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IMMOBILIZED GLUTAMATE DEHYDROGENASE: SOME CATALYTIC AND STRUCTURAL ASPECTS

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SUMMARY

1. Glutamate dehydrogenase has been immobilized by binding the enzyme to CNBr-activated Sepharose 4B particles at pH 9.0 (in buffer as well as in 10% dioxane) and at pH 7.2 in buffer. All preparations have high catalytic activities viz. 65–80% of the V value of the free enzyme. The K_m value for glutamate (at infinite NAD^+ concentration), measured in the presence of 0.2 mM ADP, is identical in the different Sepharose complexes (2.0 mM) and equal to that of free enzyme under similar conditions. The K_m' value for NAD^+ however, varies in the different preparations (0.24–0.45 mM) and is also different from that of the free enzyme (0.09 mM).

2. In the glutamate dehydrogenase–Sepharose 4B complexes one observes activation by NAD^+ and a pronounced activation by L-glutamate, although these effects are quantitatively different in the various preparations.

3. The Lineweaver–Burk plots indicate that at high concentrations of nucleotide and substrate, binding of one becomes independent of the other.

4. ADP is not able to abolish the activation by NAD^+ and glutamate, this in contrast to the situation in the free enzyme. It is proposed that at least some of the binding sites are in a conformational state similar to that induced by ADP in the free enzyme. The NAD^+ binding to these sites is not tightened by glutamate however, and the dissociation constant (0.43 mM (Prepn A) and 0.45 mM (Prepn C)) is equal to the binary binding constant of NAD^+ in the free enzyme (in the presence or absence of ADP [27]).

5. The allosteric properties of the matrix bound enzymes have been studied with ADP and GTP. The inhibition by GTP is reduced particularly when 3-acetylpyridine-NAD^+ is used as coenzyme. Although the binding of ADP is diminished, it still enhances the turnover number of the various glutamate dehydrogenase–Sepharose complexes.

6. The substrate inhibition by NADH requires higher NADH concentrations.

Abbreviation: TNBS, trinitrobenzene sulfonate.

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This and the effects mentioned under (5) correspond to the situation in glutamate dehydrogenase when lysyl residues have been chemically modified.

7. Mechanical destruction of the Sepharose matrix increases the K_m values for glutamate but decreases those for NAD^+.

INTRODUCTION

Glutamate dehydrogenase from bovine liver is affected by a variety of allosteric ligands [1] and is therefore an interesting model for the immobilization of allosteric enzymes, notwithstanding its complicated kinetics. Activation of Sepharose 4B by CNBr leads to imidocarbonate groups in this hydrophylic polymer which are reactive with unprotonated amino functions in the protein [2, 3]. We decided on a direct coupling of the protein lysyl residues to the matrix. The enzymically active monomer of glutamate dehydrogenase consists of 6 identical subunits with a molecular weight of approx. 56 100 [4]. Of the 500 residues in the bovine enzyme subunit, 32 are lysyl residues according to Moon et al. [5].

Chemical modifications with reagents primarily reactive with ε-amino groups of lysine indicate that different residues can be modified by using different reagents. For example, pyridoxal 5'-phosphate reacts primarily with lysyl Residue 126, previously called Residue 97 [5, 6], while trinitrobenzene sulfonate [TNBS] modifies preferentially Residues 422 and subsequently 419 according to Goldin and Frieden [7]. Modification of Residue 126 leads to a partial loss in catalytic activity depending on the extent of modification within the monomer while alkylation of Residue 422 has important implications for the ligand binding properties and therefore also for the kinetics of the system. Other lysyl residues are also reactive. Incorporation of 24–30 moles DNP per mole of enzyme within 1 h is possible. However without any protection of the enzyme by its ligands this results in a complete loss of activity [8]. Protection of the various nucleotide binding sites is therefore an essential part of our immobilization procedure. A similar argument has been used by Julliard et al. [9] in their immobilization experiments of glutamate dehydrogenase on collagen films as solid support.

Besides the necessity to protect essential sites during the immobilization another structural feature of the system has to be considered in this process. Namely, glutamate dehydrogenase has the potential to form lengthwise aggregated polymers; the association can be described by an indefinite self-association with one equilibrium constant [10, 11]. Under some of our experimental conditions association is irrelevant, however.

Initial rate studies of the various glutamate dehydrogenase–Sepharose 4B complexes were performed with an Aminco–Chance double beam spectrophotometer. Differences in the kinetic patterns and in the effects of the various ligands which occur as a consequence of the immobilization will be discussed.

MATERIALS

Bovine liver glutamate dehydrogenase was obtained in a 50% glycerol suspension from Boehringer Mannheim. The coenzymes NAD^+, NADH and 3-acetyl-

pyridine NAD^+ and the nucleotides GTP, ADP, were purchased from Sigma, L-glutamate from the British Drug Houses. Sepharose 4B was obtained from Pharmacia (Uppsala).

