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IMMOBILIZATION OF GLUTAMATE DEHYDROGENASE INTO PROTEIC FILMS

STABILITY AND KINETIC MODULATION BY EFFECTORS

J.N. BARBOTIN and M. BREUIL

Laboratoire de Technologie Enzymatique, E.R.A. No. 338 du C.N.R.S., Université de Technologie de Compiègne, B.P. 233 - 60206 Compiègne (France)

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Summary

Bovine liver glutamate dehydrogenase (L-glutamate:NAD(p)$^+$ oxidoreductase (deaminating), EC 1.4.1.3) was immobilised within a protein membrane using a co-crosslinking method. The retention of enzyme activity after immobilisation was dependent on the presence of NAD^+, ADP or GTP during the insolubilisation process. The highest yield (40%) was obtained in the presence of 4 mM NAD^+ and $3.5 \cdot 10^{-2}$ M ADP and the immobilised enzyme was much more resistant to thermal denaturation. The optimal pH for matrix-bound glutamate dehydrogenase was shifted towards the alkaline by 0.85 units as compared to the free form. Deviations from Michaelis-Menten behaviour towards higher activity were observed with increasing concentrations of NAD^+ (for the enzyme immobilised in absence of ADP) and glutamate (for the enzyme immobilised with or without ADP). Due to the presence of effectors during immobilisation, apparent K_m values for glutamate (at high NAD^+ concentration) measured in the presence of 3.5 mM ADP are not deeply modulated. At the opposite for glutamate dehydrogenase immobilised in presence of ADP, an important decrease in K_m value is observed (when $4.6 \cdot 10^{-5}$ M GTP is added to the reaction milieu) as compared with immobilised enzyme in absence of ADP. This observation is in favor of a conformational state similar to that induced by ADP in the native enzyme case. We suggest that a conformational state could be frozen by a tight chemical immobilisation of glutamate dehydrogenase. The allosteric properties of immobilised glutamate dehydrogenase have been studied with ADP and GTP. Due to diffusion limitations, inhibition by GTP is slightly reduced, but ADP activation is dramatically reduced. An explanation could be a modification of the ADP-binding site. The reversibility of such modulations has been observed for all presented matrix-bound enzymes suggesting that the degree of association of the enzyme does directly affect the activity.

Introduction

The properties of bovine liver L-glutamate dehydrogenase (L-glutamate: $NAD(p)^+$ oxidoreductase (deaminating), EC 1.4.1.3) are now well known [1—2], but its regulation appears to be controlled by a great variety of factors. ADP, which enhances enzymatic activity, favors aggregation of the enzyme, while GTP which inhibits the activity, favors disaggregation. It is clear that the effect of purine nucleotides results from binding at a site separate and distinct from the active site. Recently, Di Prisco [3] has suggested that the conformation of each allosteric site of native glutamate dehydrogenase is changed by pH and ionic strength.

Although the mechanisms of enzyme regulation in metabolism have attracted considerable attention [4], few advances have been made in the study of the coupling between regulatory enzyme activity and diffusion processes [5]. In view of the recent accomplishments in the immobilised enzymes field [6], it is important to examine such reactions involving association-dissociation mechanisms of oligomeric enzymes. The introduction of artificial conformational constraints by immobilisation of allosteric enzymes [7] can give new prospectives to this subject. Various studies of immobilised glutamate dehydrogenase have already been reported [8—10]. Julliard et al. [8] in studying glutamate dehydrogenase covalently bound to collagen have shown that the regulatory properties of the enzyme are preserved after coupling to a collagen matrix and a protector effect of ADP and GTP can also be observed. Havekes et al. [9] with glutamate dehydrogenase immobilised by binding to CNBr-activated Sepharose 4 B particles, have observed that ADP is not able to prevent activation by NAD^+ and L-glutamate, in contrast to the results observed with free enzyme. In these studies immobilised preparations, like native glutamate dehydrogenase, did not exhibit Michaelis-Menten kinetics. Mosbach's team [10—11] using glutamate dehydrogenase coupled covalently to porous succinamido propyl glass beads, in presence of ADP or GTP described a means for the direct determination of the reversibility of allosteric ligand-induced changes, confirming the view that the degree of association of monomers does not directly affect the activity.

