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IMMOBILIZATION OF L-GLUTAMATE DEHYDROGENASE INTO SOLUBLE CROSS-LINKED POLYMERS

ADP EFFECT AND ELECTRON MICROSCOPY STUDIES

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Summary

Active soluble cross-linked L-glutamate dehydrogenase (L-glutamate: $NAD(P)^+$ oxidoreductase (deaminating), EC 1.4.1.3) albumin polymers were produced. Electron microscopic studies and kinetic properties were studied with the polymer in solution and compared with previous published data about the enzyme immobilized inside proteic films (Barbotin, J.N. and Breuil, M. (1978) Biochim. Biophys. Acta 525, 18—27). The glutaraldehyde effect on activity yield, ADP and β-NAD^+ protection, stability and pH rate profile were studied and discussed. Apparent Michaelis constants were determined with soluble polymers produced with or without ADP during the grafting process. Experiments were performed on the regulatory properties of immobilized glutamate dehydrogenase showing the decrease of ADP activation and GTP inhibition as compared to the free form.

In other respects, electron microscopy observations showed morphological differences between the two populations of soluble polymers produced in presence of ADP, obtained after gel filtration on Sepharose 6B. Linear aggregates of high molecular weight and classical soluble polymers were obtained. Similar K_m values and regulatory properties were exhibited by the two forms, demonstrating the absence of interdependence between the allosteric control and the polymerization of enzyme monomers.

Introduction

The study of the relationship between the quaternary structure and the

biological function has been extensively reviewed in the case of the enzyme glutamate dehydrogenase (L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3) [1—6]. Yet, the control of enzyme activity by the environment of a polyatomic framework is a vast topic which could be lightened by the immobilization of allosteric enzymes [7,8]. The possible effect of molecular interactions on the catalytic behaviour of glutamate dehydrogenase has been studied using the approach of matrix-bound derivatives [9—11]. Previously, we have reported [11] the immobilization of bovine liver L-glutamate dehydrogenase. In the preparation of immobilized enzymes into such solid matrices, it is difficult to separate the effect of the chemical modification of the enzyme from the effect of the insolubilization. However, the binding of enzymes to soluble carriers has been already reported [12—15]. In our laboratory, a technique using serum albumin and glutaraldehyde as a bifunctional agent has been used to obtain active soluble polymers bearing enzyme activities [16—18]. In such cases, the enzyme is subjected to slight accessibility constraints, chemical effects being of the same order than in proteic films.

One goal of this present paper is the study of the kinetic properties of glutamate dehydrogenase grafted on these polymers. These results are then compared with those obtained formerly by immobilizing the enzyme in artificial films [11]. Significant differences between activity of the free and immobilized enzymes are discussed depending on the glutaraldehyde concentration used. Furthermore electron microscopy observations are performed to investigate the polymer structure.

On the other hand, the ADP effect on the reversible polymerization of glutamate dehydrogenase and its catalytic activity have attracted considerable interest. Especially Cohen and coworkers [4,5] have presented theoretical and experimental evidences about the important role of the aggregation process in establishing the regulatory properties of this enzyme. The present work deals with a comparison of the catalytic and structural properties of soluble polymers produced in the absence or presence of ADP. In the last case two morphologically different populations can be obtained and their kinetic properties have been studied. These observations as well as previous works by Thusius [19] and Zeiri and Reisler [20] point out the uncoupling of the catalytic activity and the polymerization of glutamate dehydrogenase.

Materials and Methods

Enzyme polymer production. A solution (5 ml) containing 60 mg · ml^{-1} bovine serum albumin (Sigma, fraction V), from 0.1 to 0.6 mg · ml^{-1} of glutamate dehydrogenase (in 50% glycerol suspension from Boehringer, used without further purification), 2 mg · ml^{-1} of β-NAD$^+$ (Sigma, Grade III), ADP from 0 to 15 mg · ml^{-1} (Sigma, Grade III) and 2.25 (polymer A)—3.25 (polymer B) mg · ml^{-1} of commercial glutaraldehyde (Merck) in 20 mM phosphate buffer (pH 6.8) was mixed and stored for 10 h at 4°C (polymer B) or 48 h at 4°C (polymer A). The soluble polymer obtained was then stabilized by adding glycine up to a final concentration of 10 mg · ml^{-1}. The preparation was dialyzed for 12 h against 20 mM phosphate buffer, pH 6.8. The existence of polymers was checked by gel chromatography. The presence of associated enzyme activity was also measured.