METHODS

Immobilization procedures

Prepn A (pH 9.0). The enzyme was dialysed extensively against 100 mM sodium phosphate buffer (pH 8.0). 10 mg of enzyme were poured over a Sephadex G-25 column (heighth 15 cm, diameter 1 cm) which previously had been equilibrated with 20 mM sodium borate buffer (pH 9.0) containing 10% dioxane and also 1 mM α-oxoglutarate, 100 μM of each ADP and NADH and 0.5 mM β-mercaptoethanol. An additional 900 μM ADP, 100 μM NADPH and 50 μM GTP was added to the enzyme fraction eluted from this column, just before starting the coupling reaction. The original amount of enzyme (10 mg) was reacted for 24 h at 4 °C with 100 mg Sepharose 4B (2.5 ml packed gel), which had been activated with 100 mg CNBr [3]. To remove unreacted enzyme a small column was made from the Sepharose reaction mixture and the gel washed with 800 ml of a 100 mM sodium phosphate buffer (pH 8.0) containing 0.1 mM EDTA. After 500 ml had been passed no residual glutamate dehydrogenase activity could be detected in the eluate. The Sepharose beads were removed from the column and suspended in a final volume of 10 ml of a 100 mM sodium phosphate buffer (pH 8.0) containing 0.5 mM EDTA and 0.2 mM ADP. No loss in activity was observed in a standard activity test over a period of 5 months when the enzyme was stored at 0–4 °C.

Prepn B (pH 9.0). Prepn B has been prepared identically except that 10% dioxane was omitted in the chromatography and immobilization steps.

Prepn C (pH 7.2). 15 mg of dialyzed enzyme were incubated with the same effector concentration described above and then linked to 8.5 ml of packed Sepharose 4B which had been activated with 40 mg CNBr per ml of packed gel. The immobilization was carried out in 30 mM sodium phosphate buffer (pH 7.2) and 0.5 mM EDTA and in the presence of the same ligands as mentioned for Prepns A and B.

Protein determinations

Free enzyme was determined spectrophotometrically at 280 nm using $E^{1\%}_{1\,cm} = 9.3$ [12]. Glutamate dehydrogenase bound to Sepharose was determined colorimetrically as described by Lowry et al. [13], but with the modification that the reaction mixture was stirred (20 min) and then filtered before determining absorbance.

Activity determination

The standard assay mixture contained 100 mM sodium phosphate buffer (pH 8.0), 0.1 mM EDTA, 0.1 mM NAD^+ and 5 mM sodium L-glutamate. Initial velocities were measured with an Aminco–Chance dual wavelength spectrophotometer as described previously [14]. Activities are expressed in moles of NADH formed per min/mg of protein. In the reactions with 3-acetylpyridine-NAD^+ as coenzyme, 363 nm and 400 nm were used [15] as measuring and reference wavelength, respectively. Kinetic characterization of the different preparations was done in a 100 mM sodium phosphate buffer [pH 8.0] in the presence of 0.1 mM EDTA and 0.5 mM β-mercaptoethanol.

These measurements were done in the presence or absence of ADP (0.2 mM). The stock solution of the immobilized preparations was stirred before making an appropriate 1 to 40 dilution in the assay buffer. This dilution was chosen so that after gently stirring the mixture to distribute the Sepharose beads homogeneously it was possible to add 0.1 ml to the cuvette (using an 0.5-ml pipette). This procedure proved to give very reproducible results.

The free enzyme was prepared in a stock solution of 0.12 mg/ml in 100 mM phosphate buffer (pH 8.0). 10–12 μg of enzyme were used in each assay, an amount which is the concentration range used by Engel and Dalziel [16] for their kinetic experiments*.

Inactivation of glutamate dehydrogenase–Sepharose 4B was performed in 10 mM Tris–HCl buffer (pH 8.0) in the presence of 0.1 mM EDTA [17]. In order to prepare different enzyme dilutions in this buffer, a measured volume of glutamate dehydrogenase–Sepharose 4B in the stock buffer was filtered over a glass filter under reduced pressure. The still damp particles were then resuspended as required.

Sonification

Gel beads were destroyed by sonification of the particle suspension for a total of 10 min at 0 °C. Pulses of sonification were given in periods of 15 s with intervals of 3–5 min between each pulse.

RESULTS AND DISCUSSION

Effect of immobilization on the general properties of glutamate dehydrogenase

Immobilization of the enzyme. The general properties of our three preparations

TABLE I

COMPARISON OF THE AMOUNT OF GLUTAMATE DEHYDROGENASE COUPLED UNDER DIFFERENT CONDITIONS AND OF SOME CATALYTIC PROPERTIES OF THESE INSOLUBILIZED ENZYMES WITH RESPECT TO THE SOLUBLE ENZYME

V values have all been determined in the presence of 0.2 mM ADP.

Coupling	Coupling conditions	mg bound per ml packed gel	V (moles/s/mg protein)	K_m (NAD^+) (mM)	K_m (Glu) (mM)
Prepn A	20 mM borate buffer (pH 9.0) + 10% dioxane	0.57**	$1.17 \cdot 10^{-7}$	0.43	2.6
Prepn B	20 mM borate buffer (pH 9.0)	0.85–1.0***	$1.2 \cdot 10^{-7}$*	0.24	2.0
Prepn C	30 mM phosphate buffer (pH 7.2)	1.05**	$0.95 \cdot 10^{-7}$	0.45	2.0
Free enzyme	–	–	$1.47 \cdot 10^{-7}$	0.09	1.9

* Based on an averaged protein concentration.
** Method of Lowry et al.
*** Aspartic and glutamic acid analysis.

* The turnover number of the enzyme preparation used in the present study was approximately 70% of the value published previously [26].

of immobilized glutamate dehydrogenase are summarized in Table I. It is clear that the coupling efficiencies at pH 9 and 7.2 are the same. This is surprising since the efficiency is expected to be lower at pH 7 [3, 18] as observed with lipoyl dehydrogenase [14]. The V values of the glutamate dehydrogenase–Sepharose complexes, determined in the presence of 0.2 mM ADP are quite similar for the three preparations and almost as high as for the free enzyme. In general the K_m values for the substrate are not affected by the coupling, in contrast to the K_m values for the coenzyme which are considerably enhanced.

