SOME MODIFICATIONS OF THE KINETIC PROPERTIES OF BOVINE LIVER GLUTAMATE DEHYDROGENASE (NAD(P)) COVALENTLY BOUND TO A SOLID MATRIX OF COLLAGEN

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1. Introduction

In order to determine the regulations exerted by the membrane and the membrane integrated enzymatic systems on the oxidation of glutamate by glutamate dehydrogenase in mitochondria, the binding of bovine liver glutamate dehydrogenase (GDH)* has been studied on a solid matrix of collagen which can be used to simulate a membrane.

Up to now, no covalent binding of GDH to a solid support and very few kinetic data concerning polymeric enzymes covalently bound to solid supports, have been reported.

In the present work, GDH is coupled to thin films of acid soluble collagen by the azide method [1] in the presence of various protectors. The specific activity is stable and makes possible the determination of kinetic parameters of the bound enzyme which differ significantly from those of the free enzyme. The affinity and cooperativity for glutamate are lowered down by the binding to collagen. The bound GDH is strictly pH dependent although the optimal pH is not affected. The GTP inhibition and ADP activation are kept. ADP seems to affect only the catalytic properties.

* *Abbreviations*:
GDH : L-glutamate:NAD (P) oxidoreductase (deaminating) EC 1.4.1.3.
EDTA: ethylene diamine tetracetic acid
β-ME : beta mercaptoethanol
Glu : potassium glutamate.

2. Materials and methods

Bovine liver GDH in 50% glycerol, NAD and NADH were purchased from Boehringer Mannheim. Calf skin collagen was prepared according to [2]. L-Glutamic acid, A grade, was purchased from Calbiochem, USA.

Thin films of collagen were prepared according to [3] and tanned with 2.1% formaldehyde [4]. GDH was covalently bound to collagen by the azide method [1] modified as follows: 5 mg of small thin film particles of collagen are treated 12 hr at room temperature with 0.05% aqueous hydrazine. Hydrazine is then rinsed out with 0.1 M sodium chloride. The acyl-hydrazide collagen is treated 2 min at 0° with 5 ml 0.3 N HCl and 0.5 M sodium nitrite. Nitrite is rinsed out with a large volume of 10^{-3} N HCl and 0.1 M sodium chloride. Then GDH, plus substrates or effectors, in borate buffer 0.02 M, pH = 9.0, 0°, is added to the acyl-azide collagen. After a variable time (see table 1) ammonium chloride is added or not, directly to the enzyme mixture (pH adjusted to 9.0 with ammonia). Then the excess GDH is rinsed out with large volumes of 0.02 M phosphate buffer, pH = 8.0, 4°. The collagen bound GDH can be kept for many weeks at 4° in 0.02 M phosphate, 0.5 mM EDTA, 0.5 mM β-ME, 1 mM ADP, pH = 8.0.

The oxidation of glutamate by GDH is followed by the appearance of NADH at 340 nm with a Pye Unicam SP 800 UV spectrophotometer which permits accurate evaluations of reaction rates of 0.16

Table 1
GDH protection during azide coupling.

Protectors added to GDH	GDH (mg) added	t°C	incubation time (hr) with: GDH	NH_4Cl	V_m × volume nmoles NADH min^{-1}
1 mM ADP; 1 mM NADH	0.6	0	2	0	6.7
1 mM ADP; 1 mM NADH	0.6	0	1	1	5.3
1 mM ADP	0.6	0	2	0	16.6
1 mM ADP	0.75	0	3	2	23
1 mM ADP	1.5	4	3	2	8.2
1 mM GTP	0.6	0	2	0	9.8
1 mM GTP	0.6	0	2	2	10
1 mM α-ketoglutarate	0.6	0	2	2	0
0.1 mM Zn^{2+}	0.6	0	2	2	0
None	0.6	0	2	2	0

Conditions are described in Materials and methods.
V are maximal rates obtained as in fig. 3 and measured at 28°.

Table 2
Influence of the binding on the kinetic parameters of GDH. Action of ADP.