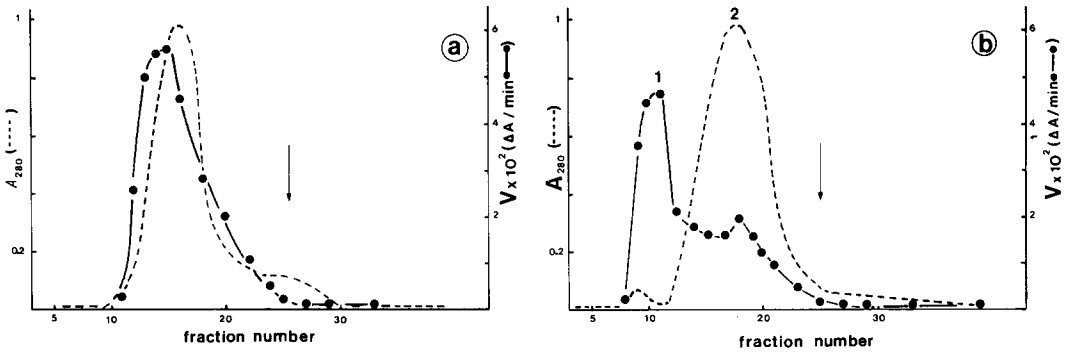

Fig. 1. Sepharose 6B gel chromatography of soluble proteic polymers bearing glutamate dehydrogenase, produced in absence (a) or in presence (b) of 15 mM ADP. Protein elution was followed continously at 280 nm (· · · · · ·). Enzyme activity was measured in each sample as mentioned in the text (●———●). The free enzyme was eluted in the fraction which was indicated by the arrow. It should be noted that in (b) two different fractions of polymers (1 and 2) bearing enzyme activity are present.

From electron microscopy observations, polymers bearing 3.5 mg · ml^{-1} of ferritin (from horse spleen, Sigma) instead of glutamate dehydrogenase were found to be produced.

Gel filtration on Sepharose 6B. A Sepharose 6B column (40 × 3.0 cm) was equilibrated with 20 mM phosphate buffer (pH 6.8) and eluted with the same buffer. Protein concentration was followed by recording ultraviolet absorption (280 nm) with an ISCO model UA 5 absorbance monitor. The elution pattern is presented in Fig. 1a (polymer B without ADP) and Fig. 1b (polymer B with ADP). 5-ml fractions were automatically collected.

Membrane production. As already described [8,11,21], enzyme membranes were produced on a plane glass surface. By using air flux, a cocross-linking reaction occurred during a 2 h period in a solution containing 50 mg · ml^{-1} of bovine serum albumin, from 2.5 mg · ml^{-1} (membrane A) to 3.5 mg · ml^{-1} (membrane B) of glutaraldehyde, 3 mg · ml^{-1} of β-NAD$^+$, ADP from 0 to 20 mg · ml^{-1} and 0.2 mg · ml^{-1} of glutamate dehydrogenase.

For electron microscopic observations, membranes bearing 10 mg · ml^{-1} of ferritin instead of enzyme were produced.

Measurement of glutamate dehydrogenase activity. For the enzyme, free in solution (F) and bound to soluble polymers (SP), measurements were carried out in a 3 ml quartz cuvette with 0.1 M (F) or 0.2 M (SP) Tris-HCl buffer, pH 8.25 (F) or 9.0 (SP), in presence of 10 mM EDTA. Enzyme concentrations were 0.002 mg · ml^{-1} (F) and 0.01 mg · ml^{-1} (SP), respectively. Concentration of L-glutamate was 12 mM (F) or (SP) and β-NAD$^+$ 4 mM (F) or 6 mM (SP). These experiments were performed in the presence and absence of ADP (0—5 mM) or GTP (0—500 μM) at 30°C.

