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SURFACE-BOUND ASPARTATE AMINOTRANSFERASE ON COLLAGEN FILMS

COMPARED PROPERTIES WITH NATIVE ENZYME

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Summary

Aspartate aminotranferase (L-aspartate : 2-oxoglutarate aminotransferase, EC 2.6.1.1) has been covalently bound to chemically activated collagen films. This enzyme had never previously been coupled to any other solid support. The coupling method, including acyl azide formation on the carrier, allowed coupling of many other enzymes.

A systematic study of coupling conditions has been performed; influence of time of coupling and of concentration of coupling solution on the enzymatic activity retained on the film. Coupling solutions could be used for several successive couplings.

To determine the yield of binding, *N*-[^{14}C]ethylmaleimide-labelled enzyme was prepared fully active and bound to collagen films.

After lyophilisation the film retained most of its activity when stored in buffer and the half-life of the enzymatic film was about ten months.

pH Dependence and activation energy were about the same for soluble and coupled enzyme. Coupling protects against thermal denaturation and increases the stability of the enzyme; the enzymatic film could be used repeatedly.

Kinetics were somewhat modified in the coupled enzyme as compared to the enzyme in solution. Glutamate appeared more available while oxaloacetate seemed to be limiting. These modifications might be due to the proteic support itself. The enzymatic films also revealed themselves as a good tool for industrial or clinical purposes as well as for studying the mechanism of enzyme action.

Introduction

For many years, our laboratory has been concerned with glutamate metabolism in pig heart mitochondria [1]. Two main enzymes are involved:

glutamate dehydrogenase and aspartate aminotransferase. In order to simulate the behaviour of these enzymes when associated with mitochondrial membranes, experiments have been performed to bind them to collagen films. As a first step, glutamate dehydrogenase was bound to ultrathin films of collagen [2], collagen being chosen for its protein nature and abilities to adsorb phospholipids. Since then, we have developed a mild method of general use in binding enzymes on films of highly polymerized collagen prepared in industrial conditions [3]. Besides the fundamental aspects, being given the very promising potential use of enzymatic films in clinical analysis as well as in bioengineering, we have conducted a systematic study of coupling conditions of aspartate aminotransferase on collagen films. This enzyme has never been immobilized on a solid support before. A comparison of properties of native and coupled enzyme shows that its behaviour is hardly affected by the coupling. The stability of enzymatic films was striking and their reuse was thoroughly tested. Lyophilization was conducted without damage to the carrier or the enzyme.

Materials and Methods

Aspartate amino transferase (EC 2.6.1.1., L-aspartate : 2-oxoglutarate aminotransferase) salt free, lyophilized powder, $E^{1\%}_{1\,cm}$ = 9.6 at 280 nm, was purchased from Miles-Seravac or from Sigma Chemical Co. Films of highly polymerized collagen [4] (100 μm thick in a dry state, and 300—500 μm when swollen) were a gift of the Centre Technique du Cuir, Lyon. L-Glutamic acid and L-aspartic acid were from Calbiochem; oxaloacetic acid from Boehringer and 2-oxoglutaric acid from Sigma Chemical Co. Other reagents were of the highest grade commercially available. *N*-[^{14}C]ethylmaleimide was obtained from C.E.A. France with a spec. act. of 4.5 Ci/mol. Soluene 100 was obtained from Packard.

Enzyme assays

Aspartate aminotransferase catalyses the reversible reaction:

$$\text{L-glutamate} + \text{oxaloacetate} \underset{\text{reverse}}{\overset{\text{forward}}{\rightleftharpoons}} \text{2-oxoglutarate} + \text{L-aspartate}.$$

