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BINDING OF L-GLUTAMATE TO GLUTAMATE DEHYDROGENASE IN THE PRESENCE OF 1,4,5,6-TETRAHYDRONICOTINAMIDE ADENINE DINUCLEOTIDE

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1. Introduction

In a previous work with Julliard, a lipid-protein complex showing a high affinity for glutamate was extracted from pig heart mitochondrial membranes; it exhibited many properties expected from a glutamate translocator [1]. From its protein composition it was suggested that enzymes implied in glutamate metabolism could play a role as glutamate binding unit (aspartate aminotransferase, glutamate dehydrogenase) [1,2]. However the reported affinities of these enzymes for glutamate are 1–3 orders of magnitude lower than the affinity of the isolated lipid-protein complex ($K_d = 62 \mu\text{M}$). If glutamate dehydrogenase[†] was the glutamate binding unit of this lipid-protein complex, it would mean that its glutamate affinity would be increased in such a complex. The purpose of this work was to find a model that could increase glutamate affinity of GDH.

GDH affinity for L-glutamate is weak but increases in the presence of NAD(P)(H) [3]. In absence of coenzyme, it is difficult to measure directly the dis-

sociation constant of glutamate and the number of binding sites. However a GDH-L-glutamate complex has been indirectly demonstrated by glutamate protection from urea denaturation [4] and from *p*-chloromercuribenzoate binding [5]; the existence of such a complex has been either postulated in transient-state kinetic studies [6,7] or directly demonstrated by spectrophotometric measurements [8]. Pantaloni and Lécuyer measured one specific binding site for L-glutamate on each protomer (mol. wt = 56 000) in presence of NAD(P)H by differential spectroscopy, optical rotatory dispersion and circular dichroism studies [9].

This paper presents the behaviour of GDH in presence of an analogue of NAD(H), namely the 1,4,5,6-tetrahydro NAD ($\text{H}_4\text{-NAD}$). It is shown here that $\text{H}_4\text{-NAD}$ was not coenzyme of the reaction and behaved mainly as a competitive inhibitor of NAD^+ and a non-competitive inhibitor of L-glutamate. It strongly increased GDH affinity for L-glutamate. Equilibrium dialysis experiments achieved in presence of $\text{H}_4\text{-NAD}$ showed that besides the low affinity binding sites for L-glutamate (6 per hexamer) there exists a high affinity binding site (1 per hexamer).

2. Materials and methods

Beef liver GDH, L-glutamate: NAD(P)⁺ oxido-

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† *Abreviation*; GDH, glutamate dehydrogenase.

reductase (deaminating) (EC 1.4.1.3.) was purchased from Boehringer, as, either a crystalline ammonium sulfate suspension (20 mg/ml), or a glycerol solution (10 mg/ml). The former suspension was used for activity measurements whereas the latter was used for binding measurements. In both cases the enzyme was used without purification. However, by sodium dodecyl sulfate gel electrophoresis [10] no contaminating protein could be detected in all samples used.

The pH values of all solutions were determined with a Methrom E 300 B pH-meter. The buffer used in the activity and binding studies was 0.067 M phosphate (K) at pH 7.60. NAD^+ was purchased from Boehringer (Grade I), L-glutamic acid from Calbiochem (Grade A) and L- ^{14}C glutamin acid uniformly labelled (156 mCi/mM) from the French C.E.A. - Depart. of Biology. Its radiochemical purity was checked by paper chromatography developed in butanol-formic acid-water (75:15:10). $\text{H}_4\text{-NAD}$ was prepared by catalytic reduction of NADH [11,12]. This compound absorbs at 288 and 265 nm and the ratio A_{288}/A_{265} was used to check its purity.

Activity studies were carried out on a Beckman 25 spectrophotometer, following the NADH absorbance at 340 nm in a 1 cm path length cuvette. The final enzyme concentration after mixing was 2.0, 4.0 or 8.0 $\mu\text{g/ml}$.

