CHEMICAL EVIDENCE FOR SYNCATALYTIC CONFORMATIONAL CHANGES IN ASPARTATE AMINOTRANSFERASE[1]

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1. Introduction

Conformational changes in proteins are well established experimentally, as the structural changes correlate with the transitions between different functional states. In enzymes, particularly, conformational alterations appear to play a fundamental role for both the regulation of activity and the mechanism of action. In a number of instances they have been found to accompany the formation of the enzyme–substrate adsorption complex [cf. 2]. However, chemical modification studies with aspartate aminotransferase have recently indicated that conformational changes may also occur during the subsequent phase of catalysis when covalent bonds are formed and broken [3]. These structural alterations have now been explored by probing the reactivity of sulfhydryl groups toward N-ethylmaleimide in different enzyme–substrate intermediates of the multistep reaction sequence. In a distinct phase of catalysis, most probably in the ketimine complex, two sulfhydryl groups are at least one order of magnitude more reactive than either in the other enzyme–substrate complexes or in the free enzyme. Blocking of these two groups reduces enzymatic activity about a hundred-fold.

[1] The term "syncatalytic" was introduced to characterize processes in enzyme–substrate complexes occurring synchronously with the catalytic reaction.

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2. Materials and methods

The α-subform of cytoplasmic aspartate aminotransferase was isolated from pig heart according to the procedure of Banks et al. [4] modified in our laboratory (Zaoralek and Christen, to be published). The specific activity of the enzyme was 280–330 units/mg (μmoles oxalacetate per min per mg) when assayed as described below. Enzyme concentrations were determined spectrophotometrically using a molar absorptivity $\epsilon_{280}^{1\,cm} = 1.3 \times 10^5$ which is based on amino acid analysis and a molecular weight of 93,000 [5]. The content of pyridoxal 5′-phosphate was > 95% of the theoretical value and was determined spectrophotometrically in 0.1 M NaOH assuming a molar absorptivity $\epsilon_{388}^{1\,cm} = 6{,}600$ [6]. L-Glutamic acid, L-aspartic acid, α-ketoglutaric acid, and N-ethylmaleimide were obtained from Fluka; α-methyl-DL-aspartic acid and L-cysteine sulfinic acid from Sigma; DL-*erythro*-β-hydroxy aspartic acid from Calbiochem; malate dehydrogenase and NADH from Boehringer; pyridoxal 5′-phosphate from Merck; 5,5′-dithiobis-(2-nitrobenzoic acid) from Aldrich; guanidine hydrochloride (ultra pure) from Mann.

Transaminase activity was determined spectrophotometrically [7] at 25° in 0.2 M aspartate – 6 mM α-ketoglutarate – 0.2 mM NADH – malate dehydrogenase (2 μg/ml) – 0.05 M sodium phosphate (pH 7.5) in a total volume of 3.0 ml. No transaminase activity could be detected in the employed quantity of the commercial malate dehydrogenase. In chemical modification experiments transaminase activities were obtained by assaying 1–20 μl of either the reaction mixture or the enzyme solution after gel filtration. Both methods gave identical results. Chemical modification

reactions were quenched by passing 0.5 ml of the reaction mixture through a Sephadex G-25 column (0.6 X 17 cm) equilibrated with 0.05 M sodium phosphate (pH 8.5). Absorption spectra were obtained with a Cary Model 15 spectrophotometer. Sulfhydryl groups were determined according to Ellman [8] at protein concentrations of about 0.005 mM with 0.3 mM 5,5-dithiobis-(2-nitrobenzoic acid) in 6 M guanidine hydrochloride – 1 mM EDTA – 0.3 M Tris-chloride (pH 8.0) using a molar absorptivity of thionitrobenzoate $\epsilon_{420}^{1\,cm} = 13{,}600$ (λ_{max} shifts from 412 nm in 0.02 M phosphate to 420 nm in guanidine hydrochloride). Absorbance at 420 nm was measured after 1 hr reaction at room temp against a control without protein. This value was corrected for the absorbance of pyridoxal 5′-phosphate dissociated from the enzyme in the course of denaturation. The pyridoxal 5′-phosphate form of the enzyme was converted into the pyridoxamine 5′-phosphate form by addition of 5 mM cysteine sulfinate [9].