The measurement of activity using malate dehydrogenase as an auxiliary enzyme is easy only in the reverse direction. In order to determine initial rates of forward and reverse reactions, direct spectrophotometric measurement of the disappearance or appearance of oxaloacetate at 257 nm was chosen [5]. At this wavelength, ϵ_M is 1180 $M^{-1} \cdot cm^{-1}$. Unless otherwise stated, the activity of aspartate aminotransferase was measured in 0.05 M Tris-HCl, pH 8.0, 30°C; for the forward reaction, 0.5 mM oxaloacetate and 50 mM L-glutamate; for the reverse, 2 mM 2-oxoglutarate and 20 mM L-aspartate [6]. Oxaloacetate solutions were kept at 0—4°C to avoid spontaneous decarboxylation; controls proved their stability under these conditions for a whole day. The reaction was initiated simply by dipping the enzymatic film or aliquots of soluble enzyme in the reaction mixture. A circulation loop enables the reaction mixture to pass through a spectrophotometric cuvette for the continuous recording of the reac-

tion by a Pye-Unicam SP 800. With enzymatic films, the reaction can be stopped at will by taking off the film.

Preparation of enzyme films

Activation of collagen films consisted of three steps: acidic methylation, action of hydrazine and formation of acyl azide by nitrous acid. After removal of all excess reagents by washing, coupling of the enzyme to the chemically activated surface of collagen films proceeded spontaneously. Details have been published elsewhere [3]. In a typical coupling experiment, pieces of chemically activated collagen film 1.5 × 2.5 cm (7.5 cm^2 available for activation on both faces) were immersed for 2 h in 1.5 ml of 0.2 M borate buffer pH 8.8 containing 2 mg of enzyme (coupling solution). Both protein concentration (absorbance at 280 nm) and activity of the coupling solution were determined before and after coupling.

Routinely, films exhibited an average enzymatic activity of 25 nmol consumed oxaloacetate · min^{-1} · cm^{-2} of film (forward reaction).

Labelling of aspartate aminotransferase with N-[^{14}C]ethylmaleimide

Aspartate aminotransferase (2 mg/ml) was incubated with 2 mM *N*-[^{14}C]-ethylmaleimide for 1 h in 0.1 M phosphate buffer pH 7.5 at room temperature [7], for reaction with -SH groups. Addition of 20 mM β-mercaptoethanol stopped the reaction by removal of excess *N*-ethylmaleimide. The reaction mixture was then passed through a Sephadex G 50 column (0.8 cm × 35 cm) equilibrated with 0.2 M borate buffer pH 8.8. The eluted labelled enzyme, free from unbound *N*-[^{14}C]ethylmaleimide, was concentrated on an AMICON cell fitted with a PM 30 membrane. *N*-ethylmaleimide treatment (Birchmeier et al. [7]) and the ultrafiltration did not diminish specific activity of aspartate aminotransferase. Coupling of this labelled enzyme to collagen films was performed as described above. After determination of their enzymatic activity, the films were dissolved for 2 h in 1 ml Soluene at 50° C. After addition of 10 ml of scintillation mixture (2,5-diphenyloxazole 4 g, 1,4-bis-2-(4-methyl-5-phenyl-oxazolyl)-benzene 0.1 g per l of toluene) and 1 ml Triton X-100, radioactivity was measured in an Intertechnique SL 40 liquid-scintillation spectrometer.

Results

Coupling conditions

Influence of time. Eight identical films, having undergone the same activation process, were each immersed in 1.5 ml 0.2 M borate buffer pH 8.8 containing 2 mg aspartate aminotransferase and allowed to react for various times. The films were taken out and their enzymatic activity measured and plotted against the time of coupling as shown in Fig. 1. After 2—3 h, maximal activity was reached. Therefore, in all further experiments films were immersed for 2 h in the coupling enzyme solution.

Influence of enzyme concentration. 7 films previously activated were immersed in coupling solutions with different enzyme concentrations: from 0.15 mg to 10 mg in 1.5 ml 0.2 M borate buffer, pH 8.8. Fig. 2 shows that the activity retained on the film increased with enzyme concentration. Maximal