As already described [11] an artificial membrane was immersed in a 15 ml batch reactor.

The rate of NADH appearance at 340 nm was recorded. (DBT Beckman Spectrophotometer and Ricken-Denshi SP G3 recorder).

Electron microscopy. Ultrathin sections of membranes were obtained with

the LKB Ultrotome III, using a previously described method [22]. Free glutamate dehydrogenase and soluble polymers bearing ferritin or glutamate dehydrogenase were negatively stained with uranyl oxalate (12 mM, pH 6.8) according to Mellema et al. [23]. Preparations were observed on carbon grids with a JEOL 100 C electron microscope operating at 80 kV.

Results and Discussion

As already described for urate oxidase [17] and ribonuclease [18], active soluble polymers bearing glutamate dehydrogenase can be obtained by using 30—35 mg · ml^{-1} bovine serum albumin and 4 mg · ml^{-1} glutaraldehyde. As pointed out under Materials and Methods, the maximum efficiency was observed with the concentrations as used for membrane production. In the absence of ADP during the grafting process, the ratio of the activity of enzyme polymer to the whole enzyme activity introduced in the reactive mixture was about 10% (Table I). It is clear that the enzyme activity yield was modulated by the bifunctional agent concentration. Such a phenomenon was of the same order of magnitude for the both immobilized forms. In presence of 2 mg · ml^{-1} of glutaraldehyde the free enzyme (without albumin) retains only 30% of its enzymatic activity. By using a lower glutaraldehyde concentration (1.0 mg · ml^{-1}) Josephs et al. [24] have indicated a small difference in V between native and cross-linked polymers. However, as shown in Table I, a yield of 30% of glutamate dehydrogenase activity was exhibited by the soluble polymer A obtained in the presence of 10 mM ADP. By using such a concentration of ADP, the yield could be highly modulated (from 10 to 30%) as a function of glutaraldehyde concentration (from 3.25 to 2.25 mg · ml^{-1}). When the low glutaraldehyde concentrations were used, the results were similar to those described with artificial films [11].

Like with glutamate dehydrogenase immobilized inside a membrane [11], β-NAD^+ was used as a protector during the polymerization process. ADP and β-NAD^+ offering a substantial protection while NADH (or $NADP^+$, NADPH, glutamate) were considerably less effective.

The pH-rate profile obtained with glutamate dehydrogenase grafted on soluble polymers is quite similar to that exhibited by the enzyme immobilized

TABLE I

ACTIVITY YIELD OF CROSS-LINKED GLUTAMATE DEHYDROGENASE

100% activity refers to the reduction of NAD into 4.9 μmol NADH/mg per min for the native glutamate dehydrogenase. Measurements were made according to Materials and Methods. Membrane A was produced with 2.25 mg · ml^{-1} glutaraldehyde, membrane B with 3.25 mg · ml^{-1} glutaraldehyde. ADP concentration used for membrane production was about 35 mM [11]. For soluble polymer, the upper concentration used was 15 mM.

Effector added during the cocross-linking process	Activities (%)			
	Membrane A	Membrane B	Polymer A	Polymer B
None	12	8	10	6
ADP	40	20	30	12

in the membrane [11]. In both cases, the optimal pH for the immobilized enzyme was shifted towards the alkaline by 0.8 unit (from pH 8.2 to pH 9) as compared to the free form. In all the cases, an increasing of ionic strength (by adding 0.1—2 M NaCl) lead to a decreasing of the enzyme activity.

Stability

The effect of immobilization on the time stability of glutamate dehydrogenase grafted on soluble polymers was tested. After one month, the enzyme retained 50% of its initial activity for the soluble polymer form and 25% for the free form. When 10 mM GTP was present during the grafting process, 40% activity retention was observed. For the enzyme immobilized in presence of 10 mM ADP, the soluble polymers exhibited 95% activity retention over a period of 2 months. In all the cases, the enzyme concentration used was about 0.1 $mg \cdot ml^{-1}$.