3. Results

The susceptibility of aspartate aminotransferase to inactivation and modification of sulfhydryl groups by N-ethylmaleimide depends markedly on the functional state of the enzyme. On addition of N-ethylmaleimide to the free pyridoxal 5′-phosphate form enzymatic activity increases within one min to 125% of the initial value (fig. 1A), and then decreases very slowly (rate constant of inactivation $k_{inact} = 7 \times 10^{-4}$ min^{-1}). However, if the substrate pair glutamate and α-ketoglutarate is subsequently added the rate of inactivation is increased almost a hundred-fold ($k_{inact} = 6 \times 10^{-2}$ min^{-1}). In the presence of α-ketoglutarate alone, when an unproductive adsorption complex with the pyridoxal enzyme is formed [10], only moderate inactivation occurs. Similarly, both the free pyridoxamine form of the enzyme ($k_{inact} = 5 \times 10^{-3}$ min^{-1}) and its adsorption complex with glutamate [10] are inactivated by N-ethylmaleimide at a rate 12 times slower than that measured in the presence of the substrate pair[2] (fig. 1B).

[2] With the enzyme concentrations employed about the same equilibrium concentration of enzyme–substrate intermediates is established by adding glutamate plus α-ketoglutarate to either the pyridoxal or to the pyridoamine form.

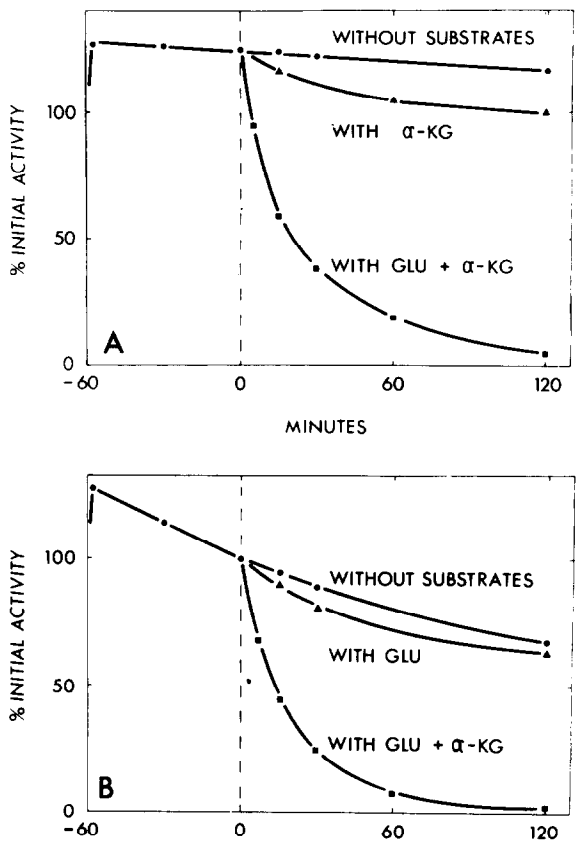

Fig. 1. Inactivation of aspartate aminotransferase by N-ethylmaleimide: effect of the substrate pair glutamate and α-ketoglutarate. (A) Inactivation of the pyridoxal form of the enzyme: 16.5 mM N-ethylmaleimide was added to 0.007 mM enzyme in 0.05 M Tris-Cl (pH 7.5) at 25°. After 1 hr the reaction mixture was divided into 3 parts. To the first part 70 mM glutamate (K_m = 4 mM) plus 2 mM α-ketoglutarate (K_m = 0.1 mM) [10], to the second part only 2 mM α-ketoglutarate was added. The third part served as control. Transamination activity was determined by direct assay of aliquots of the reaction mixture. (B) Inactivation of the pyridoxamine form of the enzyme: Conditions are the same as those in (A).

Inactivation appears to reflect modification of essential sulfhydryl groups (fig. 2). In the absence of substrates about 4 moles of sulfhydryl groups per mole of the pyridoxal form of the enzyme readily react with N-ethylmaleimide. The residual sulfhydryl groups seem to be inaccessible to the reagent. However, in the presence of glutamate plus α-ketoglutarate 2 additional moles of sulfhydryl groups are modified.

Table 1
Effect of the substrate pair glutamate and α-ketoglutarate on the susceptibility of the pyridoxal (AAT_{PLP}) and the pyridoxamine form (AAT_{PMP}) toward N-ethylmaleimide (NEM): enzymatic activity and sulfhydryl content.

	Relative activity	Sulfhydryl content: (moles/mole enzyme)
AAT_{PLP}	100	9.4[a]
+NEM	115	5.2[a]
+NEM, Glu + α-KG	5	3.6[a]
AAT_{PMP}	100	9.4[a]
+NEM	75	4.5[b]
+NEM, Glu + α-KG	5	3.5[b]

Enzyme, 0.03 mM in 0.05 M sodium phosphate or 0.05 M Tris-Cl (pH 7.5) was incubated with 15 mM N-ethylmaleimide in the presence or absence of glutamate plus α-ketoglutarate. After 90 min the reaction was stopped by gel filtration and activities and sulfhydryl contents were determined.

[a] Mean of 6 experiments, standard deviation ±0.2 sulfhydryl groups.
[b] Mean of 2 experiments.

