A RAPID PREPARATION OF THE APO–HOLO HYBRID OF ASPARTATE AMINOTRANSFERASE

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1. Introduction

The preparation of hybrids of native and modified oligomeric enzymes to study the interactions between subunits has been proven a very useful approach [1–5]. Of special interest in the case of aspartate aminotransferase, which consists of two identical subunits, is the apo–holo hybrid, containing only one pyridoxal 5′-phosphate (PLP) bound per dimer. Such a hybrid has been prepared by isoelectric focusing and its properties investigated [6] but a disadvantage of this preparation is the very long time required for zone stabilization on the focusing column (at least 5–7 days), in view of the instability of the apo subunit. In [7] a hybrid enzyme was prepared in which one of the active sites of the dimer was inactivated by reduction of the internal aldimine linkage by $NaBH_4$. The method of preparation in [7] (dissociation of the modified enzyme under denaturing conditions and recombination with dissociated native enzyme) is not convenient for the preparation of the apo–holo hybrid because of the difficulty of renaturation (especially for the apoenzyme). Affinity chromatography would have been a very suitable method of preparation of the hybrid enzyme, as in the case of beef liver aspartate aminotransferase [8], and we attempted to isolate it with a 1-6-diaminohexyl–Sepharose 4B resin coupled to PLP, according to [9]. Unfortunately, we failed to separate hybrid from apoenzyme, and we finally developed another procedure by ion-exchange chromatography on CM-cellulose. Here we describe this novel and rapid preparation of the apo–holo hybrid of aspartate aminotransferase. Some properties of the hybrid are presented.

2. Materials and methods

2.1. *Materials*

Cytoplasmic holoaspartate aminotransferase (EC 2.6.1.1) from pig heart was prepared as in [10]. Apoenzyme was obtained by a method adapted from [11], as in [12]. Enzyme concentrations were determined as in [13]. PLP and D,L-α-methylaspartic acid were purchased from Sigma, and 5,5′-dithiobis-(2-nitrobenzoate) (NbS_2) from Aldrich. CM-52 carboxymethyl-cellulose was obtained from Whatman.

2.2. *Preparation of the apo–holo hybrid*

In a typical experiment, to a solution of apoenzyme dimer 18 μM (total amount 14 mg) in 0.02 M acetate buffer (pH 5.6) was added PLP to a final concentration 18 μM. The mixture was applied on a CM-cellulose column (20 × 1.5) equilibrated in the same buffer. After washing with 20 ml 0.02 M acetate (pH 5.6) the column was eluted with 200 ml 0.06 M acetate (pH 5.6) and then 50 ml 0.2 M acetate pH 5.6 (flow rate 13 ml/h). The 4 ml fractions eluted from the column were checked for absorbance at 280 nm, activity before and after addition of PLP. The fractions of the separate peaks were pooled, concentrated by ultrafiltration and dialysed against 0.05 M Tris–HCl buffer (pH 8.3) unless otherwise stated. All experiments were performed in this buffer at 25°C.

2.3. *Enzymatic activity measurements*

Enzymatic assays were carried out as in [14]. Steady state experiments were performed with a Leres S 67 spectrophotometer, at fixed L-aspartate (10 mM) and variable 2-oxoglutarate levels (0.5–500 μM).

2.4. *Binding of α-methylaspartate*

Absorption spectra of holoenzyme (1.3×10^{-5} M) and hybrid (1.1×10^{-5} M) in the presence of D,L-α-methylaspartate (9.8–96 mM) were recorded over 320–500 nm with a Perkin-Elmer 535 spectrophotometer. Titration data at 430 nm were analyzed according to [15].

2.5. *Fluorescence emission corrected spectra*

These spectra were obtained on an absolute fluorimeter Fica 55, as described elsewhere (M. A.-D., D. V., submitted) in 0.05 M triethanolamine–acetate buffer (pH 8.3).

2.6. *Kinetic studies of NbS_2 binding*

The kinetics of the reaction NbS_2 (2×10^{-4} M) with enzyme (5×10^{-6} M) were followed by monitoring the increase of absorbance at 412 nm, using a Perkin-Elmer 535 spectrophotometer. Kinetic data were analyzed according to general methods of multilinear regression with a Wang 2200 B calculator, and the number of cysteine residues of each class was deduced from the amplitude of the different phases, using a molar absorbancy of 13 600 $M^{-1} . cm^{-1}$ [16].

3. Results

3.1. *CM-cellulose chromatography*

The elution diagram obtained after chromatography of a mixture of holoenzyme, hybrid and apoenzyme is shown in fig.1. Two well-separated peaks (I,II) are eluted with the 0.06 M acetate buffer, and a third one with the 0.2 M acetate buffer. Peak I has the specific activity of holoenzyme (250 U/mg), peak II has spec. act. 130 U/mg which on addition of PLP is increased to 240 U/mg. Peak III was identified as apoenzyme (spec. act. 10 U/mg, raising to 250 U/mg on addition of PLP). Holoenzyme and apoenzyme, when run separately, were eluted as peaks I and III, respectively. The relative distribution of the 3 enzyme species, holoenzyme, hybrid and apoenzyme, was 3.5, 3 and 3.5 (mean 4 expt), respectively.