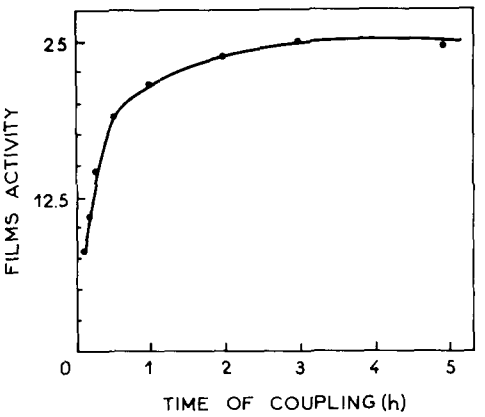


Fig. 1. Retained aspartate aminotransferase activity on films after different times of coupling from 5 min to 5 h. After being thoroughly washed with KCl 1 M, enzymatic activity of the films was tested (forward reaction). Disappearance of oxaloacetate was followed at 257 nm in the reaction mixture: 0.05 M Tris-HCl, 0.5 mM oxaloacetate, 50 mM L-glutamate pH 8.0, 30°C (3 experiments); activity in nmol oxaloacetate consumed · min^{-1} · cm^{-2} of film.

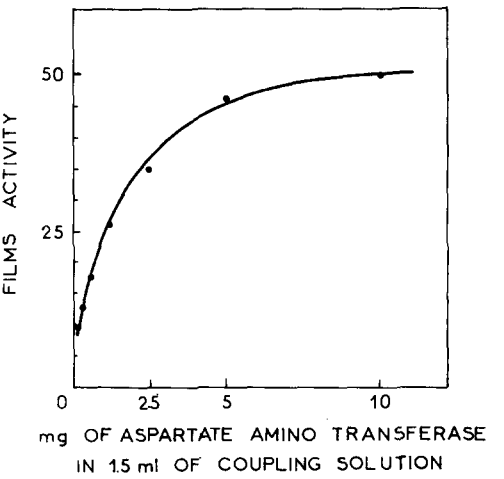


Fig. 2. Retained aspartate aminotransferase activity on films after 2 h coupling in solutions of variable enzyme concentration from 0.15 mg to 10 mg in 1.5 ml 0.2 M borate buffer pH 8.8. After a thorough washing of the films with KCl 1 M, their enzymatic activity was tested (reverse reaction). Appearance of oxaloacetate was followed at 257 nm in the reaction mixture: 0.05 M Tris-HCl, 2 mM 2-oxoglutarate, 20 mM L-aspartate pH 8.0, 30°C. Activity in nmol oxaloacetate appeared · min^{-1} · cm^{-2} of film. (2 experiments).

activity was reached when enzyme concentration was 10 mg per 1.5 ml. At 2 mg per 1.5 ml, 65% of maximal activity was obtained.

Yield of coupling

The specific activity of the enzyme in the coupling solution did not decrease during the coupling process but about 20% of the enzyme was removed from the solution. Therefore, when the coupling was performed in standard conditions (2 mg aspartate aminotransferase per 1.5 ml buffer) one can estimate that about 400 μg enzyme were removed from the solution. But the total enzymatic activity exhibited by the films after washing was equivalent to that of about 4 μg free enzyme (10 determinations). It is likely that a larger amount of enzyme was grafted onto the film partly as an inactive form. Protein concentration and enzymatic activity were difficult to evaluate in the washing solutions because of the dilution. Therefore, to determine accurately the amount of enzyme coupled to the films, labelled aspartate aminotransferase was prepared. Coupling of enzymes to acyl azides is assumed to imply amino groups. Birchmeier et al. [7] studied the reactivity of -SH groups of aspartate aminotransferase with *N*-ethylmaleimide and described conditions of *N*-ethylmaleimide binding which neither diminished specific enzymatic activity, nor affected ε-amino groups. Therefore, using *N*-[^{14}C]ethylmaleimide we succeeded in preparing aspartate aminotransferase with a specific radioactivity of 1.14×10^5 dpm/mg, corresponding to 1 mol *N*-ethylmaleimide bound per mol of enzyme.

When *N*-[^{14}C]ethylmaleimide aspartate aminotransferase was coupled to collagen films, enzymatic activity was then equivalent to 10.6 μg per standard film and the total radioactivity exhibited by the film corresponded to 104 μg of enzyme (2 different films). Therefore, the enzymatic activity of the film was 10% of the activity of a solution containing the same amount of free labelled enzyme similarly tested.