This paper deals with the catalytic properties of glutamate dehydrogenase when immobilised into albumin membranes. In contrast to the above-mentionned studies (direct covalent binding on a preformed matrix), using the cocrosslinking method [12], the enzyme is tightly immobilised within a solid phase structure. Nevertheless, even under these conditions it is found that immobilised glutamate dehydrogenase is still subject to inhibition by GTP. A diminished ability to respond to activation by external ADP is observed even though evidence is presented for an increased activity for matrix-bound enzyme when immobilised in the presence of ADP. It seems therefore that allosteric regulation does not depend upon aggregation or dissociation of glutamate dehydrogenase.

Materials and Methods

Membrane production

A previously described method [13] was used. It is a co-crosslinking process

performed with 50 mg · ml^{-1} of bovine serum albumin (from Sigma Chemical Co.), 2.5 mg · ml^{-1} of glutaraldehyde (from Merck Co.) 3 mg · ml^{-1} of NAD (Sigma grade III) ADP from 0 to 20 mg · ml^{-1} (Sigma grade III) or GTP from 0 to 20 mg · ml^{-1} (Boehringer lithium salt) and 0.2 mg · ml^{-1} (24 I.U.) of glutamate dehydrogenase (in 50% glycerol suspension from Boehringer, used without further purification) in 0.02 M phosphate buffer pH 6.8. 1 ml of such a final solution was spread on a plane glass surface. Polymerisation took place, and due to solvent evaporation total insolubilisation occurred. The film (50 μm × 16 cm^2), easily separated from the glass plate, was rinsed until the rinsing water no longer absorbed at 280 nm. No enzyme activity was detected in the water, showing that all the added glutamate dehydrogenase had been insolubilised.

Measurement of glutamate dehydrogenase activity

In solution, measurements were carried out in a 3-ml quartz cuvette with 0.1 M or 0.25 M Tris · HCl buffer (pH 8.25)/10 mM EDTA. Except for the determination of the respective Michaelis constants, the concentration of L-glutamate was 16 mM and NAD^+ 4 mM. These experiments are performed in the presence or absence of ADP (0—5 mM) or GTP (0—500 μM) and at 30°C.

One albumin-enzyme membrane was immersed in a 15-ml batch reactor, kept thermostatically at 30°C, with 0.25 M Tris · HCl buffer (pH 9.1) 10 mM EDTA. When determining the pyridine nucleotide reduction, 60 mM L-glutamate and 6 mM NAD^+ were used.

The rate of NADH appearance at 340 nm was recorded with a continuous flow quartz cuvette (DBT Beckman Spectrophotometer and Ricken-Denshi SPG3 recorder). The fluid was recirculated from the flow cell to the reactor using a peristaltic pump. After pulling the membrane out of the beaker, the absence of any enzyme leakage could be checked.

Results and Discussion

Immobilisation features

As already described for many other enzymes [12] glutaraldehyde was used for the immobilisation of glutamate dehydrogenase by covalent crosslinkage. The results show a non linear decrease in enzymatic activity as a function of the final glutaraldehyde concentration used. The optimal concentration seems to be from 2.5—4.0 mg · ml^{-1} with respect to acceptable activity and correct mechanical properties of the film. Little variation in enzymatic activity was observed between pH 6 and 7.5 for the pH-dependence of insolubilisation reaction (optimum at pH 6.8). If this procedure give a homogeneous distribution of enzyme molecules within the membrane, as shown by electron microscopy [14], we can conclude on statistical grounds that some irreversible chemical modification of the enzyme has occurred. However, the nature of glutaraldehyde reaction with proteins is not yet fully understood [15] and nothing is known about the nature and the location of the lysysl residues modifications in glutamate dehydrogenase (of the 500 residues in the bovine enzyme subunit, 32 are lysyl according to Moon et al. [16]).

