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ASPARTATE AMINOTRANSFERASE IMMOBILIZED ON COLLAGEN FILMS. ACTIVITY OF DISSOCIATED SUBUNITS

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Summary Aspartate aminotransferase has been covalently bound to insoluble films of collagen. The immobilized subunits of holoenzyme obtained after alkaline dissociation were inactive but their activity was recovered by incubation with either active enzyme, reduced holoenzyme, or apoenzyme. It was also possible to reduce the bound subunits by sodium borohydride and to reassociate them with native subunits of holoenzyme.

Aspartate aminotransferase (EC 2.6.1.1.) from pig heart cytosol is a dimeric enzyme for which the two active sites function independently (1,2). At low concentration it can be reversibly dissociated into monomers (3,4,5) but this equilibrium is displaced towards the dimer by substrates (5) so it is impossible to determine if the activity observed at low enzyme concentration is due to either active monomers or to dimers reassociated in the presence of substrates. Recently it has been shown by X-Ray diffraction that strong interactions exist between protomers (6), so it is of interest to investigate the problem of the activity of isolated monomers. To avoid the reassociation of dissociated enzyme in the presence of substrates, aspartate aminotransferase has been immobilized on an insoluble matrix of collagen.

MATERIALS AND METHODS.

Enzyme : α form of aspartate aminotransferase was prepared in the laboratory from pig heart cytosol, according to Martinez-Carrion et al. (7). Reduced

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enzyme was obtained by action of a large excess of sodium borohydride, the reagent being removed by molecular sieving on Sephadex G 25; the activity of the reduced enzyme was 1% of that of the native holoenzyme.
Immobilization of the enzyme : Aspartate aminotransferase was covalently bound to the surface of water-insoluble collagen films (generous gift of the Centre Technique du Cuir, Lyon) by a previously described method (8), slightly modified : to minimize the attachment of the enzyme to the matrix by more than one subunit, a milder activation was performed, using a shorter esterification period of 10 to 24 hours instead of 72 hours or more in previous experiments.
Enzymatic activity was determined by the formation of oxaloacetate from a mixture of 20 mM L-aspartate and 2 mM α-ketoglutarate in 0.05 M Tris-HCl buffer pH 8.3 at 25°C. 12 ml of reaction mixture were poured into a reaction vessel and a circulation loop enabled the reaction to be followed in a Cary 16K spectrophotometer at 257 nm ($\Delta\varepsilon$ = 1180 $M^{-1}cm^{-1}$). The reaction was initiated by dipping the film (usually 1.5 x 1.25 cm = 3.75 cm^2 of surface for both faces of the film) into the substrate mixture and stopped by taking the film out. Activity was expressed in µmoles of oxaloacetate formed per minute per cm^2 of film.
Dissociation of the bound enzyme in monomers was performed in an alkaline medium at 0°-4°C. The enzymatic film was dipped into 1.5 ml of dissociating solution (usually 0.02N NaOH) for a defined time and then thorougly washed for 5 minutes in 1M KCl to eliminate the dissociated subunits still adsorbed on the film. The unfolded monomer remaining covalently bound on the matrix was reactivated by soaking the film in a mixture of 0.1 mM pyridoxal-5'phosphate and 0.1 mM α-ketoglutarate in 0.05 M Tris-HCl buffer pH 8.3 , then washed 1 minute in 1M KCl and stored in Tris-HCl buffer before determining their activity as previously described. To reassociate the monomers bound to the matrix with native subunits, the film was incubated overnight with 0.5 µM aspartate aminotransferase at pH 8.3 in Tris-HCl buffer at 4°C; at this concentration few monomers are in equilibrium with dimers (5). The film was then extensively washed during 90 minutes in 1M KCl and its activity determined.

RESULTS.

Activity of the immobilized enzyme was, for 3.75 cm^2 films, equivalent to that of 2 µg of soluble aspartate aminotransferase (i.e. 0.14 µmoles of oxaloacetate per minute per film,or 37 nmoles/min/cm^2). Preliminary experiments using [^{14}C] labelled enzyme, modified on cysteine residues 45 and 82 by [^{14}C]-iodoacetate, indicated that 4 ± 1 µg of enzyme was bound to the matrix ; the averaged activity of the immobilized aspartate aminotransferase was therefore 40-60 % of the activity of the free enzyme.