	Free enzyme		Bound enzyme	
	no ADP	0.25 mM ADP	no ADP	0.25 mM ADP
V (0.5 mM NAD)	1 ± 0.09 (3)	1.8 ± 0.05 (2)	1.7 (2)	4.8 (2)
apparent K_m for glutamate (0.5 mM NAD)	0.51 ± 0.01 (2)	1.04 ± 0.03 (2)	3.14 ± 0.12 (9)	3.0 ± 0.12 (2)
n Hill number for glutamate (0.5 mM NAD)	1 (2)	1 (2)	0.52 ± 0.01 (9)	0.58 (2)

Experimental conditions as in fig. 2. V is the maximum velocity for saturating glutamate when NAD is 0.5 mM; it is expressed in nmoles NADH × ml^{-1} × min^{-1}. Numbers of assays are given in parenthesis.

nmole NADH × ml^{-1} × min^{-1}, with expanded scale. Soluble GDH is directly assayed in silica cuvettes, while the collagen bound GDH is tested outside the spectrophotometer cuvette: the bound GDH particles are kept in suspension and the incubation solution is continuously pumped into a spectrophotometer flow cuvette and back to the reaction vessel with a peristaltic pump. Reaction rates are linear for ten minutes. We have shown that reaction rates obtained with soluble GDH are the same in this device as those obtained when GDH is directly assayed in silica cuvettes. The small particles of collagen were abundantly rinsed with distilled water between each assay.

All calculus and regression tests were performed with an Olivetti desk top computer P 101.

3. Results and discussion

Table 1 gives the optimal conditions for coupling GDH. In the absence of protectors no detectable activity can be measured with the collagen bound GDH; therefore if the enzyme is actually bound, it has been inactivated by the binding process. ADP and GTP are the best protectors; this is not surprising since they are regulatory effectors of GDH; when the activity of bound GDH is tested, ADP or GTP are easily washed out before measurements. In the absence of protectors, the inactivation of GDH may be due to the acylation of active lysine residues [5]. Incubation with NH_4Cl, which reacts with excess azide during binding, seems unnecessary.

The binding mixture (GDH + protectors) must be

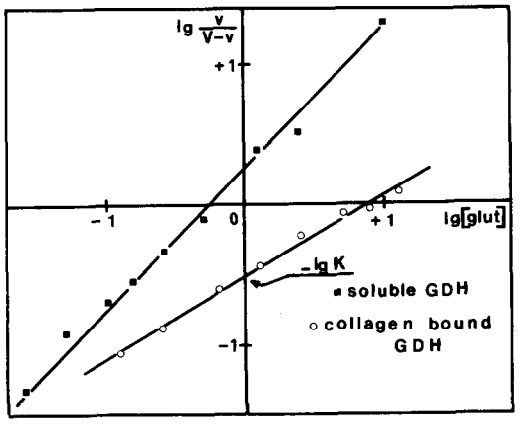

Fig. 1. Hill plot. Incubation medium: 0.02 M phosphate K, 0.5 mM β-ME, 0.5 mM EDTA, 0.5 mM NAD, pH = 8.0, 28°; soluble or collagen bound GDH: 0.1 I.U.B. unit added, final volume 4 ml. V is obtained from fig. 3.

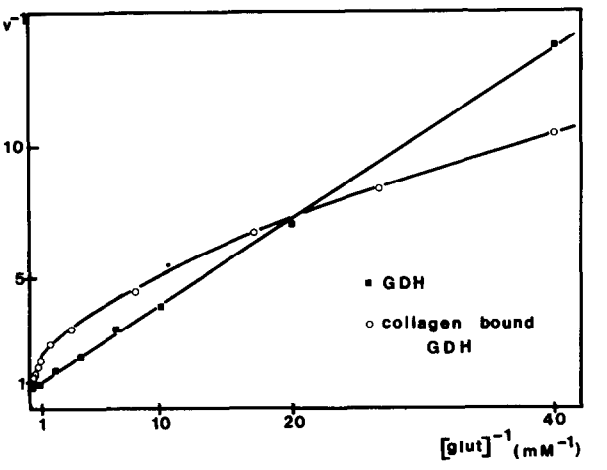

Fig. 2. Double reciprocal plot. Experimental conditions as in fig. 1. v^{-1} in nmole^{-1} of NADH × ml × min.

kept in contact with the azide collagen matrix for at least one hour and less than three hours to obtain maximum activity.The bound GDH remains stable (less than 5% variation of activity) in 0.02 M phosphate buffer, 0.5 mM β-ME, 0.5 mM EDTA, pH = 8.0, 28°, to perform 30 different assays lasting five hours; after use, the bound GDH is rinsed and kept at 4° as mentioned in Materials and methods, ready for other experiments.