The activity yield (i.e. fractional retention of enzyme activity on immobilisa-

tion) is not very high: from 6% to 12% as a function of glutaraldehyde concentration. A yield of the same magnitude was also observed for lactate dehydrogenase [17]. We have noted that glutamate dehydrogenase immobilised in the presence of its co-substrate NAD^+, is more active than the enzyme immobilised in its absence, as already described for alcohol dehydrogenase [18]. In this case, in contrast with the experiments of Scouten et al. [19] NAD^+ is used as a protector, since it is not co-immobilised. No better results were obtained by using either NADH or $NADP^+$ as protectors.

By adding ADP or GTP during the insolubilisation higher retentions of enzyme activity have been obtained. With $3.5 \cdot 10^{-2}$ M ADP or $3 \cdot 10^{-2}$ M GTP yields of 40 and 18% respectively can be observed. Such protection effects are discussed later.

pH-dependence of the activity

The optimum pH for the immobilised glutamate dehydrogenase is shifted toward the alkaline by 0.85 units as compared to the native enzyme (Fig. 1). It is well known that when an enzyme is embedded in a solid support, it is removed from its native aqueous medium, and is thus exposed to a different local microenvironment [20]. Immobilised enzyme kinetics are performed at pH 9.1. At such a pH a slight pH decrease may occur (approximatively 50% of the NH_4^+/NH_3 produced is as NH_4^+ and the rest as NH_3, the pH for NH_4^+ being 9.2). This was confirmed in unbuffered solution, as a progressive decrease in pH was observed during the course of the native enzyme reaction (0.25 units in 10 min). Due to diffusion limitations this phenomenon is enhanced in the membrane. Due to surface covalent linkage such pH dependency has not been

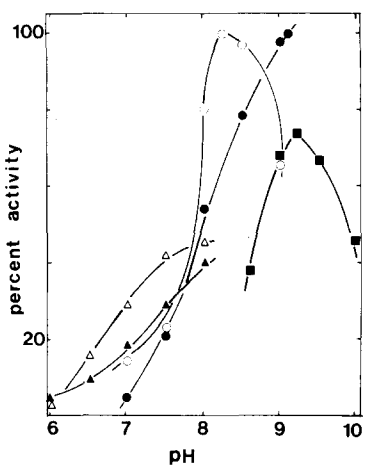


Fig. 1. Effect of pH on the activity of native enzyme in 0.1 M phosphate buffer (△———△) or in 0.1 M Tris · HCl buffer (○———○) and immobilised enzyme in 0.1 M phosphate buffer (▲———▲), in 0.1 M Tris · HCl buffer (●———●) or in 0.1 M glycine/NaOH buffer (■———■).

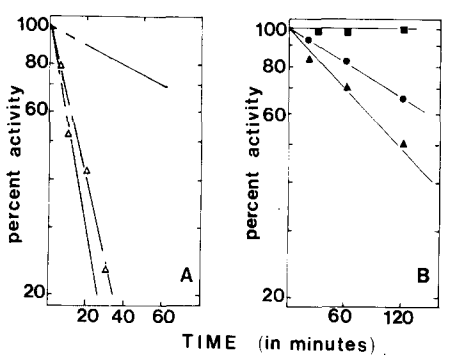


Fig. 2. Effect of immobilisation of glutamate dehydrogenase on the thermal stability of the enzyme at 52°C. A: native enzyme in absence of effector (○———○), preincubated in presence of 10 mM ADP (□———□) or in presence of 10 mM GTP (△———△). B: enzyme immobilised in absence of effector (●———●), in presence of 10 mM ADP (■———■) or in presence of 10 mM GTP (▲———▲). Enzymatic activities were measured at 30°C in absence of any effectors.

described in the other studies of immobilized glutamate dehydrogenase [8—11]. For free and immobilised enzyme, activity is found to be better in 0.1 M or 0.25 M Tris · HCl buffer than in phosphate or glycine/NaOH buffers of the same concentrations. However, below pH 8 an activating effect of phosphate ions is observed (Fig. 1) suggesting a possible ionic control even for matrix-bound enzyme.

