THE THIOL GROUPS OF ASPARTATE AMINOTRANSFERASE. REACTIONS OF SPECIFIC REAGENTS WITH ALDIMINE AND AMINIC FORMS OF ENZYME.

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SUMMARY.

The reaction of thiol groups of aspartate aminotransferase with DTNB and p-MB has been studied with the aldimine and the aminic forms of the enzyme. The result indicate that two cysteine residues by dimeric enzyme are exposed to the solvent after the transformation of the aldimine enzyme into the aminic form.

INTRODUCTION.

During the amino acid transamination reaction catalyzed by L-aspartate-2-oxoglutarate aminotransferase (EC 2.6.1.1) the substrate binding on protein could modify the location of some residues and their reactivities with specific reagents.

We have studied the reaction of 5-5'dithiobis-2-nitrobenzoate and paramercuribenzoate with the thiol groups of pyridoxal-5'-phosphate and pyridoxamine-5'-phosphate forms of AAT*.

MATERIALS AND METHODS.

Cytoplasmic aspartate aminotransferase is prepared according to the method of Jenkins, Yphantis and Sizer (4) using succinate instead of maleate. In order to protect the thiol groups and to avoid the formation of multiple forms 10^{-4} M dithiothreitol is added (2) during the purification and the

Abbreviations used : AAT : L-aspartate-2-oxoglutarate aminotransferase
DTNB : 5-5'dithiobis-2-nitrobenzoate
p-MB : paramercuribenzoate
DTT : dithiothreitol.

conservation of the enzyme. The enzymatic activities are determined by the Karmen method (5) with 2.10^{-2} M L-aspartate and 2.10^{-3} M α-ketoglutarate. The aldimine enzyme is transformed into the aminic form according to the method of Jenkins and d'Ari (6). Protein thiol groups are reduced by DTT (2). Detection of thiol groups is carried out with DTNB according to Ellman (7). The enzyme and reagent concentrations used are respectively 5.10^{-6} M and 2.10^{-4} M at pH 8.3 in 0.05 M TRIS-HCl buffer. There is only formation of mixed disulfide between free sulfhydryl group and thionitrobenzoate. After filtration of a mixture of enzyme with an excess of DTNB using a Sephadex G 25 column the enzymic form with cysteine residues accessible blocked by DTNB, called AAT_{TNB}, is obtained.

The method of Boyer (8) is used to titrate the sulfhydryl groups. At pH 5.5 in 0.1 M acetate buffer and 5 M urea, 5.10^{-6} M protein is incubated with 10^{-4} M p-MB. In order to avoid light scattering during spectrophotometric titration at 250 nm the protein solutions are centrifuged 30 minutes at 30000 rpm before and after addition of p-MB. The data are recorded after 2 hours of incubation.

Amino acids, α-keto acids and DTT are Calbiochem A grade reagents. NADH and malatedehydrogenase are purchased from Boehringer. We use urea from Merck and DTNB from Aldrich.

The number of thiol groups are expressed for a dimeric enzyme of molecular weight 90,000.

RESULTS.

It has been previously shown that during the enzyme purification and conservation, some of the 10 thiol groups of AAT (3) are rapidly oxidized (1) and modify the sulfhydryl titration.

The aldimine form of AAT, with thiol groups reduced by DTT (2) reacts with 6 thionitrobenzoate molecules per mole of dimeric enzyme. Using p-MB titration, in the presence of denaturing reagent, 9.7 cysteine residues are titrated. The protein previously reduced by DTT and converted into its

TABLE I

DTNB and p-MB reaction with different forms of AAT.

	Thiol groups detected by DTNB SHa	Thiol groups titrated by p-MB in the presence of urea	SHb
Aldimine form of reduced AAT	6	9,7	3,4
Aldimine form of oxidized AAT	3,5	7,5	4
Aldimine form of AAT-TNB	0	3,9*	3,9*
Aminic form of reduced AAT	7,3	9,4	2,1
Aminic form of oxidized AAT	3,5	5,5	2

Conditions : enzyme 5. 10^{-6}M

DTNB 2. 10^{-4}M at pH = 8.3 Tris-HCl buffer 0.05 M.

p-MB 10^{-4}M at pH = 5.5 acetate buffer 0.1 M.

The numbers are reagent moles fixed by mole of enzyme.

*at pH = 8.3 Tris-HCl buffer 0.05 M.

aminic form reacts with 7.3 thionitrobenzoate molecules. When p-MB titration is performed 9.4 thiol groups are titrated. We can detect both on the aldimine and the aminic forms of AAT 2 kinds of thiol groups : SHa groups (easily accessible) are blocked by DTNB ; as shown on Table I the number of thionitrobenzoate molecules fixed decreases with the oxidation of the enzyme ; SHb groups (buried) are cysteine residues only titrated by p-MB in the presence of urea. SHb groups correspond to the difference between the total number of thiol groups and the SHa groups accessible to DTNB. SHb groups obtained for the aldimine form and the aminic form of AAT are compared. The enzyme

oxidation does not affect the number of SHb groups. The aldimine enzyme has 4 SHb groups ; the aminic enzyme has 2 SHb groups.