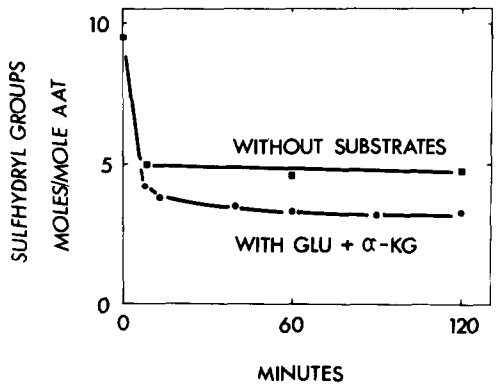

Fig. 2. Modification of sulfhydryl groups of the pyridoxal form of aspartate aminotransferase by N-ethylmaleimide: effect of the substrate pair glutamate and α-ketoglutarate. The concentration of enzyme was 0.02 mM, of N-ethylmaleimide 15 mM. Other conditions are those of fig. 1. At the indicated times an aliquot was removed and the reaction was quenched by gel filtration. Sulfhydryl groups were determined as described in the methods section.

Whereas modification of the first 4 moles of sulfhydryl groups coincides with an initial increase in activity, the modification of 2 additional moles of sulfhydryl groups in the presence of the substrate pair is accompanied by almost complete loss of activity (see table 1). The pyridoxamine form of the enzyme which is inactivated more rapidly than the pyridoxal form shows a degree of sulfhydryl modification corresponding to the degree of inactivation.

The susceptibility of the various covalent intermediates of the multistep reaction sequence toward N-ethylmaleimide was examined with substrate analogues. With α-methyl aspartate and the pyridoxal form only the first of the covalent complexes, the aldimine complex, is formed [11]. Addition of *erythro*-β-hydroxy aspartate to the pyridoxal form results in a marked accumulation of the semiquinoide complex [12]. The rate of inactivation in the presence of either one of these substrate analogues is about one order of magnitude smaller than in the presence of glutamate and α-ketoglutarate, when also the ketimine complex is formed[3] (fig. 3).

In the presence of the substrate pair N-ethylmaleimide reduces enzymatic activity to 5% of the initial value within 2 hr (fig. 1). On prolonged incubation a final constant low level of 1–2% is reached. On addition of cysteine sulfinate (see Methods) this partially active enzyme is converted completely into the pyridoxamine form (λ_{max} 328 nm at pH 8.5 instead of 333 nm in the case of the native enzyme). Subsequent addition of α-ketoglutarate (10 mM) reverses the enzyme derivative to the pyridoxal form[4] (λ_{max} 362 nm, identical to the native enzyme).

[3] Covalent intermediates are also formed using a single substrate, i.e. glutamate for the pyridoxal enzyme. However, in the equilibrium state of the reaction keto acid will be present less than equimolar to the enzyme concentration (< 0.06 mM in the present study). Since this concentration is smaller than the value of K_m = 1 mM [10], a part of the enzyme would be present in the pyridoxamine form as an unproductive complex with glutamate.

[4] This enzyme derivative has lost 20–30% of its coenzyme as evidenced spectrophotometrically. However, it can be resaturated by addition of pyridoxal 5'-phosphate with an increase in activity of less than 1%. The observed coenzyme deficiency of the inactivated enzyme might correspond to the decreased binding affinity for pyridoxamine 5-phosphate reported to result from modification of more than 4 sulfhydryl groups of aspartate aminotransferase [13].

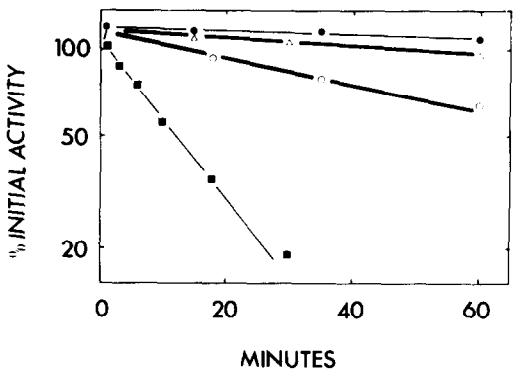

Fig. 3. Inactivation of the pyridoxal form of aspartate aminotransferase by N-ethylmaleimide: effect of the substrate analogues α-methyl aspartate and *erythro*-β-hydroxy aspartate. Enzyme, 0.03 mM, was incubated with 15 mM N-ethylmaleimide in the absence of substrates (●—●), in the presence of the substrate pair glutamate and α-ketoglutarate (■—■), in the presence of 50 mM α-methyl aspartate (○—○) or 50 mM *erythro*-β-hydroxy aspartate (△—△). The rates of inactivation are 7×10^{-4} min^{-1}, 6×10^{-2} min^{-1}, 4×10^{-3} min^{-1} and 8×10^{-3} min^{-1}, respectively. (The rates are average values from 3–5 experiments).