Stability

The stability of the native (preincubated in absence or in presence of 10^{-2} M ADP or GTP) as well as the immobilised glutamate dehydrogenase was tested for its ability to withstand heat denaturation. Increased thermal stability of enzyme chemically bound in an albumin matrix has been already described [12], and Fig. 2A and B demonstrate that immobilised enzyme is much more resistant at 52°C than the enzyme in the free form. However, differences in the stabilities of matrix-bound glutamate dehydrogenases are observed. In the case of immobilisation in presence of ADP there is a distinct increase in enzyme stability in relation to same observations made for the free form. At higher temperatures (60°C) activity of glutamate dehydrogenase immobilised with ADP is found to decrease slowly with time. The behaviour of enzyme immobilised with GTP is quite different, confirming the hypothesis of the existence of two distinct sites for ADP and GTP on the enzyme molecule.

The effect of insolubilisation on the time stability of the enzyme has been also tested. After one month of storage at 4°C, native and immobilised forms retain 25% and 35% respectively of their initial activities. The high stability of the native enzyme in dilute solutions is confirmed, as well as the protection effect of ADP. The enzyme immobilised in presence of ADP, but stored in absence of it exhibits a high stability (100% after one month) identical to that observed with free enzyme stored in presence of ADP. With the matrix-bound glutamate dehydrogenase, 100% activity retention has been observed over a period of 4 months.

Determination of K_m values

To assess the effect of crosslinking on the behaviour of glutamate dehydrogenase, apparent Michaelis constants measured for L-glutamate and for NAD^+ are summarized in Table I. Due to diffusion limitations, an increase in the apparent K_m values is generally observed. In the present case these constants seem to be slightly affected. However as already described [13], it is possible by using a diffusion-reaction device, to measure experimentally true values of K_m.

For NAD^+, deviations of Lineweaver-Burk plots towards higher activities with higher NAD^+ concentrations are shown in Fig. 3. In these data, L-glutamate was held at a fixed concentration (five times the K_m value for both enzyme forms). Such down curvature is observed for free glutamate dehydrogenase (as already discussed by Engel and Dalziel [21]) and also for immobilised enzyme (as observed by Havekes et al. [9] with Sepharose-bound enzyme). In contrast with Havekes's results, we have shown that when ADP is present during the formation of the matrix, such activation is strongly decreased like in solution when free enzyme is preincubated with ADP. Fur-

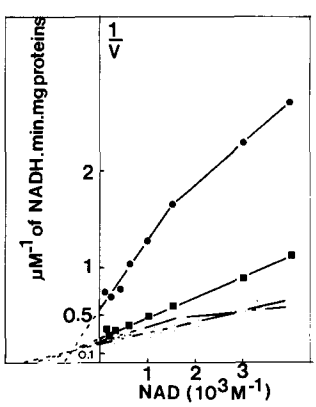
TABLE I

EFFECT OF GLUTAMATE DEHYDROGENASE INSOLUBILIZATION ON THE CATALYTIC PROPERTIES

Substrate	Native enzyme		Matrix-bound enzyme			
	K'_m (M)	Specific activity *	Without effectors		With $1.5 \cdot 10^{-2}$ M ADP **	With 10^{-2} M GTP
			K'_m (M)	Specific activity	K'_m (M)	K'_m (M)
L-Glutamate	$1.7 \cdot 10^{-3}$	4.9	$2.7 \cdot 10^{-3}$	0.36	$2.7 \cdot 10^{-3}$	$6.7 \cdot 10^{-3}$
L-Glutamate in the presence of $3.5 \cdot 10^{-3}$ M ADP	$2.7 \cdot 10^{-3}$	7.3	$2.7 \cdot 10^{-3}$	0.41	$2.9 \cdot 10^{-3}$	$4 \cdot 10^{-3}$
L-Glutamate in the presence of $4.6 \cdot 10^{-5}$ M GTP	$1.7 \cdot 10^{-3}$	0.58	$14 \cdot 10^{-3}$	0.15	$3 \cdot 10^{-3}$	10^{-2}
L-Glutamate in phosphate buffer, 0.1 M, pH 8.0	$1.1 \cdot 10^{-3}$	2.8	$1.3 \cdot 10^{-3}$	0.13	—	—
NAD^+	$6.5 \cdot 10^{-4}$	5	$1.5 \cdot 10^{-3}$	0.37	$6 \cdot 10^{-4}$	