Storage conditions

The activity of an enzymatic film of aspartate aminotransferase stored for 15 months in 0.05 M Tris-HCl buffer, pH 8.0 at 4°C, has been measured from time to time. Fig. 3 shows that there was no loss of activity during the first 5 months and after only a decrease with time. The half-life of the enzymatic film was 10 months.

Preservation of enzymatic activity of films after lyophilisation. An enzymatic film, after lyophilisation, remained in its film form and could be stored at −20°C. Replaced in 0.05 M Tris-HCl buffer pH 8.0, it swelled and recovered its original form and activity. The same film stored in this buffer exhibited 97.5% of its initial activity after 1 month. For comparison, soluble enzyme at 0.1 mg per ml stored in buffer at 4°C, lost 25% of its initial activity in 15 days; the lyophilized enzyme is stable for months at −20°C.

Compared properties of coupled and soluble enzyme

pH Dependence. Enzyme activity was determined at different pH from 5 to 10 (forward reaction). Three different buffers were used to cover the pH range investigated: 0.05 M sodium cacodylate-HCl pH 5—7.4, 0.05 M Tris-HCl pH 7—8.7, 0.05 M glycine-NaOH pH 8.4—10. Overlapping of buffer zones is necessary to control any differences due to the buffer itself. Activity of enzyme, in solution or coupled to collagen, is expressed in percent of activity at optimal pH (Fig. 4). No shift of this optimum, but a greater sensitivity to alkaline conditions, was found. Unlike assays with enzyme in solution, in which

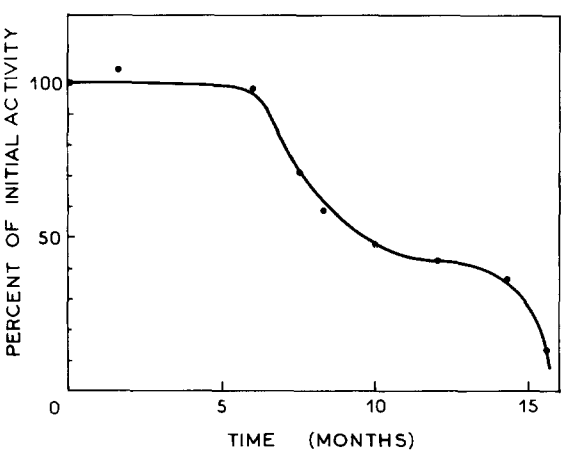
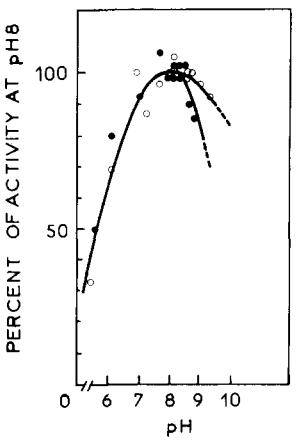


Fig. 3. Stability of enzymatic activity on storage of the films in 0.05 M Tris-HCl buffer pH 8.0, 4°C. Activity of the enzymatic film was tested from time to time over a period of 15 months (forward reaction, conditions in Fig. 1). After 10 months 50% initial activity still remained.



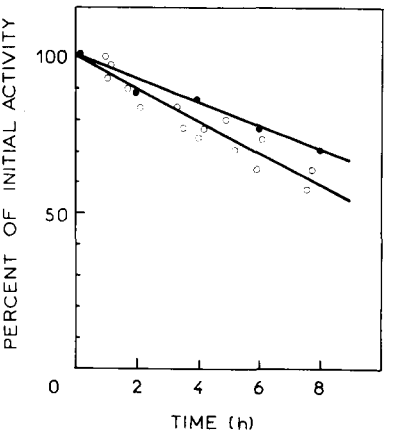


Fig. 4. Influence of pH on aspartate aminotransferase activity. ○———○, native enzyme; ●———●, collagen-bound enzyme. 3 buffer solutions were used to cover the pH range investigated: 0.05 M sodium cacodylate-HCl pH 5—7.4; 0.05 M Tris-HCl pH 7—8.7; 0.05 M glycine-NaOH pH 8.4—10. Conditions of measurement of the activity were as in Fig. 1, with variable values of pH from 5 to 10. (4 experiments).