Effectors have been added as protectors of functionally important regions of the enzyme. A complication which arises from the addition of these ligands is their influence on the degree of polymerization and isomerization of glutamate dehydrogenase (see e.g. [1, 19]). The molecular size distribution determines the amount of protein coupled on the surface and within the internal volume of the matrix. This becomes of importance when the kinetics of such a system are diffusion-controlled.

Cassman and Schachman [20] observe significant polymerization of glutamate dehydrogenase even at pH 9.0. The particular effect of the mixture of effectors and substrates used here on the polymerization is unknown* although the presence of ADP enables polymers to occur [21, 22]. In the presence of 10% dioxane [23], however, we expect mainly monomers of glutamate dehydrogenase to be present. It is important to note, however, that although the enzyme may exist in different polymeric states during the coupling, the equilibrium constant (cf. [1]) is such that after the washing procedure one expects predominantly if not exclusively monomers.

pH dependency of the activity. It is quite frequently observed that the pH optimum of an enzyme shifts on binding the enzyme to a solid support (see e.g. [24]). Part of the pH profile for the glutamate dehydrogenase–Sepharose complex (Prepn B) is shown in Fig. 1. In this case the pH optimum for our immobilized enzyme seems

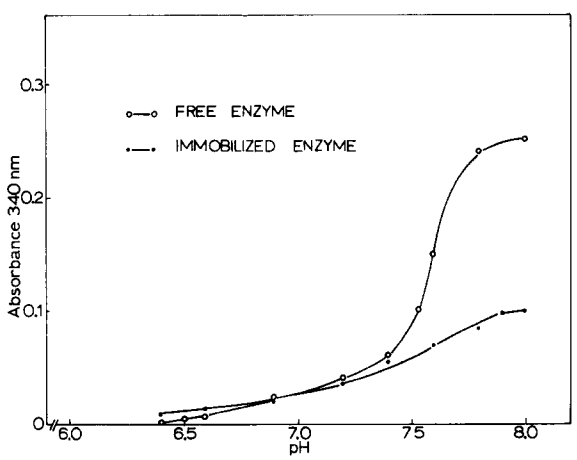

Fig. 1. pH optimum profile for matrix-bound and free glutamate dehydrogenase. The buffers used for the assay were 100 mM sodium phosphate buffers of different pH values containing 0.1 mM EDTA with standard substrate concentrations as described in Methods.

* We are aware of the fact that the conditions used may give rise to still other complications, viz. nucleotide linkage to the matrix, while still in interaction with bound enzyme molecules.

not shifted although the profile is broadened. The curve, however, has not been analysed in detail above pH 8, but the activity increases approx. 2% near to the known optimum in the region from pH 7.8 to 8.0. This is identical for both free and immobilized species which gave us confidence that it was valid to make comparative measurements at pH 8.0. It is noteworthy that in the case of glutamate dehydrogenase bound to collagen, the profile was slightly narrowed but again the optimum remained unchanged [9].

Kinetic properties of glutamate dehydrogenase–Sepharose

Activation by coenzyme and substrate and the effects of ADP.

Engel and Dalziel [16, 25] analyzed the steady-state kinetics of glutamate dehydrogenase in detail and concluded that glutamate deamination occurs through a ternary complex mechanism. They observed sharp activations by increasing concentrations of NAD^+ under different pH and buffer conditions but not in the presence of ADP [26]. Negative interactions between the subunits in the ternary complex of enzyme, coenzyme and glutamate were postulated as an explanation. These kinetic data are supported by nonlinearity in the binding of both NAD^+ and $NADP^+$ in a ternary complex with glutarate [27].

An activation by NAD^+ is also observed (Fig. 2) with our glutamate dehydrogenase–Sepharose complex (Prepn C) and the activation is at approximately the same concentration (0.2–0.3 mM) as in the soluble enzyme (0.5 mM). We were surprised to find however that the complex is also activated by high concentrations (3.0–7.5 mM) of glutamate. This activation is not observed with the free enzyme which in fact

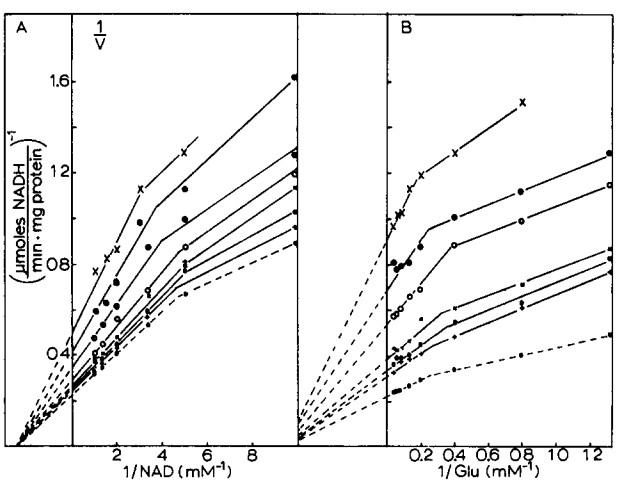

Fig. 2. Lineweaver–Burk plots for immobilized glutamate dehydrogenase–Sepharose (Prepn C). In A variable NAD^+ concentrations are used at the following L-glutamate levels: +—+, 25 mM; ○—○, 17.5 mM; ×—×, 7.5 mM; ○—○ 5 mM; ◐— ◐, 2.5 mM; ●—●,, 1.25 mM; and ×—×, 0.75 mM. In B the symbols correspond with the following NAD^+ concentrations: +—+, 1000 μM; ○—○, 750 μM; ×—×, 500 μM; ○—○, 300 μM; ◐— ◐, 200 μM and ×—×, 100 μM. — — —, represents Lineweaver–Burk plots at infinite concentration of the other substrate. The assay buffer contained 100 mM sodium phosphate buffer (pH 8.0), 0.1 mM EDTA and 0.5 mM β-mercaptoethanol. Temperature: 25 °C. The reciprocal values for the initial velocity have been expressed in μmoles NADH produced per min/mg of protein.

is inhibited by high concentrations (20 mM) of this substrate. An activation of the free enzyme by glutamate has been reported [29] but this was observed at lower concentrations.