Binding studies were carried out by equilibrium dialysis. Eleven dialysis cells were constructed in teflon. Each cell contained two compartments (diameter = 2.0 cm, thickness = 0.3 cm) filled with 0.7 ml each and separated by a semi-permeable dialysis membrane. The membrane was prepared from Visking tubing (A. H. Thomas, diameter = 2.6 cm) which had been successively boiled in 2 large vol of 5% Na_2CO_3 -5% EDTA, rinsed with distilled water and kept at 4°C in 50% ethanol solution. Membranes were rinsed with distilled water and dried with filter paper just before use. The experiment was initiated by adding to the 'inside' cell compartment 0.7 ml of a mixture of 300 μl of enzyme solution (10 mg/ml), 500 μl of both $\text{H}_4\text{-NAD}$ and L- ^{14}C glutamate (9 μM) and 100 μl of L-glutamate (0 to 1 M). The 'outside' cell compartment was filled up by 0.7 ml of a similar mixture in which enzyme was replaced by 300 μl of a glycerol 50% solution. Final buffer concentration was 0.067 M phosphate (K) in both compartments. Cells were closed by teflon tubes and incubated for 3-4 hr at

19-20°C while being rotated at 13 rev/min around the axis of the cell.

In these conditions it was checked that equilibrium was reached after 2 hr. After dialysis, all half-cells were emptied with a syringe; 150 μl aliquots were used for radioactivity determinations; 20 μl aliquots were diluted into 2 ml of buffer, and GDH activity, $\text{H}_4\text{-NAD}$ concentration were determined on aliquots of the latter dilutions. Enzymic activity was not significantly modified during dialysis. This was also the case for $\text{H}_4\text{-NAD}$ spectrum, since its A_{288}/A_{265} ratio indicated that there was no significant splitting of this compound into ADP-ribose.

Radioactivity determinations were made in either 10 ml Instagel Packard plus 0.8 ml distilled water or 10 ml Bray solution (1250 ml toluene, 750 ml ethanol, 5.02 g 2,5-diphenyloxazole Packard scintillation grade and 126 mg 1,4-bis-2 (4-methyl-5-phenyloxazolyl)-benzene, Packard scintillation grade). Countings were performed with a liquid scintillation spectrometer ABAC SL 40, associated with a Teletype. The concentration of free L-glutamate (X_f) was calculated from the radioactivity in the 'outside' compartment. The concentration of bound L-glutamate (X_b) was calculated from the difference in radioactivity between the two compartments. The average number of ligand molecules bound per molecule of enzyme (X_b/E_t) was calculated from the concentration of bound ligand and the input GDH concentration.

3. Results

3.1. Effect of $\text{H}_4\text{-NAD}$ on GDH activity

$\text{H}_4\text{-NAD}$ as shown for alcohol dehydrogenase [11] could not serve as coenzyme. It inhibits NAD^+ reduction by L-glutamate in presence of GDH. Double reciprocal plots obtained with various NAD^+ and $\text{H}_4\text{-NAD}$ concentrations (fig.1) in the presence of 19.5 mM L-glutamate give a maximum rate of 5 $\mu\text{mol per min per mg protein}$, independent of $\text{H}_4\text{-NAD}$ concentration. With NAD^+ concentration higher than 0.1 mM, $\text{H}_4\text{-NAD}$ behaves as a competitive inhibitor; when lower NAD^+ concentrations are also considered, the Lineweaver-Burk plots are not linear, as previously reported [3,13] and $\text{H}_4\text{-NAD}$ is then a partially competitive inhibitor; the Dixon's plot of the same data (fig.2), where the 0.02 mM NAD^+ line is out of