Fig. 5. Thermal stability of aspartate aminotransferase. Test tubes containing native enzyme or enzymatic film in 0.05 M Tris-HCl buffer pH 8 were incubated at 55° C. Aliquots or enzymatic films were withdrawn and the residual activity determined at 30° C (forward reaction, conditions as in Fig. 1). (3 experiments). Free (○———○) and collagen-bound enzyme (●———●).

enzyme cannot be reused after the assays, the same film was used for all values of pH; so it was checked that the activity of film was not affected by its immersion in reaction mixtures at different pH. That means that no denaturation or leakage of the enzyme was observed in the studied pH range.

Thermal stability. Enzymatic films and soluble aspartate aminotransferase (80 μg/ml) were incubated in 0.05 M Tris-HCl buffer pH 8.0 at 55° C for 7 h and their activity tested at intervals (forward reaction, 30° C). Results are shown in Fig. 5. Denaturation occurred more slowly with bound aspartate aminotransferase. Extrapolated times corresponding to a residual activity of 50% of the initial were about 14 h for the supported enzyme and 10 h for the enzyme in solution.

Activation energy. Activity of enzyme, in solution or coupled to collagen, was measured at different temperatures from 15° C to 40° C. The logarithms of initial rates were plotted versus $1/T$ (Fig. 6). In both cases, a straight line was obtained and values of activation energy were not significantly different: 11.7 and 11.5 kcal per mol for coupled and soluble enzyme respectively.

Kinetics. With the enzyme in solution the initial rates were measured at several concentrations of each substrate. In the range $6.6—33 \cdot 10^{-5}$ M oxaloacetate and $6.6—33 \cdot 10^{-3}$ M glutamate, the primary double reciprocal plots were linear and parallel, typical of a "ping-pong" mechanism as expected for aspartate aminotransferase [5,8]. The following values, from the secondary plots, were calculated: $V = 39.5$ μmol oxaloacetate $\cdot$ min^{-1} $\cdot$ mg^{-1} protein; K_m oxaloacetate $= 5.2 \cdot 10^{-5}$ M; K_m glutamate $= 5 \cdot 10^{-3}$ M. However, Fig. 7 shows that if lower concentrations of oxaloacetate were used, reciprocal primary plots did not fit with one single straight line; this was constantly observed, indicating changes in the affinity of the enzyme for oxaloacetate.

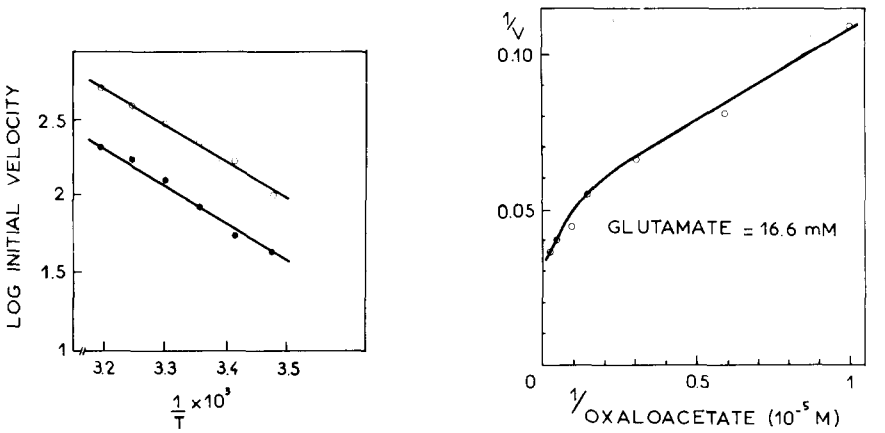


Fig. 6. Arrhenius plots for native (○———○) and collagen-bound (●———●) aspartate aminotransferase (forward reaction). Reaction rates were determined at different temperature from 15°C to 40°C. Logarithms of their values were plotted against reciprocals of temperatures (°K) and activation energy deduced from the slope. No significant difference was found (3 experiments).