Table 2 shows the influence of the covalent binding on the GDH parameters towards glutamate oxidation for a constant concentration of NAD. The apparent K_m is increased 6-fold by the binding. The apparent maximum velocity of bound GDH cannot be compared to that of the free enzyme owing to the great difficulties to determine small concentrations of GDH fixed on a relatively big matrix of collagen. The Hill number (fig. 1) for glutamate is 1 in the free enzyme and becomes close to 0.5 after the binding; ADP is and 'uncompetitive' activator for the free GDH towards glutamate oxidation: the apparent V_m is increased 1.8-fold and glutamate affinity is decreased 2-fold. With the bound GDH, ADP seems unable to affect the affinity for glutamate but still increases the reaction rate about 3-fold.

Fig. 2 shows that the double reciprocal plot linearity of v^{-1} versus [glutamate] $^{-1}$ is lost when GDH is bound, whatever the NAD concentration is.

In fig. 3, on the contrary, we see that the variations of v^{-1} versus [glutamate] $^{-0.5}$ are perfectly linear as tested by the regression procedure. This is in agreement with the fact that the Hill number for glutamate varies from 1 in the free enzyme to 0.5 in the bound GDH. The apparent K_m calculated from this plot is the same as that calculated from the Hill plot vertical intercept (fig. 1).

Fig. 4 shows the pH dependence of GDH. We see that the binding does not affect the optimal pH value of 8.15 ± 0.05, but the pH curve is sharpened indicating greater dependence of catalytic properties towards pH.

4. Conclusions

One can suggest that during the coupling, the azide groups formed on the aspartyl and glutamyl residues of the tanned collagen matrix [4] react with GDH amino groups, among them, with some specially reactive lysine [5] necessary for GDH activity.

The fact that the regulatory properties of GDH have been preserved after coupling on the collagen matrix, suggests that in the matrix the enzyme still possesses an oligomeric structure.

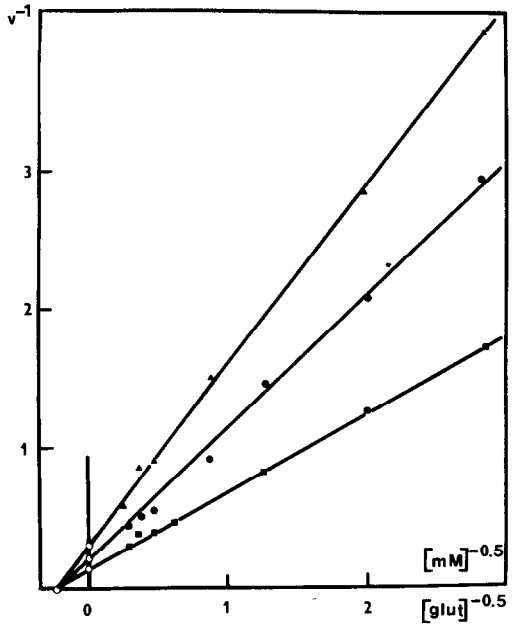

Fig. 3. Plot of v^{-1} versus [glutamate] $^{-0.5}$ for three different preparations. Experimental conditions as in fig. 1. v^{-1} in nmole^{-1} of NADH × ml × min.

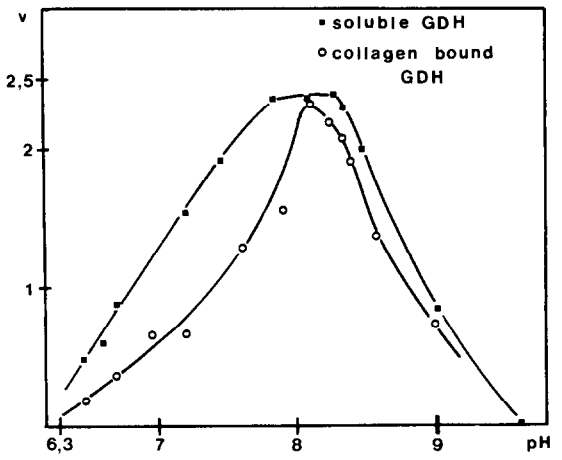

Fig. 4. Optimal pH. Experimental conditions as in fig. 1 except for 18.75 mM Glu and pH is fitted on the complete medium before addition of GDH.

As a general conclusion, this collagen covalently bound GDH seems a good model to study the behaviour of GDH integrated in a membrane and its regulation.

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