group (1-4). The latter group can be blocked selectively by p-MB* at pH 5 with 95% inactivation of the enzyme (2). This SH group, belonging to residue Cys-390, is accessible to modification

*Abbreviations: p-MB, p-mercuribenzoate; NEM, N-ethylmaleimide; DTNB, 5,5'-dithiobis(2-nitrobenzoate); TNM, tetranitromethane; SDS, sodium dodecyl sulfate.

by NEM, DTNB or TNM only in the presence of a substrate pair (so called "syncatalytic" modification, associated with 95% inactivation) (3). Reaction of pig heart Asp-transaminase with TNM results in oxidation of SH groups and nitration of 3 tyrosine residues; Tyr-40 appears to react most readily, particularly in the presence of substrates, with inactivation of the enzyme (4). It has been suggested that a cysteine and a tyrosine residue are part of the active site of Asp-transaminase (4-6).

Comparative investigation of a homologous, but phylogenetically remote, enzyme may provide additional information on the significance of cysteine and tyrosine residues for transaminase activity. Any functionally important residue should be conserved and display similar properties in the enzyme derived from different species whereas its replacement by another amino acid indicates non-essential nature of a given residue. A study of the reactivity and role of cysteine and tyrosine residues in Asp-transaminase from chicken heart cytosol was therefore undertaken.

METHODS

Homogeneous Asp-holotransaminase was prepared from chicken heart cytosol according to the procedure of Kochkina and Torchinskii (7). Concentration of the enzyme in solutions was measured spectrophotometrically, taking $A^{1\%}_{1cm} = 14.2$ at 280 nm (8). SH groups were determined with p-MB (9), DTNB (10) and 4,4'-dipyridyl disulfide (11). Modification with TNM was carried out by adding aliquots of a 0.65 M ethanolic TNM to protein solution in 0.05 M Tris-HCl buffer, pH 8.0. The reaction was stopped by gel filtration on Sephadex G-25, equilibrated with 0.05 M Tris buffer, pH 8.5. The nitrotyrosyl content was determined spectrophotometrically at pH 8.5 assuming $\varepsilon_{428} = 4100\ M^{-1}cm^{-1}$ (12). All modification experiments were

Table 1: Estimations of SH Groups in Asp-Transaminase

Reagent and pH[a]	Number of Accessible SH Groups per Subunit	
	native enzyme	enzyme denatured with 0.5% SDS
p-MB, pH 8.0	1.0	3.8
p-MB, pH 5.2	2.0	-
p-MB, pH 4.6	2.1	3.9
DTNB, pH 8.0	1.0	4.0[c]
4-DPD[b], pH 8.0	1.0	-
4-DPD, pH 5.2	1.2	-

[a] Estimations were carried out in 0.05 M or 0.1 M Tris-HCl buffer (pH 8.0) and in 0.2 M acetate buffer (pH 5.2 or 4.6). [b] 4-DPD, 4,4'-dipyridyl disulfide. [c] 3.7 SH groups were estimated with DTNB in enzyme denatured by 6 M guanidine·HCl.

carried out at 22°. The number of SH groups and nitrotyrosine residues was calculated per protein subunit of mol. wt 50,000; the enzyme consists of two such subunits (13).

RESULTS AND DISCUSSION

Number and reactivity of SH groups. In SDS or guanidine HCl solution a total of 4 thiol groups per subunit was found in chicken Asp-transaminase (Table 1). In the native enzyme, two SH groups react with p-MB at pH ~ 5, whereas only one reacts with p-MB, DTNB or 4,4'-dipyridyl disulfide at pH 8. DTNB does not react with the SH group at pH 5. 4,4'-Dipyridyl disulfide reacts with one SH group at both pH values: the second-order rate constants at pH 8.0 and 5.2 are 328 $M^{-1} s^{-1}$ and 7.2 $M^{-1} s^{-1}$, respectively.

Incubation of Asp-transaminase with 10 mM iodoacetamide (pH 8.0; 1.5 h), 50 mM NEM (pH 7.5; 1.5 h) or 1.5 mM TNM (15-fold molar excess, pH 8.0, 5 min) results in modification of one SH group; the modified enzyme lost the ability to react with DTNB. Thus, iodoacetamide, NEM and TNM modify the same exposed SH group which readily reacts with DTNB. Alkylation or oxidation of the exposed SH group has almost no effect on enzymic activity; blocking of the same group with p-MB or DTNB resulted in decrease of activity by 5-10%. Blocking with p-MB of two SH groups - the exposed and the semi-buried one - reduces activity to 70% of the initial value. Addition of more than two eqs of p-MB to the enzyme does not increase the amount of mercaptide formed (Fig. 1), but induces some addi-