Determination of the apparent Michaelis constants

Table II gives the apparent Michaelis constants determined for native and immobilized glutamate dehydrogenase, in Tris-HCl buffer. Like for the free enzyme [25,26] both immobilized forms (membrane and soluble polymer) in absence of ADP, exhibited non-linear double-reciprocal plots of the steady-state velocity against substrate and coenzyme concentrations. In all the cases, the K_m values corresponding to the matrix-bound enzyme as compared to the which corresponded to the higher concentrations (such values were given for saturated concentration of the fixed substrate). Table II shows an increasing of the K_m values corresponding to the matrix-bond enzyme as compared to the free form. It should be noted that the K_m values obtained with soluble polymers were close to those exhibited by the enzyme immobilized inside the membrane when low glutaraldehyde concentration was used. In presence of glutaraldehyde (2 $mg \cdot ml^{-1}$) the free enzyme (at pH 8) exhibits an increase in the values of K_m for glutamate (4 mM) and for NAD^+ (1.6 mM) suggesting a chemical modification of the enzyme affinity towards the substrates. Yet, by

TABLE II

APPARENT MICHAELIS CONSTANTS OF IMMOBILIZED GLUTAMATE DEHYDROGENASE

Values for the native enzyme were determined in Tris-HCl buffer (0.1 M at pH 8.2).

Substrate	Native enzyme (mM)	Matrix-bound enzyme in absence of ADP (mM)			
		Membrane A *	Membrane B *	Soluble polymer * A	Soluble polymer * B
L-Glutamate	1.7	2.7	5	3	3
β-NAD^+	0.65	1.5	2	1.6	1.6
		Matrix-bound enzyme in presence of 15 mM ADP (mM)			
L-Glutamate	—	2.7	5	3 **	3 **
β-NAD^+	—	0.6	1.1	0.97 **	1.3 **

* See Materials and Methods section or Table I.

** Same K_m values were also exhibited by the both fractions obtained after gel filtration (Fig. 1b).

using phosphate buffer (0.1 M, pH 8), the apparent K_m values for glutamate were found to be identical (1.1 mM) for immobilized (membrane A and soluble polymer A) and free forms. Some investigators [27,28] have already outlined that pH, ionic strength, and ionic compounds have a marked influence on the catalytic properties of glutamate dehydrogenase.

As shown in Table II, the K_m values for glutamate, obtained with soluble polymers produced with or without ADP, are similar. However, some differences were observed for β-NAD^+, especially as a function of the glutaraldehyde concentration used during the polymerization process. As already described with glutamate dehydrogenase immobilized in a membrane in presence of ADP [11], the biphasicity of the Lineweaver-Burk plots disappeared with the corresponding soluble polymers. In other respects, K_m values were not deeply modulated by the addition of variable ADP concentrations during the kinetic data.

When ADP is present during the polymer production, the elution pattern in Sepharose 6B shows two separated fractions (1 and 2) bearing glutamate dehydrogenase activity (Fig. 1b). Some details about their structural features are given below. However, the most interesting implication of the present results is that the apparent K_m values measured with the eluted fractions (1 and 2) for both substrates are the same (Table II). These results indicate that the degree of association has no evident effect on the enzyme affinity.

Effect of immobilization on regulatory properties

ADP seems to offer a great protection for the activity of the enzyme when it is present during immobilization. However, as shown in Table I, such increasing of the enzyme activity could be observed only with low glutaraldehyde concentrations. These results may suggest a competition between ADP and glutaraldehyde for two different polymerization processes: glutamate dehydrogenase polymers and classical soluble polymers. It should be noted that, glutaraldehyde is also capable of forming intramolecular cross-links which could be interpreted as a freezing of a conformational state.

As described with the enzyme immobilized in a membrane [11], the enzyme grafted to soluble polymers did not show the response to ADP as that displayed by the free enzyme when added to the reaction mixture. The kinetic effects of the nucleotides (ADP and GTP) when added to the reaction milieu are illustrated in the Fig. 2a and b. These results indicate that enzyme activation by external ADP can be modulated by the glutaraldehyde content of the soluble polymer. The role played by the buffer does not produce a clear enough explanation of the diminished ability for ADP to activate the immobilized enzyme. As shown in the Fig. 2a the activation of the native enzyme in solution was decreased in Tris-HCl (a maximal activation of 215% has been observed in 0.1 M phosphate buffer (pH 8.0) with 1.5 mM ADP in the milieu [11]).