The downward curvature in the Lineweaver–Burk plots of Fig. 2 for the immobilized enzyme might be explained in terms of diffusional barriers, as has been pointed out recently [29], but this explanation is not very likely in our case as will be discussed later.

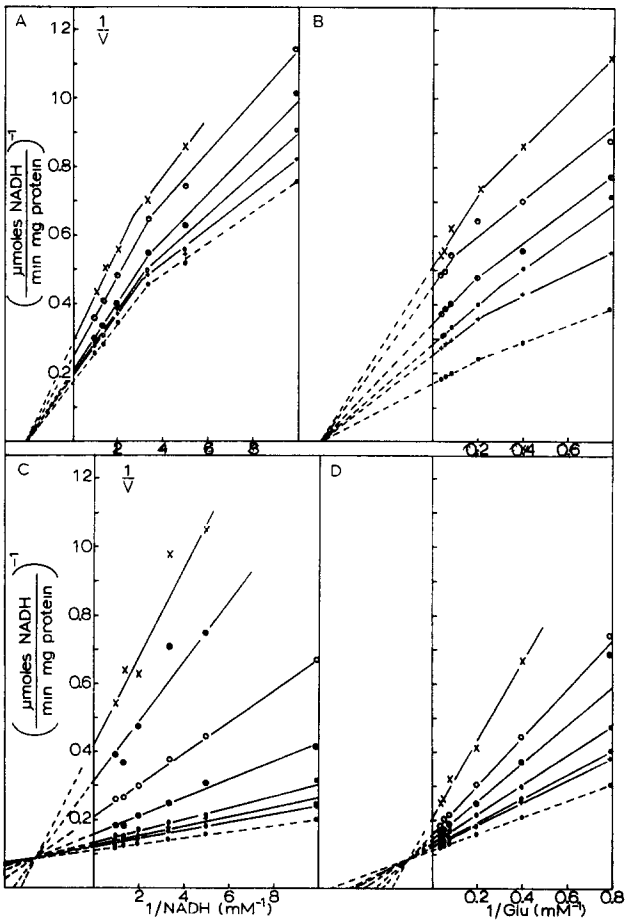

Fig. 3. Lineweaver–Burk plots for immobilized glutamate dehydrogenase-Sepharose (Prepn C) (A,B) and free enzyme (C,D) respectively. Conditions as in Fig. 2, except for the presence of 0.2 mM ADP in the assay. In A NAD^+ is used as a variable substrate. The symbols correspond with the following L-glutamate concentrations: +—+, 25 mM; ○—○, 17.5 mM; ◐—◐, 12.5 mM; ◓—◓, 5 mM and × – ×, 2.5 mM. In B L-glutamate is varied at different NAD^+ levels viz.: + – +, 1000 μM; ○—○, 750 mM; ◐—◐, 500 μM; ◓—◓, 300 μM; ×—×. 200 μM NAD^+. In C the Lineweaver–Burk plots for the free enzyme at variable NAD^+ concentrations are shown at the following L-glutamate levels: ○—○ 25 mM; +—+, 17.5 mM; ●—●, 12.5 mM; ◐—◐, 5 mM; ○—○, 2.5 mM; ●—●, 12 5 mM; ×—×, 1.25 mM. In D the symbols correspond with the following NAD^+ levels: +—+, 1000 μM; ○—○, 750 μM; ●—●, 500 μM; ◐—◐, 300 μM; ○—○, 200 μM; ×—×, 100 μM NAD^+.

TABLE II

KINETIC COEFFICIENTS FOR THE OXIDATIVE DEAMINATION OF L-GLUTAMATE IN 100 mM SODIUM PHOSPHATE BUFFER pH 8.0 CONTAINING 0.3 mM EDTA AND 0.5 mM β-MERCAPTOETHANOL

The kinetic coefficients used are those used in the initial rate equation

$$\frac{e}{v_0} = \varphi_0' + \frac{\varphi_1'}{[S_1]} + \frac{\varphi_2'}{[S_2]} + \frac{\varphi_{12}'}{[S_1][S_2]}$$

S_1 symbolizes coenzyme, S_2 substrate.