Fig. 7. Lineweaver-Burke plot for aspartate aminotransferase in solution as fixed concentration of glutamate for variable concentration of oxaloacetate. Initial rates are expressed in nmol oxaloacetate consumed per min/1 μg enzyme in 3 ml pH 8.0, 30°C (3 experiments).

Fig. 8 shows the well known inhibition due to high oxaloacetate concentrations at saturing concentrations of glutamate (30 mM). We see that much higher concentrations of oxaloacetate were necessary to inhibit bound aspartate aminotransferase as compared to the soluble enzyme.

From Fig. 8, the concentration of oxaloacetate giving half-maximal velocity for the bound enzyme appears at about $10 \cdot 10^{-5}$ M, slightly higher than the $5 \cdot 10^{-5}$ M estimated for the enzyme in solution.

In Fig. 9B we see that lowering glutamate concentration from 30 to 6 mM barely modified the reaction rates obtained with bound enzyme at increasing

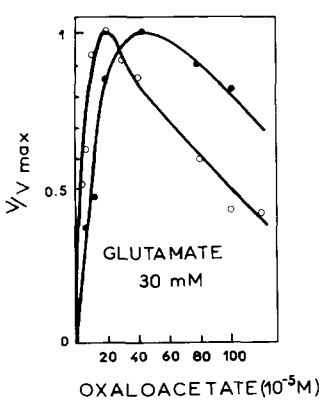


Fig. 8. Normalized Michaelis-Menten plots for free (○———○) and collagen-bound (●———●) aspartate aminotransferase at fixed concentration of glutamate and variable concentration of oxaloacetate. Initial rates expressed in nmol oxaloacetate consumed per minute/assay (1 μg/3 ml for the free enzyme and 9 ml for the collagen-bound enzyme) were normalized in view to compare kinetics of free and collagen-bound enzyme. pH 8.0, 30°C (3 experiments).

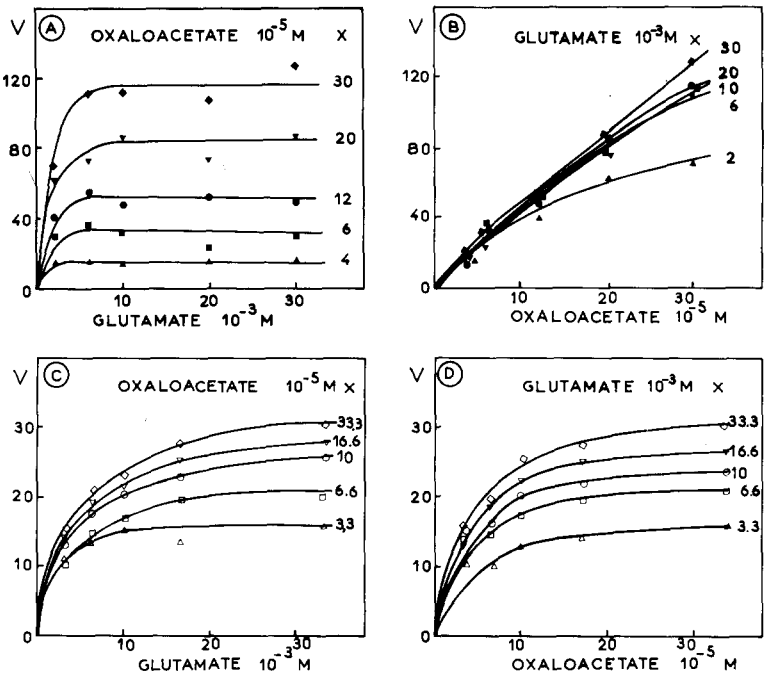


Fig. 9. Michaelis-Menten plots for collagen-bound and free aspartate aminotransferase: parts A and B and C and D respectively. Initial rates expressed in nmol of oxaloacetate consumed per minute/assay (1 μg enzyme in 3 ml for the free enzyme and 9 ml for the collagen-bound enzyme) are plotted against oxaloacetate concentration at a series of fixed concentration of glutamate or against glutamate concentration at a series of fixed concentration of oxaloacetate. pH 8.0, 30°C.