* Specific activity expressed in μmol NADH/min per mg protein.

** In absence of any effectors during the kinetic, specific activity was 0.79 μmol NADH/min per mg protein.



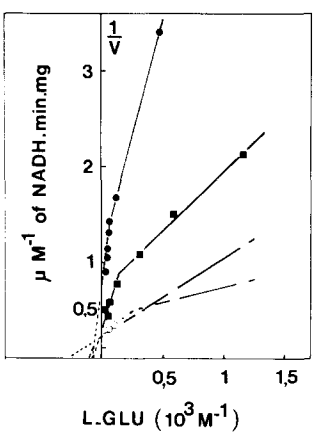


Fig. 3. Lineweaver-Burk plot of native glutamate dehydrogenase in absence of effector (○———○), preincubated in presence of 10 mM ADP (□———□) and glutamate dehydrogenase immobilised in absence of effector (●———●) or in presence of 10 mM ADP (■———■). Variable NAD^+ concentrations are used with fixed L-glutamate concentrations (10 mM in solution, 25 mM in batch). Enzymatic activities were measured at 30°C in absence of any effectors. The reciprocal values for the initial velocity have been expressed in μmol NADH produced/min per mg of protein.

Fig. 4. Lineweaver-Burk plot of native glutamate dehydrogenase in absence of effector (○———○), preincubated in presence of 10 mM ADP (□———□) and glutamate dehydrogenase immobilised in absence of effector (●———●) or in presence of 10 mM ADP (■———■). Variable L-glutamate concentrations are used with fixed NAD^+ concentrations (2 mM in solution, 12 mM in batch). Enzymatic activities were measured at 30°C in absence of any effectors. The reciprocal values for the initial velocity have been expressed in μmol NADH produced/min per mg protein.

thermore, with glutamate dehydrogenase immobilised in the presence of ADP a lowering of apparent K_m value is observed. Our results with matrix-bound glutamate dehydrogenase agree with those obtained in solution [22]; ADP still appears to prevent negative cooperative interaction between the active coenzyme binding sites and to lower the affinity of these sites for NAD^+.

Deviations of Lineweaver-Burk plots are also obtained for L-glutamate (Fig. 4), NAD^+ being held at a fixed concentration (five times the K_m value for both enzyme forms). Similar observations to those mentioned above with NAD^+ can be given for the effect of ADP on the free enzyme. In contrast maxtrix-bound glutamate dehydrogenase is always activated by high concentrations of L-glutamate, independently of the presence of ADP during the immobilisation process.

Kinetic behaviour of the immobilised enzyme is differently modulated by the presence of effector during the matrix formation. For NAD^+ binding experiment the phenomenon can be explained by the ability of ADP and NAD^+ (which are present at the same time during insolubilisation) to induce (and protect) a given conformation for the enzyme.

Effect of ADP and GTP

It is important to distinguish between the presence of nucleotides during the formation of the matrix and the kinetic effects of nucleotides when added to reaction mixture.