However, no modification was observed in phosphate buffer with the immobilized form. It should be noted that the presence of glutaraldehyde (0.8 $mg \cdot ml^{-1}$) in solution, dramatically abolished such activation with the native enzyme (115% activity was obtained in presence of 1 mM ADP).

The inhibition by GTP of the glutamate dehydrogenase grafted on soluble

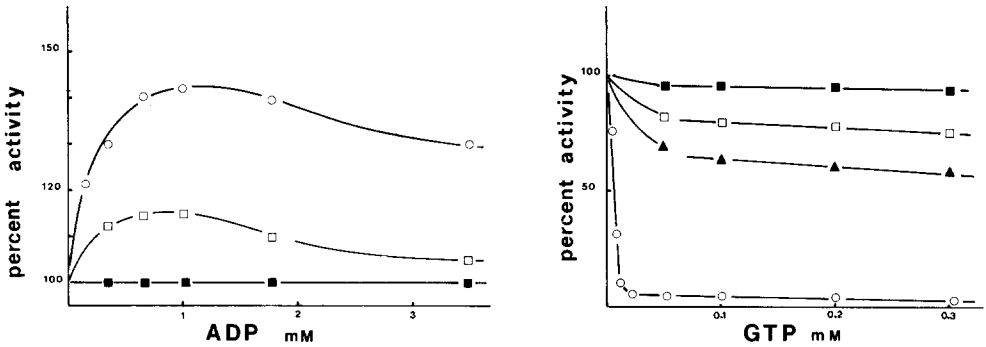

Fig. 2. Glutamate dehydrogenase activity as a function of external ADP (a) or GTP (b) when added to the reaction mixture. (a) ○———○, native enzyme; □———□, soluble polymer with or without ADP during the cocross-linking process (low glutaraldehyde content), and ■———■, idem (high glutaraldehyde content). (b) ○———○, native enzyme; ▲———▲, soluble polymer with ADP during the cocross-linking process (low glutaraldehyde content); □———□, idem without ADP, and ■———■, high glutaraldehyde content.

polymers was considerably diminished (Fig. 2b). A modulation of such effect was obtained by the presence of ADP during the polymer production.

Electron microscopy

The regular aspect and the absence of pores in artificial enzyme membranes have been already established [8,22]. Similar micrographs were obtained for membranes bearing glutamate dehydrogenase produced in presence or in absence of ADP. Direct ferritin staining provided an excellent means to visualize the enzymatic sites inside the membrane. When ferritin is present instead of the enzyme, an homogeneous repartition of such molecule inside the membrane was obtained, as illustrated on the Fig. 3. Even if higher ferritin concentrations were used, no aggregation of such molecules was detected.

Due to their lower molecular weight (from $2 \cdot 10^6$ to $5 \cdot 10^6$ [16]), the soluble polymers could be directly examined by using negative staining techniques. Due to the high protein background (99% of serum albumin), it was not possible to detect the enzyme molecules. Furthermore hydrophobic and self-aggregation properties of serum albumin (Fig. 4a) provide some difficulties to test the homogeneity of such preparations. When ferritin is grafted on soluble polymers instead of the enzyme, such molecules can be easily detected (Fig. 4b). As illustrated the aggregates observed have a heterogeneous aspect, and their size was not correlated with the estimated molecular weight of soluble polymers. However, the ferritin molecule can be visualized as a protein bound to the surface of proteinic aggregates (Fig. 4c). After elution on Sepharose 6B, the structural appearance of these polymers was quite identical. No better results were obtained by using shadow-coating techniques.