4. Discussion

In chemical modification studies substrates may protect the enzyme against reaction of essential amino acid residues. However, the present investigation has revealed quite an opposite behaviour in aspartate aminotransferase. *In the presence* of the substrate pair glutamate and α-ketoglutarate 2 sulfhydryl groups become at least one order of magnitude *more* reactive toward N-ethylmaleimide than in the free enzyme. Modification of these groups reduces enzymatic activity 100-fold. Thus, this novel type of enzyme modification, recently termed "syncatalytic" [1], seems to reflect an increase in reactivity of essential sulfhydryl groups in a particular phase of the catalytic process.

In the preparations of the α-subform of aspartate aminotransferase employed in this study, an average value of 9.4 sulfhydryl groups per dimeric enzyme molecule was determined with the Ellman method in 6 M guanidine hydrochloride. A sulfhydryl content of about 10 residues calculated for a molecular weight of 93,000 [5] has also been determined by other analytical methods [13–17, 4]. On the basis of their reactivity toward N-ethylmaleimide, a selective sulfhydryl reagent [18, 19], at least 3 classes of sulfhydryl groups may be recognized (fig. 2). First, about 4 apparently non-essential sulfhydryl groups readily react under the present conditions and concomitantly transaminase activity is slightly enhanced. The second class comprises 2 essential sulfhydryl groups. Their modification reduces the enzymatic activity to 1–2%. The reactivity of these groups strictly depends on the functional state of the enzyme. It is increased very markedly when the enzyme is engaged in the transamination cycle. The other sulfhydryl groups are unreactive under the present conditions and constitute the third class. The differentiation of a special class of sulfhydryl groups particularly susceptible to syncatalytic modification refines the previous classification based on conventional chemical modifications [13–16]. Modification of 2 essential sulfhydryl groups together with nitration of tyrosyl residues [3] has also been found to accompany syncatalytic inactivation of aspartate aminotransferase by tetranitromethane [20].

The residual activity after syncatalytic modification of the 2 essential sulfhydryl groups with N-ethylmaleimide could imply either incomplete reaction of a fraction of the enzyme or a homogeneous population of modified enzyme molecules with decreased activity. However, the complete interconvertibility of the syncatalytically modified enzyme from the pyridoxal to the pyridoxamine form and *vice versa* indicates a true residual activity of each individual enzyme molecule.

In the presence of the substrate pair glutamate and α-ketoglutarate finite equilibrium concentrations of all enzyme-substrate intermediates involved in transamination are formed. With substrate analogues the relative concentrations of these complexes can be varied, thus allowing to probe the susceptibility of the individual intermediates toward N-ethylmaleimide. The multistep reaction of aspartate aminotransferase is shown in the following scheme [21–23, 15].

$$\begin{array}{lcccc} AAT_{PLP} + \text{glutamate} & \underset{\leftarrow}{\rightarrow} & \text{adsorption complex} & \underset{\leftarrow}{\rightarrow} & \text{aldimine complex} \\ & & & & \downarrow\uparrow \\ & & & & \text{semiquinoide complex} \\ & & & & \downarrow\uparrow \\ AAT_{PMP} + \alpha\text{-ketoglutarate} & \underset{\leftarrow}{\rightarrow} & \text{adsorption complex} & \underset{\leftarrow}{\rightarrow} & \text{ketimine complex} \end{array}$$

The reactivity of the 2 essential sulfhydryl groups as reflected by the rate of inactivation appears to vary in the different intermediates within a range of at least 2 orders of magnitude (fig. 3). These sulfhydryl groups are least reactive in the pyridoxal form of the enzyme (AAT_{PLP}). Their reactivity increases in the adsorption complex (fig. 1A) and is enhanced further when the aldimine complex is formed. It seems to be somewhat lower in the semiquinoide complex but reaches a maximum in the ketimine complex (fig. 3). It attains an intermediate degree in the pyridoxamine form (AAT_{PMP}) and its adsorption complex (fig. 1B). Most probably, these syncatalytic changes in reactivity of the essential sulfhydryl groups reflect transient structural changes of the enzyme during the termination cycle. Conformational changes of protein molecules induced by adsorption of ligands are amply established by both X-ray crystallographic evidence and chemical modification studies [2, 24, 25]. However, the most significant conformational changes of aspartate aminotransferase as revealed by chemical probing with N-ethylmaleimide appear to occur *after* the binding of substrate during the actual bond forming and breaking processes. Similar flexible enzyme–coenzyme–substrate intermediates in aspartate aminotransferase have been postulated on the basis of other lines of evidence [23]. Thus, conformational changes of the enzyme protein might well be integral features of both the adsorption and the ensuing "covalent" phase of the catalytic reaction.

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