As shown above, higher activities have been observed when the matrix is synthetized in the presence of nucleotides. Fig. 5 illustrates the activity retained after immobilisation as a function of ADP or GTP concentration during the membrane formation. A 4-fold increase can be obtained in presence of $3.8 \cdot 10^{-2}$ M ADP as compared with the activity in absence of ADP. This

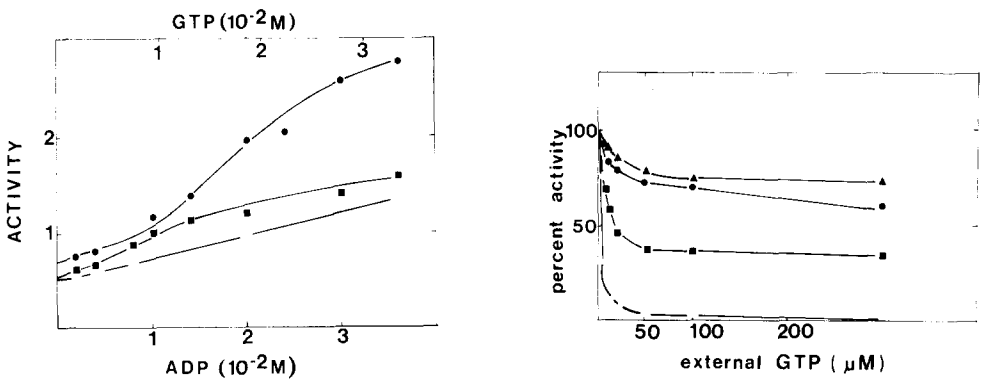


Fig. 5. Enzymatic activity (in arbitrary units) of matrix-bound glutamate dehydrogenase as a function of effectors (ADP or GTP) concentration during the insolubilisation process. ●———●, enzyme immobilised in presence of ADP with 2.5 mg/ml glutaraldehyde; ■———■, enzyme immobilised in presence of ADP with 3 mg/ml glutaraldehyde; ○———○, enzyme immobilised in presence of GTP with 3 mg/ml glutaraldehyde.

Fig. 6. Influence of external GTP (added to the reaction mixture) on glutamate dehydrogenase activity. Native enzyme (○———○); immobilised enzyme without effector (●———●); enzyme immobilised in presence of 10 mM ADP (■———■) and enzyme immobilised in presence of 10 mM GTP (▲———▲).

effect is largely dependent on the glutaraldehyde concentration. It is possible, according to Kappor and Parfett [23], in the case of the native enzyme, that the addition of ADP to our system results in a high stabilisation of the glutamate dehydrogenase structure. As shown by the same authors this interpretation is not inconsistent with the ability of ADP to induce a conformation change in the enzyme. A lower protection effect can also be obtained in the presence of GTP during the membrane production (Fig. 5). Such a matrix immersed in 1 M NaCl for 30 min decreases its activity by 35%. In contrast, enzyme immobilised in the presence of ADP completely retains its activity. These results suggest the independence of the purine nucleotide sites of the glutamate dehydrogenase molecule. This is in agreement with the reports of other investigators [22—25].

In Fig. 6 the inhibitory effect of GTP when added to reaction milieu is pictured for free and immobilised enzyme in absence of effectors. Glutamate dehydrogenase activity is considerably less sensitive to the inhibition inside the membrane. This result can be explained by the rule controlling heterogeneous enzyme kinetics: any modification of the reaction rate gives rise to an effect on the diffusion limitations [26]. We have also observed that matrix-bound glutamate dehydrogenase is more sensitive to inhibition when ADP is present during immobilisation process. No modification in the inhibition is observed in absence of ADP or in presence of GTP during the immobilisation. The conformation induced by the nucleotides could be frozen during the immobilisation (cf. above results). In the present work, no investigation of concomitant effects of ADP and GTP is presented.

Fig. 7 shows an important decrease of activation by external ADP on immobilised glutamate dehydrogenase as compared to the native enzyme. Due to steric hindrance or chemical modification, the ADP-binding site could not be identical after immobilisation. The data reported here show no protective effect of this site when ADP is present during the insolubilisation process. As described in the literature, modified glutamate dehydrogenase (after covalent binding with adenosine analog [27] or spin-labelled [28]) does not exhibit the activation by ADP, inhibition by GTP being not altered. For membrane kine-