Fig. 5a shows an unfixed commercial suspension of glutamate dehydrogenase (0.05 mg $\cdot$ ml^{-1}). As already described by Horne and Greville [29], the enzyme subunits show uniform triangular shape and size. From our data, ADP in phosphate buffer seems to play a role in the enzyme assembly. When glutamate dehydrogenase (2 mg $\cdot$ ml^{-1}) was incubated in the presence of 30 mM ADP in

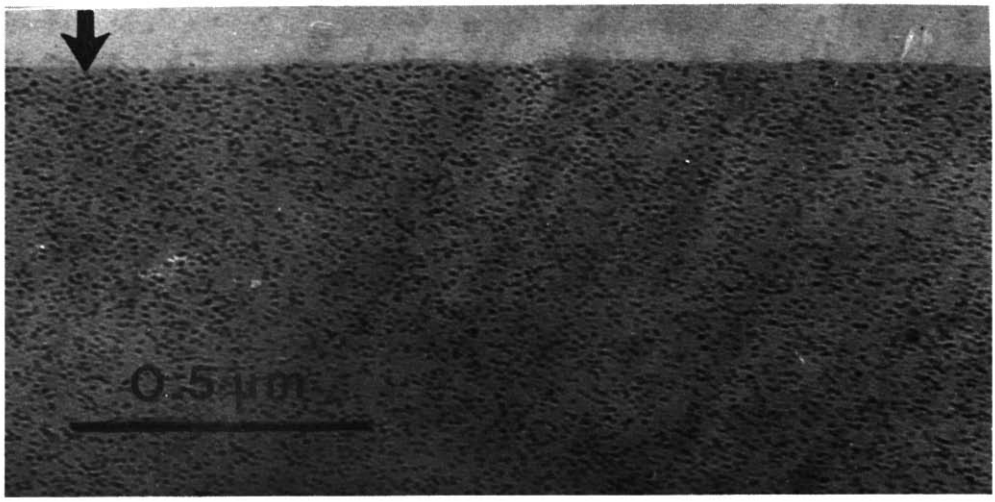

Fig. 3. Ultrathin section of a proteic membrane bearing ferritin instead of the enzyme. An homogeneous repartition of the ferritin molecules inside the matrix is clearly shown. The arrow indicates the edge of the membrane. The section was post-stained with uranyl acetate and lead citrate.

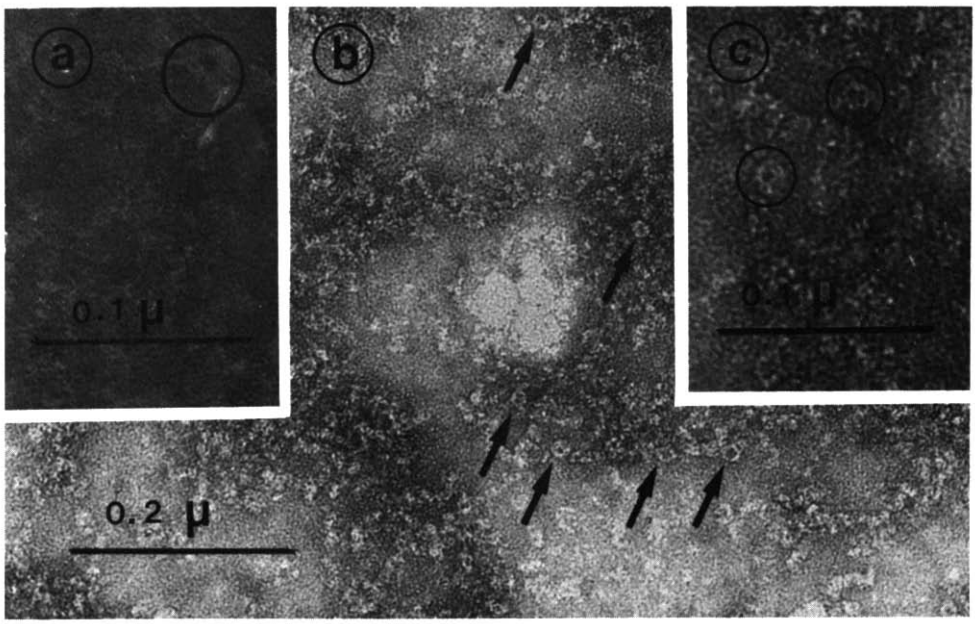

Fig. 4. (a) High magnification of bovine serum albumin (0.2 mg · ml^{-1}) negatively stained, showing spontaneous aggregates of monomers. In the circle some albumin molecules can be distinguished. (b) Negative staining of soluble polymers bearing ferritin. The arrows indicate ferritin molecules immobilized on proteic aggregates. The dispersity of aggregates can be noted. (c) High magnification of (b) illustrating ferritin molecules grafted on polymers (○).