Prepn	ADP, 0.2 mM	$\varphi_0'(\cdot 10^{-6})$ (s·mg·l·M^{-1})	$\varphi_1'(\cdot 10^{-3})$ (s·mg·l)	$\varphi_2'(\cdot 10^{-3})$ (s·mg·l)	φ_{12}' (s·mg·l·M)	φ_1'/φ_0' (μM)	φ_2'/φ_0' (μM)	φ_{12}'/φ_2' (μM)	φ_{12}'/φ_1' (μM)
A	+	8.55	3.6	22	9.6	430	2630	430	2630
B	+	8.33	2.0	18	4.2	240	2000	240	2000
C	+	10.50	4.7	21	9.5	450	2000	450	2000
C	−	13.60	5.2	25	9.5	380	1850	380	1850
Free enzyme (0.5–1.0 mM NAD^+)	+	6.60	0.6	13	5.5	90	1910	430	9560
Engel and Dalziel [26] (0.2–0.5 mM NAD^+)	−	6.00	1.1	4		180	700		
A, sonified	+	12.30	2.9	77	18.4	240	6250	240	6250
C, sonified	+	8.00	2.0	35	9.0	250	4450	250	4450

The experiments of Fig. 2 were done in the absence of ADP. When ADP (0.2 mM) is added to the free enzyme the coenzyme activation as well as the L-glutamate inhibition are completely eliminated (Figs 3C and 3D). With the immobilized enzyme (Prepn C) on the other hand both coenzyme and substrate activation remain. Moreover the Michaelis constants for NAD^+ obtained from the high concentration data in Prepn C are not strongly influenced by ADP (0.45 and 0.38 mM, respectively) as shown in Table II.

The V value of Prepn C is still stimulated in the presence of ADP which is an activator of the glutamate dehydrogenase reaction [20] at lower concentrations but which may become a competitive inhibitor for the coenzyme binding site [26] at higher concentrations with a K_i value of 6 mM [30].

Nucleotide binding of free and immobilized glutamate dehydrogenase. The kinetic parameters φ'_{12}/φ'_2 may be used to calculate the dissociation constant of the binary enzyme–coenzyme complexes assuming a rapid equilibrium random mechanism (cf. [25]). We have not included data for the free enzyme in the absence of ADP but Engel and Dalziel [25] report a value of 0.5 mM at pH 8.0 under almost identical assay conditions as used in this study*. The binary dissociation constant of the free enzyme in the presence of ADP ($\varphi'_{12}/\varphi'_2 = 0.43$ mM), is hardly affected (Table II). This is not true for the binding of the oxidized coenzymes in the ternary complex with substrate (L-glutamate) or its analogues (glutarate). In the absence of ADP Dalziel and Egan [27] observe a strong binding particularly at low coenzyme levels ($K_{diss} \approx 6\,\mu M$) with negative cooperativity whereas in the presence of ADP a single binding constant is obtained for the coenzyme. The value is higher (60 μM) but still well beyond the binary binding constant at pH 7.0. The Michaelis constant under our assay conditions

* We use a slightly higher ionic strength phosphate buffer and also 0.5 mM β-mercaptoethanol.

(pH 8.0) is 90 μM, a value which is well in accordance with previous data and which suggests that K_m and dissociation of coenzyme from the ternary complex are identical (cf. [27]).

When one applies similar calculations in the case of the immobilized enzymes (e.g. Prepn C, Table II) the dissociation constants calculated for the coenzyme both in the absence and presence of ADP (0.38 and 0.45 mM, respectively) are similar to the binary dissociation constant of the free enzyme in the presence or absence of ADP (0.43 mM (Table II) and 0.5 mM [25], respectively). It is clear from Figs 2, 3A and 3B that these values only refer to the situation where the coenzyme concentration is high since ADP does not abolish the discontinuities in the $1/v$ vs $1/NAD^+$ plots. For the other two immobilized enzyme preparations the calculated values turn out to be very similar.

ADP still causes the V value of glutamate dehydrogenase–Sepharose 4B complex C to increase to the same extent as in the free enzyme. This suggests an identical effect on the rate-limiting step regardless whether this is the dissociation of NADH from the E–glutamate–NADH complex at high glutamate concentrations [31] or the isomerization of this complex as more recently proposed [32].

L-*Glutamate binding of free and immobilized glutamate dehydrogenase.* The free enzyme in the presence of ADP has a larger φ'_2/φ'_0 value in the presence of ADP (1.9 mM) than the free enzyme in the absence of ADP (0.7 mM) (cf Table II). This is also true for the φ'_{12}/φ_1 value although an exact comparison is impossible since this figure is not presented in the paper of Engel and Dalziel [25]. ADP relieves the substrate inhibition by glutamate due to a decrease in glutamate affinity. This is evident in the ternary complex but particularly pronounced in the binary complex.

With the immobilized enzyme (Prepn C) no substrate inhibition is observed under the conditions used. Moreover, since the Lineweaver–Burk plots intersect on the negative X-axis, the K_m values in all our immobilized glutamate dehydrogenase–Sepharose complexes are all very likely to represent the dissociation constants for the binary as well as ternary complexes. The values (2.0 mM for C and B, and 2.6 mM for A) are surprisingly close to values in the literature (1.3–1.65 mM) [32] and to the ternary complex value for the free enzyme in the presence of ADP (1.9 mM).

The arguments to suggest that downward curvature in Fig. 2 is not primarily caused by diffusional limitations are the following.

(1) The observed discontinuities are rather sharp which one would not expect in a diffusion controlled process. Moreover, such discontinuities are known to occur in the glutamate dehydrogenase kinetics in the same concentration range of the coenzyme.

(2) Extrapolation of the lower concentration data in Fig. 2 at infinite concentration of the second substrate results in K_m values of approx. 160 and 670 μM for NAD^+ and L-glutamate, respectively. These figures are identical to those reported by Engel and Dalziel [25] for the free enzyme over a 0.2–0.5 mM nucleotide concentration range. In the presence of ADP both in Prepns C and A only the K_m value for glutamate (at the low concentration side of the transition) is affected and amounts 1.25 mM.