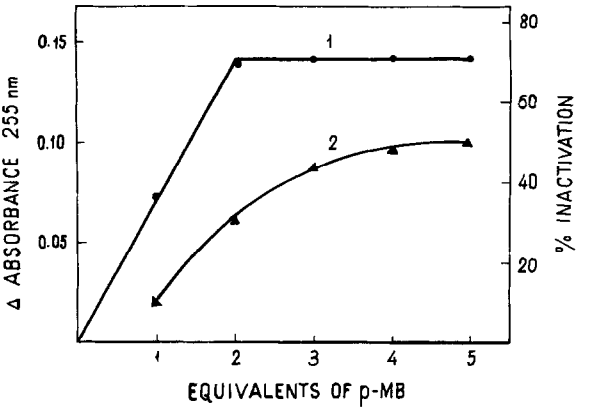

Fig. 1:

Inactivation of Asp-transaminase by p-MB and amount of mercaptide formed in 0.2 M acetate buffer, pH 5.25. Enzyme (0.56 mg per ml) was incubated with varying numbers of eq of p-MB for 20 min; then absorbance at 255 nm and enzymic activity were measured. Curve 1 represents the increase of absorbance; curve 2 - inactivation in per cent of initial value.

tional loss of activity, evidently owing to non-specific interaction between excess p-MB and the protein. The fact that p-MB reacts with the semi-buried SH group at pH 5, though not at pH 8, is probably explained by the increase in the reactivity of p-MB upon acidification (14).

The data presented above allow to distribute the 4 thiol groups of chicken Asp-transaminase as follows: one exposed SH group, readily accessible to various reagents; one semi-buried SH group accessible to p-MB at pH 5, and two fully buried SH groups, accessible only upon protein denaturation. It was of interest to ascertain whether any of SH groups of the chicken enzyme exhibits syncatalytic reactivity similar to that of the SH group of residue Cys-390 in the porcine enzyme. To solve this question, the enzyme was incubated with NEM or iodoacetamide (2,000-fold molar excess, 3h, pH 8.0) or with DTNB (500-fold molar excess, 17h, pH 7.5) in the presence of 70 mM L-glutamate and 2 mM α-ketoglutarate. It was found that the reagents mentioned blocked only the exposed SH group of the enzyme either in the absence or presence of the substrates; no additional loss of activity was observed in the presence of the substrate pair.

Thus, in contrast to the porcine Asp-transaminase the chicken enzyme does not appear to possess a syncatalytically reactive SH group. The chicken enzyme has one SH group per subunit less than the pig enzyme, and blocking one or both its accessible SH groups with p-MB does not induce as sizable loss of activity as occurs in the case of porcine enzyme. Hence it may be inferred that no cysteine residue homologous to the syncatalytically reactive Cys-390 is present in chicken Asp-transaminase. This confirms the conclusion (3) that

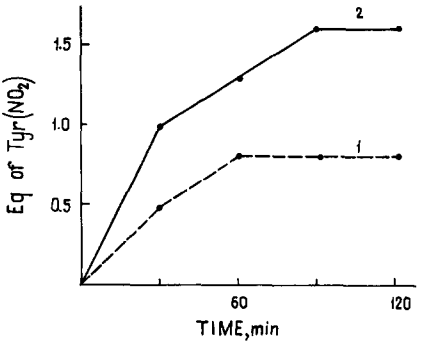

Fig. 2:

Nitration of Asp-transaminase (0.18 mM) with a 60-fold molar excess of TNM (10.8 mM) in 0.05 M Tris-HCl buffer, pH 8.0. Curve 1 represents the time course of tyrosyl nitration in the absence of substrates; curve 2 - same as 1, but in the presence of 70 mM L-glutamate and 2 mM α-ketoglutarate.

Cys-390 in the pig enzyme is non-essential, although its modification with bulky or charged reagents results in very marked loss of catalytic activity.

Reaction with tetranitromethane. Treatment of chicken Asp-transaminase with a 15-fold molar excess of TNM at pH 8.0 or 5.2 leads to oxidation of 1,0-1,2 SH group and to formation of only 0.14 eq of nitrotyrosine, without any decrease of enzymic activity. In higher molar excess TNM induces increasing tyrosyl nitration. The time course of the nitration reaction was recorded with a 60-fold excess of TNM at pH 8.0 (Fig. 2). Under such conditions maximally 0.8 eq of nitrotyrosine was formed in the absence of a substrate pair. Addition to the reaction mixture of L-glutamate and α-ketoglutarate had a marked effect on nitration: 1.65 eq of nitrotyrosine was formed after 90 min of incubation (Fig. 2). Simultaneously with tyrosyl nitration, slow oxidation of the se-

cond (semi-buried) SH group was observed with a 60-fold excess of TNM. Upon nitration in the absence or presence of substrates, enzymic activity was lowered to 90% or to 65% of the initial value, respectively.

Treatment of the apoenzyme with a 60-fold excess of TNM (1h, pH 8.0) led to nitration of ~ 0.7-0.9 tyrosine residue, oxidation of two SH groups and 35-50% inactivation. The moderate inactivation observed may result from a conformational change of the protein induced by modification of residues located outside the active site. Thus, our data provide no evidence supporting presence of an essential tyrosine residue in the chicken enzyme. It was found earlier that pig heart Asp-transaminase is almost completely inactivated by TNM in the presence of substrates, but this inactivation is mainly caused by oxidation of residue Cys-390 and not by nitration of tyrosine (15).

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