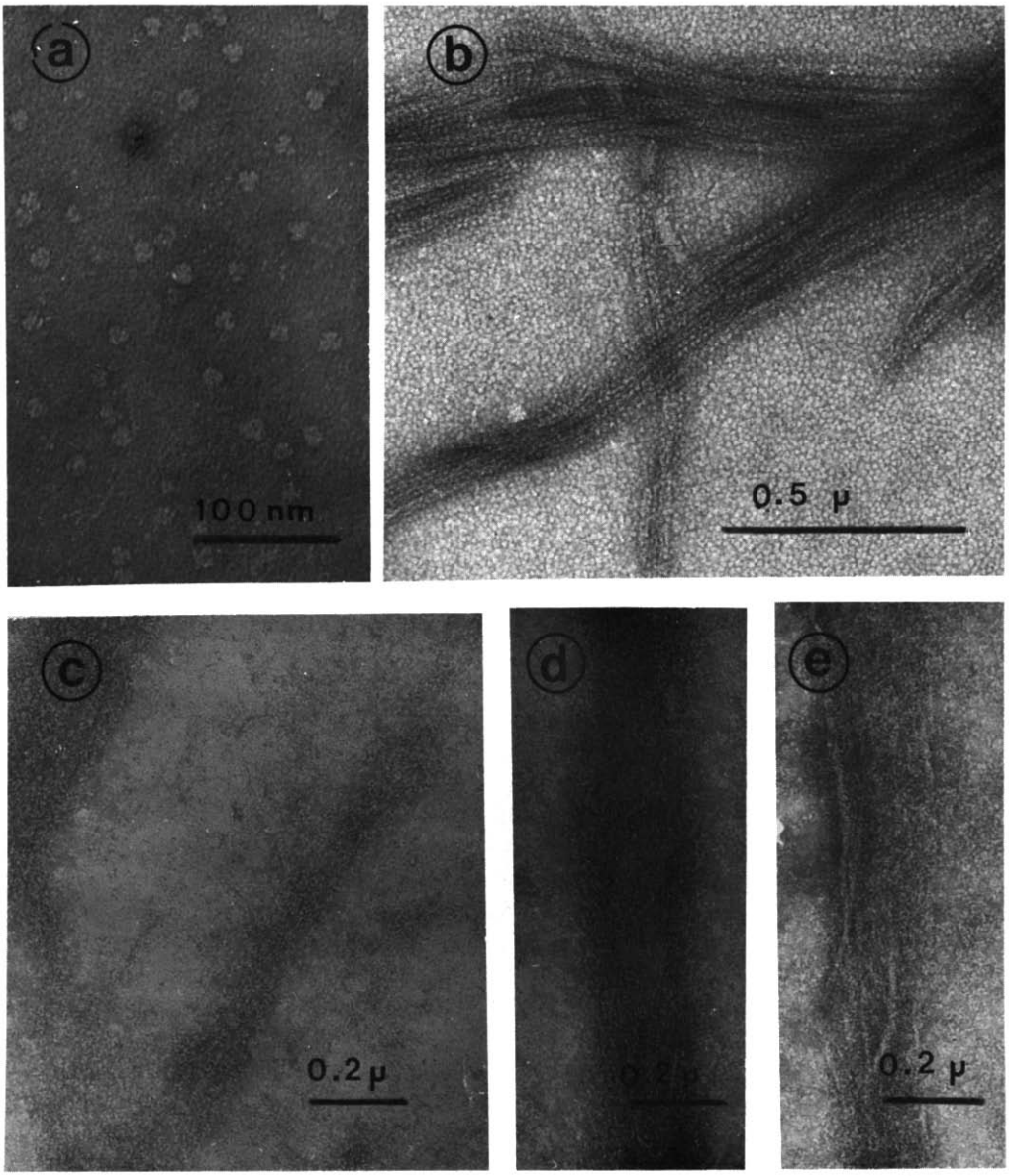

Fig. 5. (a) Negative staining of glutamate dehydrogenase (0.05 mg · ml^{-1}). (b) Negative staining of polymeric glutamate dehydrogenase obtained in presence of 10 mM ADP with a high concentration of enzyme (2 mg · ml^{-1}). Rod-like structures are clearly observed. (c—e) Some typical negative staining of soluble polymers produced with glutamate dehydrogenase and ADP corresponding to the fraction 1 eluted by gel filtration (Fig. 1b). Linear aggregates can be observed.

20 mM phosphate buffer, pH 6.8, an opalescence began to appear within 1 h at 4°C. A typical view of such a suspension is presented in Fig. 5b. The rods measure 500 nm across and up to 3 μm long. Such observations are similar to those described by Munn [30] and Josephs [31].

As can be seen in the Figs. 5c—e, a particular aspect is observed for soluble

polymers produced in the presence of 10 mM ADP. In fact, such micrographs correspond only to the fraction 1 obtained after gel filtration (Fig. 1b). The fraction 2 exhibits identical aspects to those obtained with ferritin (Fig. 4b). It seems clear that in the present case, a linear packaging of proteins is distinguished. Such polymerized structures may correspond to those illustrated in the Fig. 5b.

From this last observation, it can be concluded that two morphological different populations were produced: glutamate dehydrogenase polymers with a low content of serum albumin (fraction 1) and soluble polymers bearing glutamate dehydrogenase with a high content of serum albumin (fraction 2). The interaction with glutaraldehyde of the fraction 1 can perhaps explain the disturbed appearance of such polymers.

The two different polymers exhibiting similar kinetic properties, the non-incidence of the molecular structure on the catalytic properties of the grafted glutamate dehydrogenase can be suggested. However, the absence of substructures inside the membranes produced in presence of ADP should be noted. Such a structural difference may be explained by the cross-linking time (2 h for membranes, from 6 to 48 h for soluble polymers) and also by the viscosity of the polymerization media.

Concluding remarks