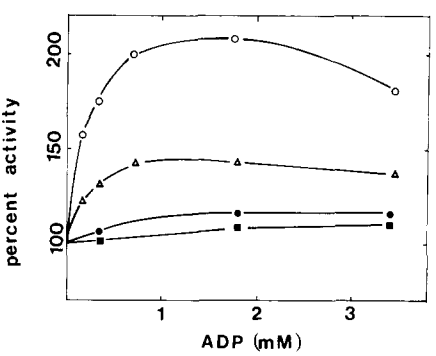


Fig. 7. Glutamate dehydrogenase activity as a function of external ADP (added to the reaction mixture) for native enzyme (○———○: in 0.1 M phosphate buffer pH 8.0 and △———△ in 0.2 M Tris · HCl buffer pH 8.2) and for matrix-bound enzyme (●———●: without effector during insolubilisation process; ■———■: in presence of 10 mM GTP or ADP during insolubilisation process).

tics no effect of buffer influence has been observed on ADP action.

As described by Horton at al. [10] with glass-bound glutamate dehydrogenase, we have always observed the reversibility of allosteric modulation by ADP or GTP. In the future, it will be of importance to know if ADP is able to cause aggregation and GTP dissociation during the step preceding immobilisation in co-crosslinking process. Preliminary data have shown that activities of glutamate dehydrogenase immobilised in the presence of nucleotides do not depend on the order of products addition or on time incubation of enzyme with nucleotides.

Table I illustrates the influence of nucleotides, when added to the reaction milieu, on the apparent K_m values of glutamate dehydrogenase for L-glutamate. For the enzyme immobilised in presence of $1.5 \cdot 10^{-2}$ M ADP, small changes in K_m values are observed as a function of external modulators. Similarly immobilised glutamate dehydrogenase, with or without effectors, exhibits little difference in glutamate K_m values, in any case when ADP is present in the reaction mixture. As described by Julliard et al. [8] with collagen-bound enzyme, external ADP seems unable to affect the apparent affinity of immobilised glutamate dehydrogenase for L-glutamate. This result suggests again a conformation freezing due to the immobilisation. In contrast a large increase in apparent K_m value is observed when GTP is present during kinetic measurement for enzyme immobilised in absence of effectors. This phenomenon is considerably retarded when $1.5 \cdot 10^{-2}$ M ADP is present during membrane formation. This suggests a protective role of ADP.

Concluding remarks

The present research confirms the impact of enzyme immobilisation procedures for kinetic studies, especially for amplifying structure-function relations. However, it is difficult to separate the effect of chemical modification of the enzyme from the effect of the microenvironment. The apparent loss in ADP activation observed with matrix-bound glutamate dehydrogenase cannot be explained only in terms of diffusion limitations (GTP being always able to inhibit the activity). It probable that the effect involves charge phenomena or conformational changes. Due to the tight chemical immobilisation of glutamate dehydrogenase inside an artificial membrane, it seems reasonable to suggest that some of the conformational states could be frozen. However, the possibility that some allosteric interactions are reversible and still present after immobilisation is confirmed by Mosbach's experiments [9—10]. Such observation agrees with kinetic properties of cross-linked glycogen phosphorylase microcrystals reported by Kasvinsky and Madsen [29]. As described for pyruvate kinase [7] immobilised enzymes do not exhibit so deep modification of conformation. The immobilisation of glutamate dehydrogenase in presence of effectors makes it possible to separate the effect of purine nucleotide from the degree of the polymerisation of the enzyme. It probably seems that allosteric control and polymerisation are independent phenomena as already described for rat liver enzyme [1]. New accomplishments for production of soluble polymers with a co-crosslinking method [30—31] bearing enzyme activity seems to be a good tool to assess whether the kinetic modulations related in the present

work are only due to the chemical fixation of glutamate dehydrogenase. Utilisation of such a technique can also raise a question regarding the relation between association-dissociation and catalytic activity.

Furthermore, due to the possibility to introduce phospholipid in a protein matrix [17], the interaction of phospholipids and glutamate dehydrogenase [32—33] can be also investigated in such a system.

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