Dissociation . To dissociate oligomeric proteins, denaturing mediums such as urea or guanidine are usually used, but aspartate aminotransferase is very difficult to reactivate after denaturation (9). It has been shown by Polyanovsky et al. (10) that a reversible dissociation can be obtained

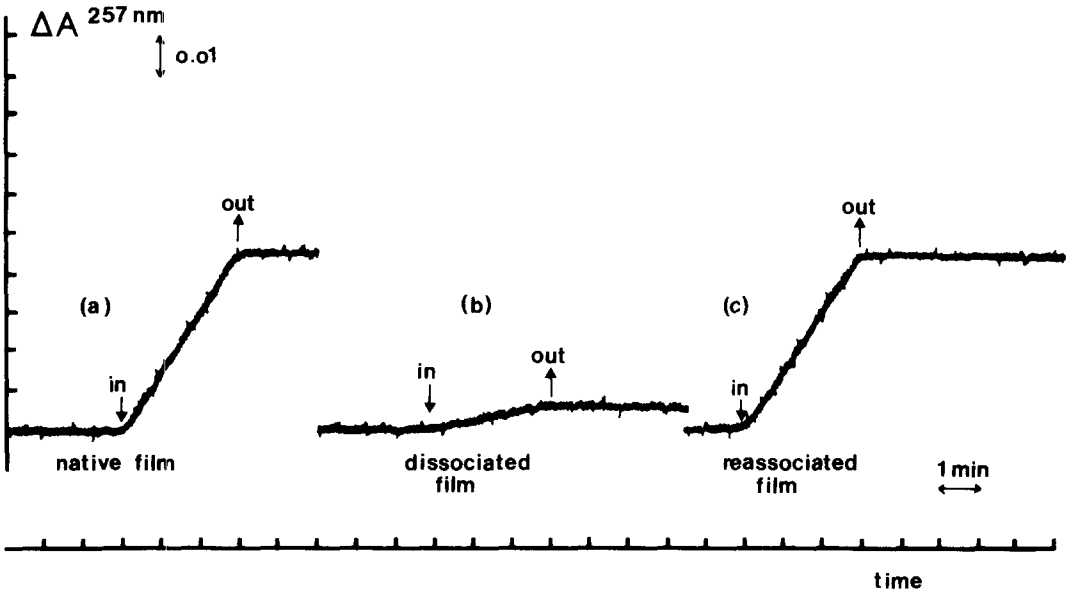

Fig. 1 : Spectrophotometric recording at 257 nm of the apparition of oxaloacetate in the reaction mixture : the baseline is recorded and the reaction initiated by dipping the enzyme-film into the medium (arrow in) and stopped by removing it (arrow out) ; a parallel to the baseline indicates that the reaction stops after this removal and that no enzyme leakage is detectable. (a) corresponds to the activity of a native membrane, (b) to its activity after dissociation in 0.02 N NaOH and (c) the activity restored after incubation with native aspartate aminotransferase.

in an alkaline medium at pH 11-12 ; 1 M urea and 0.2 mM pyridoxal-phosphate were necessary to obtain good reversibility. As the matrix of collagen and the covalent bond formed with the enzyme were found to be resistant to alkaline medium up to pH 13, we used an alkaline pH to study the dissociation of the immobilized enzyme.

A typical result of dissociation is shown in fig. 1. The activity of the native membrane was tested prior to its dissociation : in standard conditions, the activity was found to be 39 nmoles/min/cm^2(a). When the dissociation was performed in 0.02 N NaOH for 5 minutes, according to the process described , the remaining activity was 2.6 nmoles/min/cm^2, i.e. 7% of the initial activity (b). After an overnight incubation with 0.5 µM native enzyme in Tris-HCl buffer and a thorough washing by strong stirring in 1 M KCl to eliminate

the non specifically adsorbed enzyme, the membrane exhibited an activity of 38 nmoles/min/cm^2, i.e. 98 % of the initial activity (c); after removal of the film from the reaction mixture, a parallel to the baseline indicated that the reaction had been stopped and that no enzyme leakage was detectable.

If the dissociation was performed in a more alkaline medium (0.2N NaOH for example) the reactivation by native subunits was not always observed. In other experiments performed during shorter times in 0.02N NaOH or in a less alkaline medium, a residual activity was observed after dissociation and the addition of pyridoxal phosphate was not necessary to recover this residual activity.

Properties of the matrix-bound subunits. From the experiment shown in fig.1, it appears that the subunits which remain covalently bound to the support after dissociation are inactive and recover their activity after reassociation with native subunits. To confirm this result, three different experiments were performed, based upon the previous observations (1,2) that for dissymetrical dimers composed either of holo- and apoenzyme or holoenzyme and reduced holoenzyme, half of the activity of the symmetrical holoenzyme dimer was observed in both cases. In the two first experiments, bound subunits of holoenzyme were incubated with inactive enzyme to reconstitute these dissymetrical dimers on the film. In the first experiment (Table 1) the matrix-bound subunits were incubated with reduced holoenzyme or with native enzyme as a control (membranes 1 and 2). After dissociation in alkaline medium the remaining activity was only 3% of the initial activity and a comparison of the results obtained after incubation with either native or reduced enzyme showed in the first case that 98% of the activity is recovered and 48% is recovered in the second case.(It was also verified that a non-treated membrane did not adsorb native enzyme and that dissociated subunits bound to collagen were not further inactivated during the time required for the incubation).This experiment shows that inactive subunits of holoenzyme bound to collagen could recover their activity by reassociation with reduced inactive holoenzyme.