(3) These kinetic deviations occur in Prepn C even more pronounced than in Prepn A. In the latter glutamate dehydrogenase–Sepharose 4B complex however, one would expect less surface-coupling and therefore more diffusional interferences.

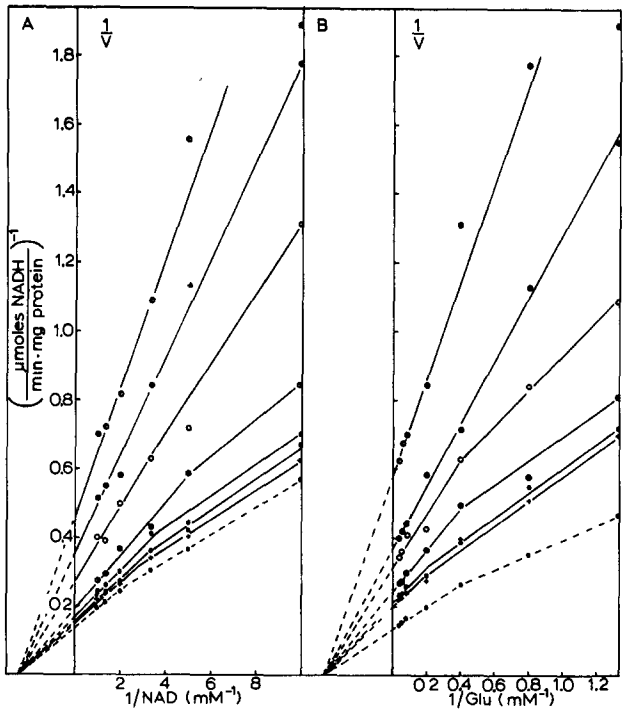

Fig. 4. Lineweaver–Burk plots for immobilized glutamate dehydrogenase–Sepharose (Prepn A), under the same conditions as described in Fig. 2. At variable NAD^+ concentrations (A), the symbols correspond with the following L-glutamate substrate levels: +—+, 25 mM; ●—●, 17.5 mM; ○—○, 12.5 mM; ◑—◑, 5 mM; ○—○, 2.5 mM; ●—●, 1.25 mM; ◐—◐, 0.75 mM. In B the symbols correspond with different NAD^+ levels viz.: +—+, 1000 µM; ○—○, 750 µM; ◑—◑, 500 µM; ○—○, 300 µM; ●—●, 200 µM; ◐—◐. 100 µM NAD^+.

The kinetic properties of the glutamate dehydrogenase–Sepharose coupled at pH 9.0 (A and B) are only slightly different from those of C (Fig. 4). At high concentrations both substrate and coenzyme binding are also mutually independent as in C whereas the dissociation constants are identical (Table II) in all cases.

Immobilization of glutamate dehydrogenase under different conditions thus affects the enzyme in a uniform manner. Activation by NAD^+ is insensitive to ADP while activation by L-glutamate becomes pronounced. Although heterogeneity of the covalent linkages to the solid support cannot be excluded the similarity in the properties of the various glutamate dehydrogenase–Sepharose complexes suggests a rather uniform coupling. An influence of the coupling on the subunit interactions would provide us therefore with a more attractive explanation for the kinetic observations. ADP added to the free enzyme binds to all six subunits and thereby equalizes the affinities of both NAD^+ and glutamate for the different subunits, although substrate and coenzyme still promote each other's binding considerably under these conditions (cf Table II). However, in the various glutamate dehydrogenase–Sepharose complexes some of the subunits seem to remain permanently in a different conformational state. The sites with low affinity are characterized by an ADP-like conformation if one considers the binding constants of the substrate, whereas the ability to promote nu-

cleotide binding is lost. The affinity of NAD^+ for the low affinity sites is equal to the dissociation constant of the binary complex free enzyme–NAD^+.

Dalziel and Egan [27] report a value of 0.47 mM in their direct binding studies while indirect data (cf Table II) indicate a value of 0.45 mM in the presence of ADP. The agreement between our indirect glutamate binding data and the binary binding constants for glutamate as reported by Di Franco and Iwatsubo [32] suggest that the kinetic properties are partially determined by subunits which have lost cooperativity. Whether the influence of the interactions between the subunits restricts itself within the two sets of trimeric subunits composing the active monomer, remains an interesting hypothesis.

Influence of destruction of the matrix on kinetic properties

Changes in kinetic parameters of insolubilized systems upon mechanical or enzymatic destruction of a matrix may reveal the importance of diffusional barriers or matrix-protein interactions. Thus Axén et al. [33] demonstrated that the kinetic properties of insolubilized chymotrypsin return upon enzymatic degradation of their dextran matrix. We could not destroy our Sepharose 4B matrix enzymically (agarase from Calbiochem), but a mechanical destruction by sonification was possible. The kinetic parameters of the sonified Prepns A and C are also presented in Table II. Both preparations show larger K_m values for glutamate and smaller ones for NAD^+. The V value increases and decreases slightly in Prepns C and A, respectively. We cannot provide a basic explanation for these data. In some respects they confirm our earlier observations with matrix-bound lipoamide dehydrogenase (unpublished results) where we observed an increase in K_m lipoamide and in V on sonification. However, in the case of lipoamide dehydrogenase there is also an increase in the K_m value of NAD^+. It is possible that residual charges on the matrix cause these effects. This explanation would fit our glutamate dehydrogenase data but is unlikely in the case of lipoamide dehydrogenase which uses a neutral substrate. An important area for future studies is the question of whether hydrogen bond formation between matrix and protein occurs and thus influences the kinetic properties. Apart from fragmentation of the polymer, sonification of the agarose backbone may lead to the formation of new hydrogen bonds between backbone and protein.