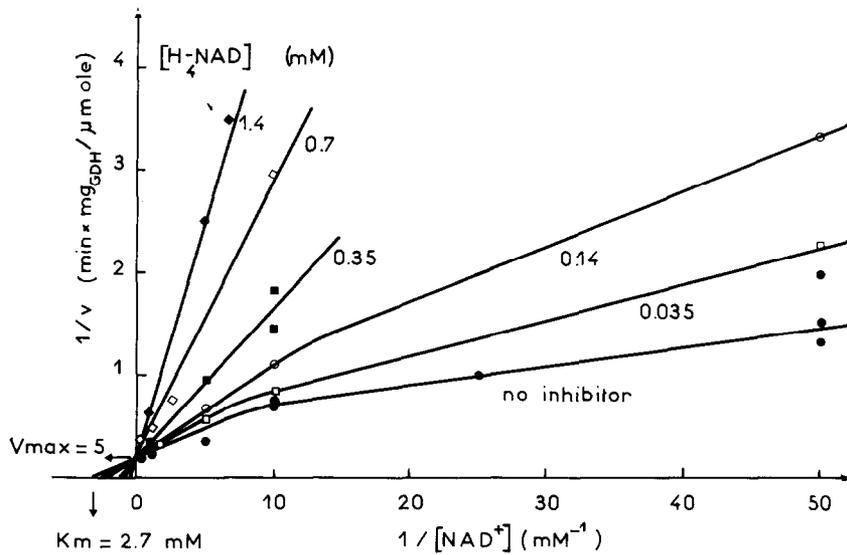


Fig.1. Lineweaver and Burk's representation of inhibition by H_4 -NAD of the reduction of NAD^+ with L-glutamate in presence of GDH. Reaction mixture: 0.067 M phosphate (K), pH 7.6, 19.4 mM L-glutamate and enzyme 4 μ g/ml, at room temperature.

phase with the others shows more clearly this partial competitive inhibition. The K_i was 0.12 ± 0.03 mM whereas K_M of NAD^+ equals 2.7 mM in the same conditions. H_4 -NAD is a non-competitive inhibitor of L-glutamate: in presence of 0.2 mM NAD^+ , K_i

(H_4 -NAD) determined on the Dixon's plot (fig.3) is independent of L-glutamate concentration and equals 0.35 mM whereas, K_M of L-glutamate determined on the corresponding Lineweaver and Burk's plot was 1.5 mM. Intrinsic value of K_i depen-

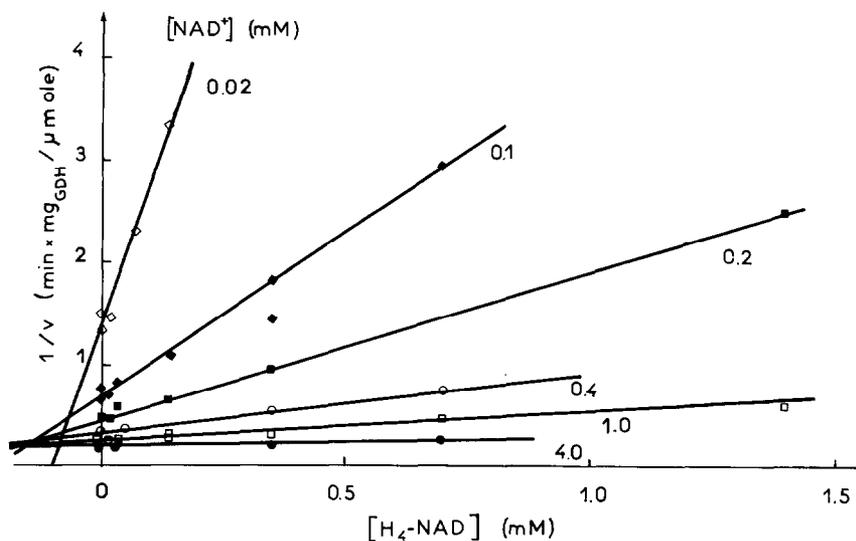


Fig.2. Dixon's representation of inhibition by H_4 -NAD of the reduction of NAD^+ with L-glutamate in presence of GDH. Reaction mixture: 0.067 M phosphate (K), pH 7.6, 19.4 mM L-glutamate and enzyme 4 μ g/ml, at room temperature.