TABLE 1

Incubation of matrix-bound monomers of aspartate aminotransferase with reduced-enzyme.

N° of membrane	1	2	3	4
Initial activity (µmoles oxaloacetate/ minute/film	0.14	0.135	0.15	0.15
% activity	100	100	100	100
Treatment	7 min. 30 in 0.02N NaOH	7 min. 30 in 0.02N NaOH	none	7 min. 30 in 0.02N NaOH
% activity	3	3	100	4
Incubation with (see methods)	native enzyme $5\ 10^{-7}$M	reduced enzyme $5\ 10^{-7}$M	native enzyme $5\ 10^{-7}$M	none
% initial activity	98	51	100	4
recovered activity due to incubation	95	48	0	-

The activity could also be recovered by incubation with apoenzyme (second experiment, Table 2).

In a third experiment (Table 3) it has been shown that for matrix-bound subunits of holoenzyme the bond between transaminase and pyridoxal might be reduced by sodium borohydride. After dissociation in 0.02N NaOH during 4 minutes, the remaining activity of membranes 7 and 8 was 8%. Membrane 7 was reduced by 2 mg of sodium borohydride in 15 ml of Tris-HCl buffer during 30 minutes and thoroughly washed in the same buffer at pH 8.3 ; the activity of this membrane still decreased to 4% of the initial activity. Then both membranes were incubated overnigth with native enzyme. The reduced subunits could be reassociated with native subunits and half of the initial activity was recovered. For the film which was not treated with sodium borohydride, the recovered activity was equal to the activity of the bound subunits plus that of the reassociated native subunits i.e. the activity of the membrane before dissociation in alkaline medium.

TABLE 2

Incubation of matrix-bound monomers of aspartate aminotransferase with apoenzyme.

N° of membrane	5	6
Initial activity (µmoles oxaloacetate/ minute/film)	0.13	0.13
% activity	100	100
Treatment	5 min. in 0.02 N NaOH	5 min. in 0.02 N NaOH
% activity	4	4
Incubation with (see methods)	native enzyme $6\ 10^{-7}$M	apoenzyme $6\ 10^{-7}$M
% activity	99	51
% recovered activity due to incubation	95	47

These three experiments confirm our previous assumption that the dissociated subunits of aspartate aminotransferase are inactive and that the dimeric structure is necessary to obtain enzymatic activity.

DISCUSSION.

In 1970, Chan proposed the use of immobilized derivatives to prevent the spontaneous association between subunits of an oligomeric protein (11). This method has been used to study the activity of isolated subunits of several enzymes which were found either active or inactive (12).

The present study demonstrates that a film of insoluble collagen is an excellent tool to study the properties of isolated subunits of oligomeric proteins. With this matrix in a film form, which is very simple to handle and avoids filtration or centrifugation in the recovery of the enzyme conjugate, it is very easy to control the conditions of dissociation ; medium, pH , time,

TABLE 3

Reduction by sodium borohydride of the matrix-bound dissociated aspartate aminotransferase.

N° of membrane	7	8
Initial activity (μmoles oxaloacetate/ min/film)	0.09	0.09
% activity	100	100
Treatment	4 min. in 0.02 N NaOh	4 min. in 0.02 N NaOH
% activity	8	8
Treatment	reduction by sodium borohydride	none
% activity	2	8
Incubation with : (see methods)	native enzyme $5\ 10^{-7}$M	native enzyme $5\ 10^{-7}$M
% initial activity	50	100
recovered activity due to incubation	48	92

temperature. The only problem is the precise determination of the amount of bound enzyme, due to the proteic nature of the film itself. A radioactive labelling of the enzyme is necessary for this determination.

For aspartate aminotransferase, we observed that isolated subunits were inactive. Recently, it has been shown that strong interactions exist between the subunits of the enzyme from chicken heart cytosol (5). The role of these interactions is to form a functional active site and to hold together all the residues of the protein responsible of the enzymatic activity. Since the holoenzyme may be dissociated into monomeric holoenzyme (3,4,5) and since it has been shown in this study that pyridoxal-phosphate is accessible to sodium borohydride in the immobilized subunits, it can be concluded that the residues

involved in the binding of the coenzyme are well positioned in the monomers. Nevertheless it seems likely that the residues responsible of the binding of the substrates are not correctly positioned ; indeed the substrates displace the equilibrium monomer-dimer by several order of magnitude in favor of the dimer (5) and a low affinity of the substrates for the monomer could explain this result.

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