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L-ASPARTATE AMINOTRANSFERASE: PROTECTION AGAINST THE FORMATION OF MULTIPLE FORMS

M.ARRIO-DUPONT, I.COURNIL and P.DUIE

Laboratoire d'Enzymologie Physico-chimique et moléculaire 91, Orsay, France

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1. Introduction

Martinez-Carrion et al. [1] have shown that cytoplasmic aspartate aminotransferase contains three or more forms, indicated by α, β, γ, according to their increasing electrophoretic mobility. These multiple forms have a very similar structure [2–4] and differ by their catalytic activity.

The present work shows that the multiple forms are not due to the binding of some ligand, nor the aggregation or dissociation of subunits. They behave as "conformers" irreversibly produced after the oxidation of some SH residues and their formation can be prevented by thiol protecting reagents.

2. Materials and methods

Aminoacids, α-ketoacids and DTT are Calbiochem A grade reagents, urea is a Merck product for biochemistry and DTNB an Aldrich product. Cytoplasmic AAT is prepared from pig heart by the method of Jenkins, Yphantis and Sizer [5], using succinate instead of maleate. The multiple forms are separated by electrofocusing on ampholine L.K.B. at pH 5 to 7, the method used is very similar to that described by Marino [6]. The identification of the subforms is performed by electrophoresis on polyacrylamide gel; the gels are stained either with amino black 10 B to determine protein or by aspartate, α-ketoglutarate and azoene fast violet B to determine the aspartate transaminase activity [7]. The molecular weights on the gels are determined by the method of Hedrick and Smith [8] serumalbumin and ovalbumin are used as standards. The AAT concentrations are determined by absorbance at 280 nm; we have found an ϵ_{280nm} value of 135,000 for the holoenzyme and 128,000 for the apoenzyme, this value refers to dimeric enzyme of molecular weight 90,000. The enzymatic activities are determined with L-aspartate 2×10^{-2} M and α-ketoglutarate 2×10^{-3} M (maximum velocity conditions); the oxaloacetate formed is measured either by absorbance at 280 nm or by NADH and malate dehydrogenase.

3. Results and discussion

The hypothesis that the subforms are due to the binding of ligand or to the aggregation or dissociation of subunits can be eliminated. Indeed, we observe that the deionization of a mixture of α, β, and γ forms by chromatography on Dowex 50 H form and Amberlite IRA 400 OH form in mixed bed gives the spectral change described by Bergami [9] without change in the subform composition, and that the apparent molecular weight obtained on the acrylamide gel by the method of Hedrick and Smith [8] is the same for all the forms: about 60,000.

The last hypothesis is that the multiple forms are "conformers" [10, 11]. This fact is confirmed by aging [3] and urea effect.

A mixture of α, β, and γ forms is treated for one hr by 8 M urea at pH 8.5 (enzyme 10^{-6} M). Much of

Abbreviations:

DTT : dithiothreitol;

DTNB: 5-5′-dithiobis-2-nitribenzoate;

AAT : L-aspartate aminotransferase (L-aspartate-2-oxoglutarate aminotransferase, EC 2.6.1.1).

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Table 1
Results of the treatment of a mixture of α, β, γ forms by urea and DTT.

Treatment at pH 8.3 (α, β, γ)	Effect (analyzed after elimination of the reagents)			
	Subforms	Activity (V_m arbitrary units)	SH titratable by DNTB	SH residual titratable by pMB
–	α, β, γ,	100[a]	3.9[b]	4
Urea 8 M	α↘ β↗ γ	80[c]	N.D.	N.D.
Urea 8 M + DTT 10^{-3} M	no change	100	N.D.	N.D.
DTT 5 × 10^{-2} M (at 4°)				
3 hr	no change		5.7	4
18 hr	no change	100	6.1	4
DTT 5 × 10^{-2} M + urea 8 M 18 hr at 4°	no change	100	5.3[d]	4

[a] α and β forms, prepared by electrofocusing, have almost the same activity.
[b] This value is variable with time and varies in different preparations (4.7 to 3).
[c] This value decreases with incubation time.
[d] Bad imprecision due to some light scattering.
N.D. = not determined.