Ligand binding to glutamate dehydrogenase–Sepharose and their kinetic effects

Since modification of amino groups affects the kinetic response of glutamate dehydrogenase to purine nucleotides, the kinetics of our immobilized enzyme preparations have to be compared to similar patterns obtained with chemically modified soluble enzymes. Such a kinetic analysis with chemically modified glutamate dehydrogenase is still in progress. As an initial approach the ligand binding model developed by Cross and Fisher [34] has been used as a frame of reference to identify changes. In Fig. 5 the inhibitory effect of GTP is pictured for both 3-acetylpyridine-NAD^+ and NAD^+ as coenzyme. The glutamate dehydrogenation by the free enzyme using 3-acetylpyridine-NAD^+ is twice as fast as with NAD^+ [35] which according to Cross and Fisher is due to an increased rate of the rate-limiting step. The activity of the immobilized enzymes (Prepns A and C) is also higher with 3-acetylpyridine-NAD^+ as a coenzyme. With both coenzymes the free enzyme is 65% inhibited by 0.1 mM GTP under our experimental conditions. The immobilized Prepns A and C are only 28 and 32% inhibited, respectively, with 3-acetylpyridine-NAD^+ as coenzyme. The inhibition is slightly higher with NAD^+ as coenzyme (35 and 50%, respectively). The

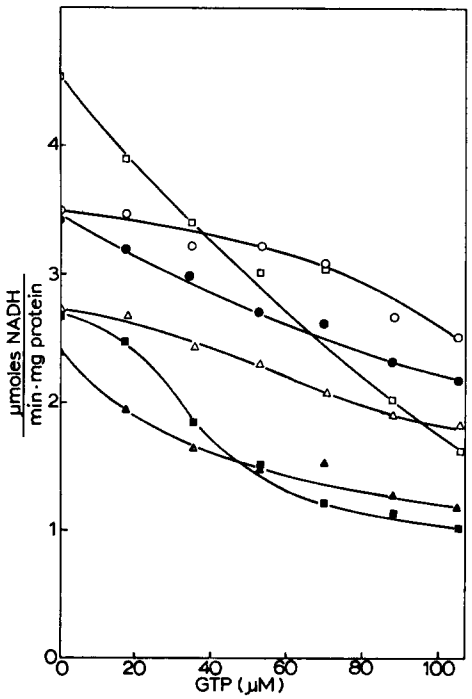

Fig. 5. Initial velocities of free and immobilized glutamate dehydrogenase (Prepns A and C), at different GTP levels. The assay conditions are 37.5 mM L-glutamate and 116 μM 3-acetylpyridine-NAD^+ in 100 mM sodium phosphate buffer containing 0.1 mM EDTA and 0.5 mM β-mercaptoethanol. □—□, free enzyme; △—△, Prepn C; ○—○, prepn A. Also with NAD^+ (0.55 mM) as coenzyme: ■—■, free enzyme; ▲—▲, Prepn C; ●—●, Prepn A.

shape of the inhibition curves for the free enzyme is quite different and GTP is a stronger inhibitor at relatively low concentrations when NAD^+ (0.54 mM) is used as coenzyme instead of 3-acetylpyridine-NAD^+. The effect may be complex since at the NAD^+ concentration used, NAD^+ activates.

The reduced inhibitory effects of GTP could be due to linkage of certain lysyl residues to the Sepharose since acetylation and other modifications to lysyl residues lead to an increase of K_i for GTP in the free enzyme [7, 8, 36, 37].

Although ADP and GTP bind to different sites they have opposite effects on the catalytic activity of GDH (cf. [1]). Steric hindrance and partial overlap of the sites have been postulated to explain these effects [34]. The effects of ADP in the absence and presence of GTP on free and immobilized enzymes are compared in Fig. 6. Both NAD^+ and 3-acetylpyridine-NAD^+ have been used. With the latter coenzyme the GTP inhibition is completely overcome by ADP. There is a slight shift in the effective concentration of ADP to higher concentrations with the immobilized enzymes.

The results with NAD^+ are more complex since the GTP inhibition is not completely abolished, even with the soluble enzyme under the assay conditions used. In addition there is hardly any stimulation of the activity of Prepn A (1.05 X) by ADP. It is clear from Fig. 6 that Prepns A and C differ with respect to the concentration of ADP which is effective, suggesting that the effector affinity in Prepn C is lower.

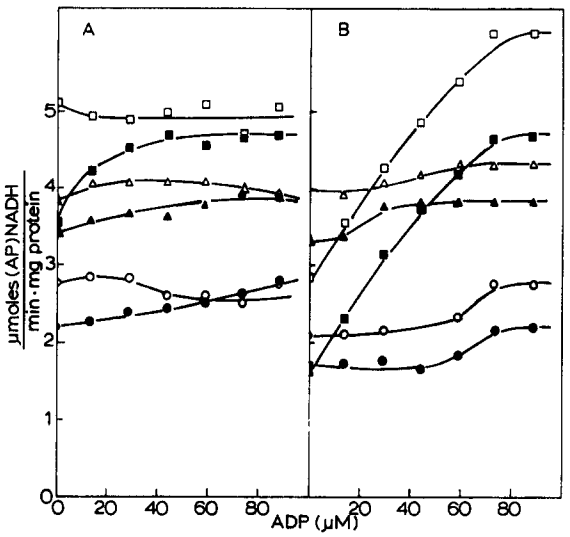

Fig. 6. A and B. ADP effects on initial velocities of free and immobilized glutamate dehydrogenase in the presence and absence of GTP (using 3-acetyl-pyridine-NAD$^+$ and NAD$^+$ as coenzymes). A Experimental conditions 37.5 mM L-glutamate and 11.6 μM 3-acetylpyridine-NAD$^+$. □—□, free enzyme; △—△, glutamate dehydrogenase–Sepharose Prepn A; ○—○, glutamate dehydrogenase–Sepharose Prepn C. Similarly, in the presence of 44 μM GTP: ■—■, free enzyme; ▲—▲, Prepn A and ●—●, Prepn C. B. Experimental conditions 37.5 mM L-glutamate and 540 μM NAD$^+$. Symbols as in A.