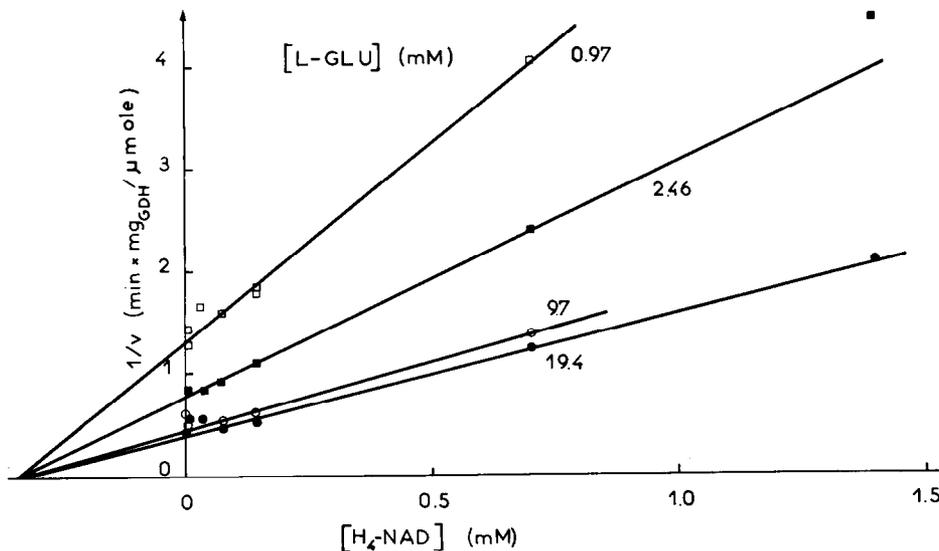


Fig.3. Inhibition by H₄-NAD of the oxidation of L-glutamate with NAD⁺ in presence of GDH (Dixon's plot). Reaction mixture: 0.067 M phosphate (K), pH 7.6, 0.2 mM NAD⁺ and enzyme 4 μg/ml, at room temperature.

ded on respective NAD⁺ and L-glutamate concentrations.

3.2. Binding of L-glutamate on GDH in presence of H₄-NAD

In absence of NAD(P)(H) the affinity of GDH for L-glutamate is so weak that equilibrium dialysis experiments gave no reliable data. H₄-NAD significantly enhances association between GDH and L-glutamate and allows a direct determination of the number of binding sites and the dissociation constant of the GDH-L-glutamate complex. Four series of independent experiments were conducted in the presence of L-glutamate in the range 6 μM–11 mM. Fig.4 shows that a biphasic Scatchard plot is obtained. Two different dissociation constants of L-glutamate can be determined: low affinity sites of $K_{d1} = 1.6$ mM and a high affinity site of $K_{d2} = 90 \pm 10$ μM. Increasing the H₄-NAD concentration from 0.7 to 2.1 mM did not change significantly these values.

Despite the low accuracy of such experiments we can estimate the number of L-glutamate binding sites in these conditions (fig.4): about 6 low affinity sites and about 1 high affinity site per unit of active enzyme (336 000 daltons).

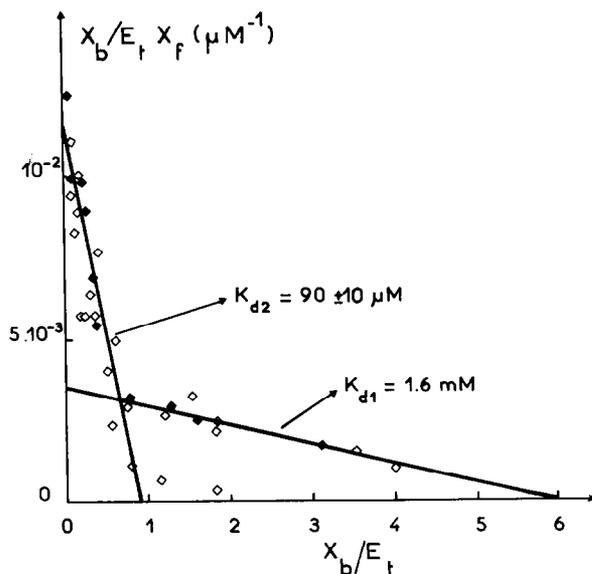


Fig.4. Binding of L-glutamate by GDH in presence of H₄-NAD. Scatchard plot. Reaction mixture: 0.067 M phosphate (K) pH 7.6, (◇) 0.7 and (●) 2.1 mM H₄-NAD, enzyme: 3.3 mg/ml (2 different samples). Temperature 19–20°C. L-[¹⁴C]glutamate 6 μM (156 mCi/mM), L-glutamate 0 to 11 mM. 4 independent series of determinations.

Table 1.
Comparison of the published and obtained K_d values for GDH-L-glutamate complex.