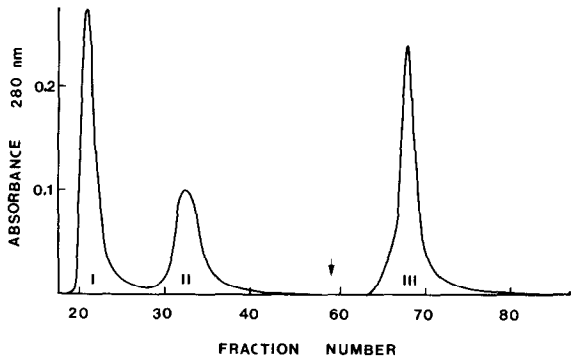

Fig.1. Typical elution pattern obtained after chromatography of a mixture of holoenzyme, hybrid and apoenzyme on CM-cellulose at pH 5.6. Acetate buffer (0.06 M) was applied at fraction 14 and 0.2 M acetate buffer at fraction 59 (→).

3.2. *Steady-state kinetics and binding of α-methylaspartate*

The experimental results are summarized in table 1. No difference can be seen between holoenzyme and hybrid with respect to the app. K'_m for 2-oxoglutarate (13 μM) and the K_d for α-methylaspartate (3 mM). The molar absorbancy of the hybrid dimer complex with α-methylaspartate is 1/2 that of the holoenzyme complex.

3.3. *Fluorescence emission*

The fluorescence spectrum of the hybrid enzyme (excitation at 295 nm) shows that the emission is less intense than the arithmetic mean between the emissions of the symmetrical dimers. At the fluorescence maximum (335 nm), if the emission of the apoenzyme dimer is taken as 100 (arbitrary units), the emission of the holoenzyme dimer is 27. The arithmetic mean of the two is 63.5, whereas the emission of the hybrid is only 46.5.

Table 1
Comparison of some properties of hybrid and symmetrical dimers

Property	holo–holo enzyme	apo–holo enzyme	apo–apo enzyme
Spec. act. (U/mg)	250	130	10
Spec. act. after addition of PLP (U/mg)	250	240	250
K'_m (μM) (2-oxoglutarate)	13	12.5	
α-Methylaspartate binding parameters			
K_d (mM)	3.1	2.9	
ϵ ($M^{-1} . cm^{-1}$)[a]	7200	3600	

[a] Molar absorbancy of the enzyme–α-methylaspartate complex at 430 nm

Table 2
Reactivity of the cysteine residues with NbS_2

Class of the cysteine residue	Number of reactive groups			Rate constant (s^{-1})		
	holo–holo	apo–holo	apo–apo	holo–holo	apo–holo	apo–apo
I (Cys-45)	2.1	1.9	2.0	n.d.	n.d.	n.d.
II (Cys-82)	2.1	2.0	2.3	81	65	51
III (Cys-390)	0	0.2	1.7	–	4	2.3

[a] n.d., not determined

3.4. *NbS_2 reaction*

As can be seen from table 2, the number of reactive thiol groups of the classes I and II is almost 2 for the 3 enzyme species, and the rate constant for class II decreases slightly from holoenzyme to hybrid and then apoenzyme. On the contrary, it appears that the cystein residues of the third class in the hybrid do not react with NbS_2 as in holoenzyme, whereas 1.5–2 residues are slowly reactive in the apoenzyme dimer.

4. Discussion

The chromatography of a mixture of apo–apo, apo–holo and holo–holo dimers on a CM-cellulose column enabled us to obtain a pure hybrid species in 1 day. The protein yield was the same for all species, so that the relative distribution of the forms (3.5, 3, 3.5) is very different from the binomial distribution (2.5, 5, 2.5) which is expected for independent binding sites, and which was obtained in preparations of the apo–holo hybrid [6,8] or of modified hybrids [7,17]. The discrepancy probably arises from the higher pH at which the semi-reconstitution was carried out in all these experiments. Our result suggests strong cooperativity between the subunits for the binding of PLP at pH 5.6.

Some properties related to the active site region were compared in the hybrid and in the holoenzyme (table 1), and no difference could be detected. The hybrid enzyme thus seems to be very similar to those prepared in [6,8,18], and the subunits appear to function independently.

On the basis of fluorescence titration data, it was assumed [19] that energy transfer could occur from some tryptophan residues of one subunit to the coenzyme of the neighboring subunit. The low fluorescence emission of the tryptophan residues in the hybrid is direct evidence that the hypothesis in [19] is valid.

The 3 classes of reactive sulfhydryl groups can be identified as Cys-45, Cys-82 and Cys-390, respectively [20,21]. Cys-45 and Cys-82 are exposed and readily reactive, whereas Cys-390 is semi-buried, susceptible to syncatalytic modification and located near the active site [22]. The two Cys-45 react rapidly with NbS_2 in the 3 enzyme species, whereas the reactivity of the Cys-82 appears to be very similar (table 2). The lack of reactivity of the Cys-390 in the apoenzyme subunit of the hybrid molecule confirms the result obtained with a carboxymethylated hybrid [23]. Since crystallographic studies [24,25] have shown that the active sites are 30 Å apart, it is not likely that the binding of one coenzyme blocks directly the Cys-390 of the other subunit. More probably, this binding induces a conformational change which is transmitted via subunit interactions to the other active site, so that the Cys-390 cannot react with NbS_2. This result provides a direct evidence of an interaction between the subunits, as does the 'abnormal' distribution pattern obtained after chromatography.

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