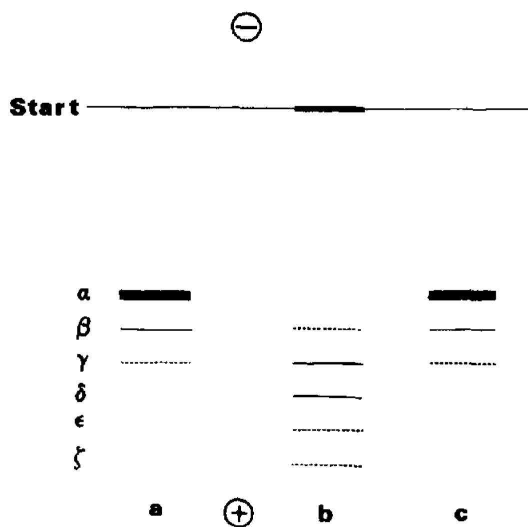

Fig. 1. Electrophoresis on polyacrylamide gel at pH 8.1 of aspartate aminotransferase, α form.
a) initial enzyme;
b) enzyme after treatment by 8 M urea;
c) enzyme after treatment by 8 M urea and DTT 10^{-3} M.
The results are the same for the specific coloration and for the protein coloration.

the protein precipitates after dialyses and concentration by ultrafiltration; the soluble protein is richer in β and γ forms than the initial mixture. This treatment is repeated on an almost pure α form. The same quantity of protein is analysed before and after the treatment. According to fig. 1, electrophoresis shows that α form is transformed to β and γ forms and that some polymerized and non-migrating material, with transaminase activity, is formed. These results indicate a transformation α → β → γ →... As it is known that some of the SH residues of the enzyme, titratable either by iodoacetate [12] or by DTNB [13] are easily oxidizable, another urea treatment has been performed in the presence of SH protecting reagents, mercaptoethanol 10^{-1} M or DTT 10^{-3} M: no change is observed in the subform composition (fig. 1), the enzymatic activity remains constant and at pH 4.5 there is no change in the coenzyme absorption at 430 nm. The thiol protecting reagents prevent the formation of multiple forms and the irreversible inactivation that are induced by urea.

If it is assumed that aging proceeds by the same mechanism as the urea effect, perhaps the thiol reagents can prevent the aging effect. AAT is prepared with DTT 10^{-4} M containing reagents, as soon as possible after the death of the pigs: the obtained enzyme is almost pure α form. We also try to reverse

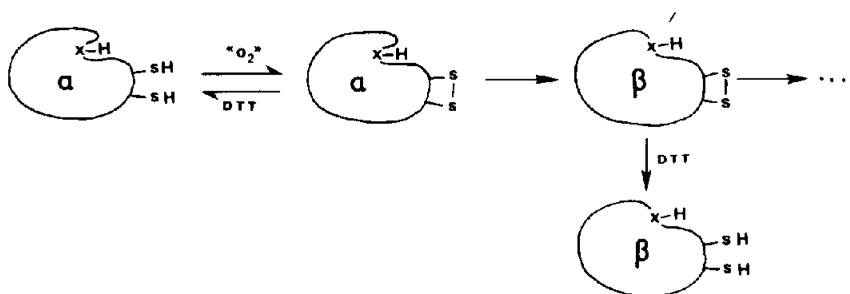

Fig. 2. Scheme of formation of the multiple forms.

the forms to α form, with unfolding and reducing reagents. A mixture of α, β and γ forms is treated by DTT and urea. For each assay the autoxidizable SH groups are titrated with DTNB after elimination of the reagents by filtration on Sephadex G 25 and it is verified that there are always four residual SH groups titratable by pMB [13]. For a native holoenzyme these residual SH groups can be titrated in about 17 hr [13] without addition of urèa [14], the effect of urea being only to accelerate the titration rate. The results are summarized in table 1: DTT treatment can reverse the oxidation of the SH, but the unfolding effect of urea is insufficient to reverse the forms to the α form.

The multiple forms of cytoplasmic AAT behave as conformers irreversibly formed. The primary step is monitored by SH oxidation and one can propose the following scheme of formation (fig. 2).

The group indicated as XH is that postulated by Banks [3], this group comes in more intimate contact with the solvent when β, γ are formed.

From a practical point of view, if only the α form is to be prepared, the use of DTT 10^{-4} M allows one to avoid the chromatography on CM Sephadex or the electrofocusing.

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