oxaloacetate concentration. This is more evident in Fig. 9A, where the rates obtained at fixed oxaloacetate concentrations depended only slighly on glutamate concentrations. If glutamate was lower than 2 mM, the rate with bound enzyme was too low to be measured. Therefore, it was not possible to determine precisely the K_m for oxaloacetate and glutamate for bound enzyme. Since maximal rates were obtained at 6 mM glutamate whatever the oxaloacetate concentration was ($4—30 \cdot 10^{-5}$ M), it seems that the affinity for glutamate was increased in bound enzyme.

Fig. 9 (C and D) shows that in the same conditions the soluble enzyme gave typical patterns of rate variations which were used in reciprocal plots to calculate V and K_m (c.f. above).

Discussion

Aspartate aminotransferase, which had never been coupled before to a solid support, has been successfully bound to a protein carrier: collagen films. Although in optimal conditions of coupling a greater enzymatic activity was obtained by immersing the film in a solution of 10 mg enzyme per 1.5 ml (Fig. 2), we have routinely used a solution containing only 2 mg enzyme/1.5 ml since it gave a good activity and was more economical in view of industrial preparation of enzymatic films. The preparation process is not denaturing and therefore the same enzymatic solution can be recycled to make several films; the enzyme concentration can be kept constant by adding lyophilized enzyme.

Properties of the enzyme appeared hardly modified by the coupling on collagen support: pH dependence and activation energy were identical; stability was greatly increased and enzymatic films could be repeatedly used and lyophilized with only minor loss of activity. Therefore, the bound enzyme is not only a very interesting stable catalyzer to be used for industrial or clinical purposes, but could also be a valuable tool to obtain informations on the mechanism of the enzyme.

Experiments with labelled aspartate aminotransferase seemed to indicate that only 10% of the enzyme retained on the film would be active. This may reflect either an inactivation of 90% of bound molecules or a fixation of enzyme on the films that hides the active centers in a random fashion leading to a 10% detectable activity. Moreover, when the labelled enzyme was bound, a greater activity was retained per standard film: 10 μg equivalent of the soluble enzyme, instead of 4 μg obtained with the unlabelled enzyme. However, our experiments do not allow us to decide whether the binding of one molecule of *N*-ethylmaleimide per molecule of enzyme favors the coupling of a greater amount of aspartate aminotransferase on the film or the setting up of enzyme molecules in a vectorial way giving better accessibility of active centers.

The nonlinearity observed in varying oxaloacetate concentration at fixed glutamate concentrations was not reported by earlier investigators [5,8] working with the enzyme in solution. The oxaloacetate concentrations used by Henson and Cleland [5] did not cover the range where the phenomenon was observed. Velick and Vavra [8], while working in the same range of oxaloacetate concentrations as ours, used lower concentrations of glutamate and obtained brief lag-phases, always difficult to interprete, when measuring initial rates. To decide whether experimental conditions or true cooperative mechanisms of the enzyme are involved in the observed differences would need an extensive study of the enzyme in solution that was not the purpose of the present work.

The differences observed in compared kinetic studies between soluble and collagen-bound enzyme could be explained either in terms of changes of affinity of the enzyme for a substrate or rather by modifications of the true concentration of substrates at the level of the film. If the film is able to bind glutamate when glutamate appeared to be more accessible to bound enzyme, it may be that locally, its true concentration was higher than in the solution; the glutamate amino group could be responsible for this concentration effect in the film. On the contrary, oxaloacetate appeared always limiting for maximal activity of bound enzyme (Fig. 9) and higher oxaloacetate concentrations were necessary to observe enzyme inhibition by excess of substrate; oxaloacetate concentration in the film could then be lower in the film than in the solution.

These interpretations are made all the more likely since the inhibitions by excess oxaloacetate were explained by a competition between oxaloacetate and glutamate to bind to the pyridoxal form of the enzyme [8,9].

Acknowledgements

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