Number of L-glutamate binding sites per hexamer (336 000 daltons)	K_d (mM)	Method	Effector (mM)	[GDH] (mg/ml)	Buffer (M)	pH	t °C	Authors	Ref.
>100		A		0.17	0.1 Tris-HCl	7.5	22	Di Franco	[4]
7.5		B		3.3	0.1 phosph.	7.3	15	Cosson	[5]
1.5		C		1.0	0.1 Tris-acetate	7.5	25	Di Franco, Iwatsubo	[6]
36 ± 10		D		1.0	0.1 phosph. K	7.6	25	Colen, Prough, Fisher	[7]
46 ± 10		D		1.0	0.2 phosph. K	6.5	25	Colen, Prough, Fisher	[7]
47 ± 5		E		0.2-1.6	0.1 phosph. K	7.6	20	Prough, Colen, Fisher	[8]
38 ± 8		E		0.2-1.6	0.1 phosph. K	7.6	25	Prough, Colen, Fisher	[8]
7.3		F		5	0.1 Tris-HCl	7.3	20	Pantaloni, Lécuyer	[9]
1.8		F	0.3 (NADH)	5	0.1 Tris-HCl	7.3	20	Pantaloni, Lécuyer	[9]
4.2		F		5	0.1 Tris-HCl	7.3	20	Pantaloni, Lécuyer	[9]
0.75		F	0.37 (NADPH)	5	0.1 Tris-HCl	7.3	20	Pantaloni, Lécuyer	[9]
6 ± 2	1.6	G	0.7-2.1 H ₄ NAD	3.3	0.067 phos. K	7.6	19-20	This paper	
0.9 ± 0.2	0.09 ± 0.01	G	0.7-2.1 H ₄ NAD	3.3	0.067 phos. K	7.6	19-20	This paper	

Methods used:

- A glutamate protection from urea denaturation
- B glutamate protection from *p*-chloromercuribenzoate fixation
- C thermodynamic calculations from transient-state kinetic studies
- D transient-state kinetic studies: analysis of the initial slopes at 340 nm
- E direct binding measurements by ultraviolet differential spectrophotometry (A_{283} , A_{287})
- F thermodynamic calculations from equilibrium difference spectroscopy, optical rotatory dispersion and circular dichroism studies
- G direct binding measurements by equilibrium dialysis in presence of H₄-NAD 0.7 and 2.1 mM;

4. Discussion

As expected, H₄-NAD was a non-competitive inhibitor of glutamate with GDH, and mainly a competitive inhibitor towards NAD⁺; this partial competition, especially at low NAD⁺ concentration, might be related to the fact that NAD⁺ behaves simultaneously as substrate and effector [3,13] and could indicate a positive co-operativity of H₄-NAD antagonistic to the negative co-operativity induced by NAD⁺ [13]. We do not know as yet, if H₄-NAD binds as NAD(H) to 'active or non-active sites' [14] of glutamate dehydrogenase, but this was not the purpose of our work.

Table 1 compares the dissociation constant obtained for the complex L-glutamate-GDH in the presence of H₄-NAD to other previously reported values. All K_d values calculated in absence of coenzyme by indirect methods [4–7,9] or determined by uv differential spectroscopy [8] ranged between 1.5–100 mM; in presence of NAD(P)H, calculated K_d values reached 0.75–1.8 mM [9].

Our direct binding measurements with radioactive glutamate in the presence of H₄-NAD revealed 1 site/GDH monomer, mol. wt 56 000, with a K_d = 1.6 mM, very comparable to that reported by Di Franco and Iwatsubo [6] and by Pantaloni and Lécuyer [9]. Unexpectedly also, H₄-NAD revealed one high affinity site for glutamate per GDH hexamer (Mol. wt 336 000), of K_d = 90 ± 10 μM. These experiments do not prove that this unexpected high affinity site is of some significance in the catalytic properties of GDH; this point remains hypothetical. However it should be recalled that, up to now, the smallest reported active unit of mammalian GDH was the hexamer; this high affinity glutamate binding site, present only in the hexamer and revealed in the presence of H₄-NAD, could be related to the enzymatic activity of the hexamer by inducing a favourable, but minor or very localized conformational change. Then we cannot exclude that this high affinity site also exists in the presence of the usual coenzyme and could have escaped to other investigations because this high affinity glutamate binding may not modify the chemical and physical enzyme characteristics previously studied [4–9].

The K_d of this unique high affinity site 90 ± 10 μM is similar to that measured for the glutamate-binding

lipid-protein complex isolated from pig heart mitochondria, 62 μM [1,2] and also to the K_M of entry of glutamate into pig heart mitochondria 76 ± 15 μM [1]. These results do not prove that GDH is the binding unit of glutamate in the lipid-protein complex (HGAP) and that it could play a significant role in glutamate translocation in mitochondria. However they show that GDH affinity for glutamate can be strongly increased, and this is compatible with our hypothesis [1,2].

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