The methods of chemical modifications have often been used as a tool to investigate the relationship between the chemical mechanism of catalysis and regulation of the enzyme. The substitution of the lysyl residues of glutamate dehydrogenase is one subject which has especially been reviewed already [32,33]. Such perturbations can lead to numerous effects showing the different reactions of the 32 lysyl residues of the enzyme subunit.

In aqueous solution, glutaraldehyde condenses to a mixture of polyunsaturated aldehydes which cross-link proteins by Schiff base formation with free NH_3^+ groups of the protein. Nevertheless, such a reaction with the lysyl residues of a protein is complex and not fully understood [34,35]. However, some freezing of active enzyme conformation have been described with glycogen phosphorylase [36] and dCMP aminohydrolase [37]. Furthermore, the glutaraldehyde cross-linking of glutamate dehydrogenase monomers and polymers have been described before by Josephs et al. [24] and more recently by Zeiri and Reisler [20]. In such a case, all the cross-linked preparations display identical catalytic and allosteric responses. However, it is difficult to confirm the existence of covalent bond between the protein molecules in the protein aggregates formed after the treatment of the enzyme with glutaraldehyde.

Under our experimental conditions, due to the chemical effect of the glutaraldehyde, the regulatory properties of the enzyme grafted on soluble polymers have been dramatically diminished. However, in the presence of ADP during the grafting process, a competition between glutaraldehyde and ADP for a polymerization process can be assessed. The resulting polymers exhibiting similar kinetic properties, it seems clear that the catalytic behaviour of the immobilized enzyme is independent of the degree of polymerization. Without

evoking an allosteric model, the use of soluble albumin polymers can light the problem of the relation between association-dissociation and the activity. As described by Josephs et al. [24], Thusius [19] and Zeiri and Reisler [20], it seems clear that allosteric control and polymerization of enzyme monomers are not interdependent. It will be now of interest to study the hydrodynamic properties of glutamate dehydrogenase grafted on soluble albumin polymers by using active enzyme sedimentation method [38].

Acknowledgements

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