The enzymatic activity has been followed during DTNB titration of SHa groups. In the presence of an excess of reagent, the SHa groups are very rapidly blocked (less than 1 minute). To detect if one of these fast reacting groups can play an important role during the enzymatic catalysis, progressively small aliquots of DTNB are added to the 5.10^{-6} M protein solution to block the thiol groups one by one (5.10^{-6} M of DTNB in the titration mixture, every 3 minutes). Simultaneously enzymatic activities are determined, under conditions of substrates concentration giving a reaction rate equal to the V_{Max} for transamination reaction. An inactivation of 15 % to 20 % of pyridoxal-5'-phosphate form of AAT is obtained when 2 SHa groups are blocked by DTNB. The observed inhibition does not vary after binding of other thionitrobenzoate molecules on the protein (figure 1). The pyridoxamine-5'-phosphate form of AAT loses 35 % to 40 % of initial activity of the enzyme when SHa groups have reacted with DTNB (figure 1).

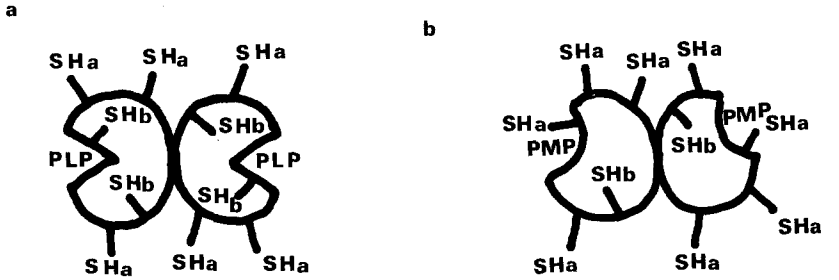

Figure 1 : variation of the enzyme activity as a function of the number of thiol groups blocked by DTNB.
● aldimine AAT
□ reduced aldimine AAT
× aminic AAT

DISCUSSION.

The cysteine residues reactivity of aldimine and aminic forms of AAT have been compared, using two different reagents p-MB and DTNB. On the reduced protein, in the presence of urea, p-MB reacts with 9.7 and 9.4 thiol

groups, among 10 cysteine residues by mole of dimeric enzyme. Therefore all the thiol groups are titrated on the pyridoxal-5'-phosphate enzyme. The number of 9.4 groups obtained for pyridoxamine-5'-phosphate enzyme could be explained by a small reoxidation during the transformation of the aldimine form into the aminic enzyme.

In the aminic enzyme 2 more SHa groups reacts with DTNB, and these 2 cysteine residues are buried in the aldimine form of AAT, and only titrated by p-MB (figure 2). The apoenzyme of AAT shows the same number of SHa accessible to DTNB as the aminic form of AAT.

Probably the protein undergoes a conformational change during the transformation of aldimine form to aminic one. According to Karpeisky et al.

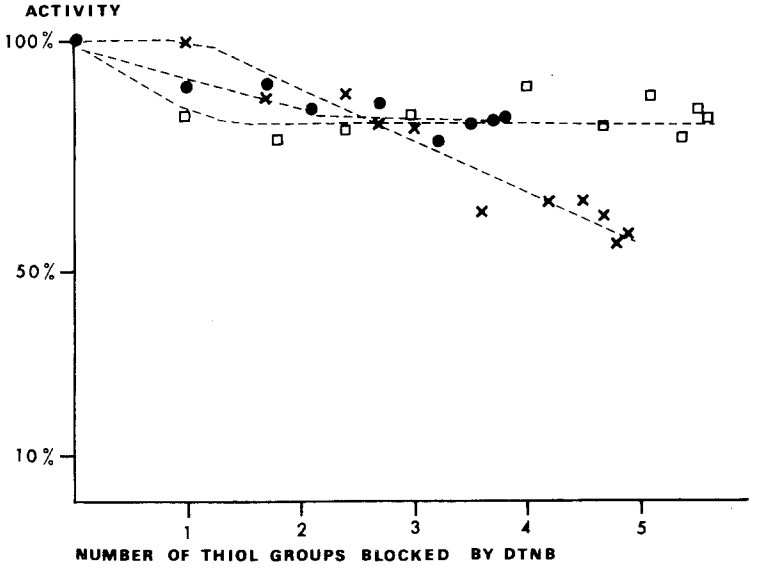

Figure 2 : Schematique representation of the location of thiol groups on the dimeric AAT
a - aldiminic form
b - aminic form

(10) a rotation of the coenzyme happens during the transamination. Very likely the rotation of coenzyme is associated with the displacement of some amino acid residues which makes accessible 2 more cysteine residues. When these groups are blocked by DTNB there is an important loss of activity of

the enzyme suggesting that these 2 residues are located in the vicinity of the 2 active centers. It has been shown that carboxymethylation of these SHa groups on the aldimine enzyme does not affect the activity of the enzyme (9)(11). The aldimine enzyme inactivation by DTNB is not explained by a lack of some essential thiol group but by steric hindrance introduced by thionitrobenzoate molecules in the protein V_{Max} decreases ; the K_M apparent value increases by a factor of two both for aspartate and α-ketoglutarate.

The accessible SHa groups in the pyridoxal-5'-phosphate enzyme does not seem to play a role in the activity nor in the conformation of the enzyme. In the pyridoxamine-5'-phosphate enzyme, two more SH groups become accessible and by blocking of these groups an important decrease of the activity is observed.

The effect of the different substrate on the thiol groups reactivity is now under investigation. We are also trying to elucidate the inhibition mechanism which results from the DTNB reaction with the two forms of the enzyme AAT.

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