Inhibition by NADH

Modification of glutamate dehydrogenase by pyridoxal phosphate [7] is characterized by loss in catalytic activity, an increased dissociation constant for GTP and,

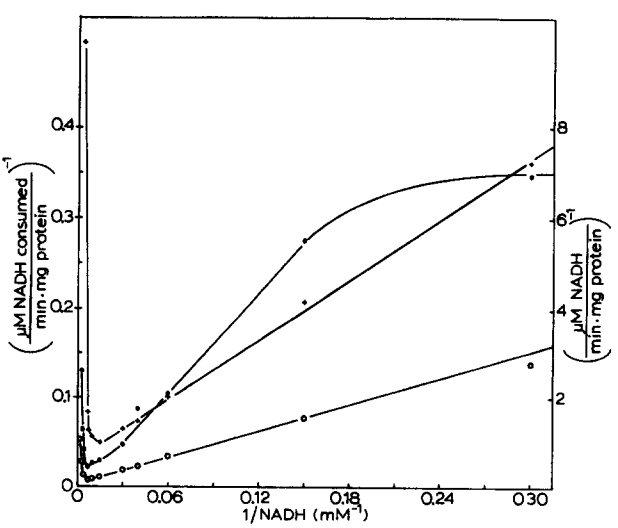

Fig. 7. Plots of reciprocal NADH concentrations vs reciprocal initial velocities. Experimental conditions: assay buffer as in Fig. 2, 5 mM α-oxoglutarate, 50 mM NH_4Cl and NADH concentrations as indicated. Symbols: ×—×, free enzyme; ○—○, Prepn A; ●—●, Prepn C. The left-hand axis indicates the reaction velocity for the free enzyme, the right hand axis those of A and C.

in the reverse reaction, by a decrease of inhibition by NADH. Similar effects are produced by trinitrobenzene sulfonate [37]. Goldin and Frieden [7, 37] emphasize the importance of changes in subunit–subunit interactions since modifications at different lysyl residues lead to similar kinetic effects. As expected from the foregoing the immobilized preparations demonstrate also clearly a decrease in substrate inhibition as shown in Fig. 7. Moreover, the immobilized enzyme C has nonlinear Lineweaver–Burk kinetics at low concentrations of the reduced coenzyme, as for the oxidized nucleotide (cf Fig. 2). A conclusion with respect to turnover number is not justified since the two other substrates were not varied, but the activities in the amination reaction seem quite low compared to the deamination reaction.

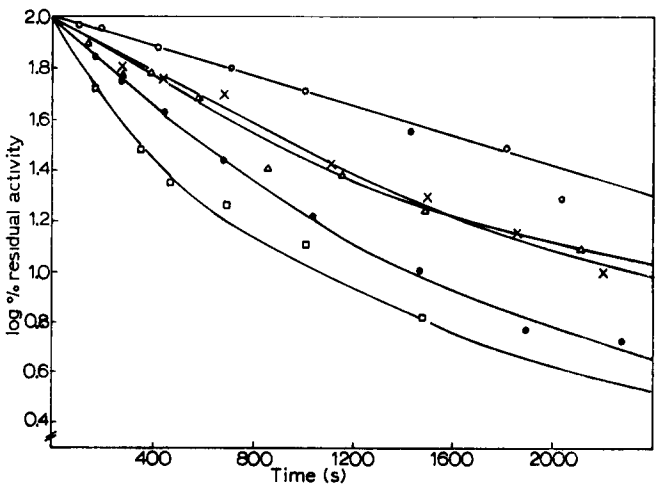

Fig. 8. Effect of glutamate dehydrogenase–Sepharose-4B concentration (Prepn C) on the inactivations, conditions see Fig. 2. Further details are described in Methods. The different symbols correspond with the following dilutions of the matrix–enzyme complex: ○—○, 10 times diluted; ×—×, 25 times diluted; △—△, 50 times diluted; ●—●, 100 times diluted; □—□, 200 times diluted.

Inactivation of matrix-bound glutamate dehydrogenase

The linear relationship between the rate of inactivation of glutamate dehydrogenase under low ionic strength conditions and the amount of monomer present in solution indicate that this species is involved in the inactivation [38]. Matrix-bound glutamate dehydrogenase (Prepn C) is also inactivated at low ionic strength, but in contrast to the free enzyme the inactivation does not follow first order kinetics (Fig. 8). Such a first order dependency would be expected if the inactivation depends on a conformational transition or a dissociation process. There is still a pronounced concentration dependency however. Thus, either our assumption is wrong that only monomers are left on the matrix or this is indicative for intermediates and some reversibility of the process.

So far our attempts to reactivate such inactivated preparations of glutamate dehydrogenase–Sepharose failed.

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