# EVIDENCE FOR TWO NICOTINAMIDE BINDING SITES ON L-GLUTAMATE DEHYDROGENASE

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SUMMARY

Circular dichroism saturation in the nicotinamide band of NADH, provides direct evidence for the binding of two nicotinamide rings per protomer of L-glutamate dehydrogenase. These two binding sites are titrated by NADH in the presence of both the substrate (L-glutamate) and an allosteric effector (GTP or $Zn^{2+}$) while only one reacts in the absence of the effector. We suggest that the second binding site, not accessible to NADPH, is demasked by a conformational change of the protein induced by the allosteric effector.

## INTRODUCTION

The number of coenzyme binding sites on L-glutamate dehydrogenase has been for a long time very controversial. Frieden (1,2), to interpret the steady state kinetic data, proposed the existence of two types of binding sites, one active and one regulatory. The active one appears accessible to both NAD and NADP, which is not the case for the regulatory one, since no activation at high concentration is detected for NADP. Moreover a competition on this site between ADP and NAD supported the idea that ADP could bind at the same place. Up until now, Frieden's hypothesis is in agreement with all the later studies even though no unequivocal demonstration has ever been given. Consequently the correlation of the effects of NAD(H) and NADP(H) can be considered as a test of the involvement of each site ; when positive, the active site only is implicated ; when negative, the regulatory site is also involved.

By observing the modifications provoked on the enzyme following the addition of both NADH (or NADPH) and GTP (3), two functionally different sites for the coenzyme were once more evident. One, accessible to both reduced coenzymes, thus the active site, provokes the inhibition and a rapid dissociation of the polyhexamer while the other one accessible only to NADH, thus the regulatory site, slows down this process. Moreover a slower modification of NADH environnement, but not of NADPH, is due to a conformational change which involves the regulatory site.

This two site hypothesis needed more quantitative support. Such experiments were carried out by Yielding and Holt (4) among others. By equilibrium dialysis, they found only one binding site for NADH. Pantaloni and Dessen (5) analyzed spectrophotometric saturation curves which are interpreted in terms of a total of two NADH sites per protomer, one binding through the adenine moiety, the other through the nicotinamide moiety.

Here we provide direct evidence of a binding through the nicotinamide moieties of one molecule of NADPH and two molecules of NADH per protomer. The site common to both coenzymes interacts with the substrate L-glutamate and the second site found for NADH interacts with the allosteric effectors such as GTP and $Zn^{2+}$. Since the effectors GTP and $Zn^{2+}$, while entirely different, produce the same effects on the catalytic activity and the quaternary structure of L-glutamate dehydrogenase (6,7), we have performed parallel experiments in circular dichroïsm on various enzyme complexes in order to obtain some insight on the type of ligand-ligand interactions.

## MATERIAL AND METHODS

The method of Kubo et al. (8) was used to prepare GDH from beef liver. The enzyme used for the experiments has been crystallized six times with $Na_2SO_4$ 5 % at pH 6.4. The protomer concentration (E) was determined from the extinction at 279 nm (E = 0.97 $cm^2/mg$). A value of 56,000 for the protomer molecular weight based on Smith's (9) later determination has been used. Nucleotides (NADH, NADPH, GTP) are purchased from Sigma. The complexes involving GTP were studied in 0.1 M Tris HCl pH 7.5 and those with $ZnCl_2$ in 0.1 M phosphate buffer pH 7.5, in which the enzyme is more stable. Parallel studies of the complexes involving GTP in Tris-Cl and phosphate buffers did not show any difference. The circular dichroïsm measurements were performed on the dichrograph II Roussel-Jouan at room temperature. Ellipticities are expressed in units of degree x $cm^2$ / decimole.

## RESULTS AND DISCUSSION

The fact that NADH shows a very characteristic optical activity in various enzyme complexes in the presence of the substrate L-glutamate and/or the effector GTP has been shown in the laboratory of Pantaloni (10). Presented here are the circular dichroïc spectra of NADH in these same complexes, but in the presence of $Zn^{2+}$ (fig. 1). Our measurements with GTP confirm the data of Pantaloni et al (10). The divalent cation $Zn^{2+}$ affects similarly as does

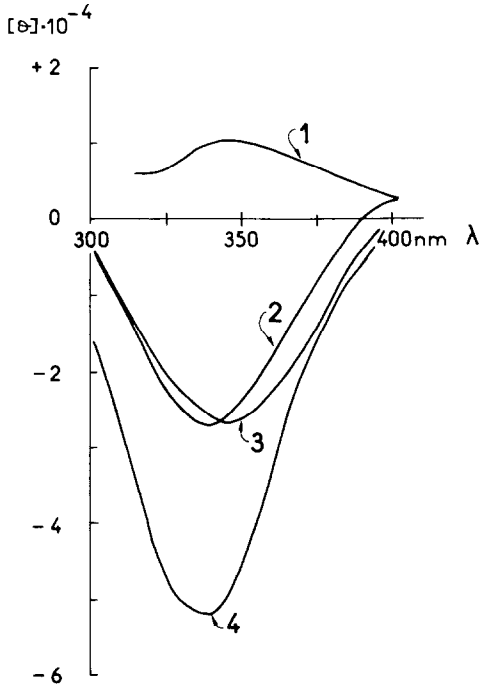


Fig. 1 : Circular dichroïc spectra of various enzyme - reduced coenzyme complexes
Curve 1 : E-NADH ; curve 2 : E-$Zn^{2+}$-NADH ; curve 3 : E-Lglutamate-NADH ; curve 4 : E-Lglutamate-$Zn^{2+}$-NADH.
Conditions : E 80 µM ; $ZnCl_2$ 140 µM ; L-glutamate 20 mM ; NADH 280 µM ; room temperature.

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GTP, the dichroïsm of the coenzyme in its reduced nicotinamide band. As it appeared that the substrate and the effectors induced specific modifications of the ellipticity, we have tried to correlate these changes with the existence of two distinct binding sites per protomer for the nicotinamide rings of NADH (only one for NADPH) and to titrate them.

First a study was carried out in the absence of the substrate. The variation of the ellipticity at 340 nm is shown in fig. 2 as a function of NADH concentration in the E-GTP-NADH and E-$Zn^{2+}$ -NADH complexes. These saturation curves pass through a maximum of small positive amplitude and then show a quasi hyperbolic decrease. The saturation levels, at high negative values, are slightly different ( ~ 10 %), which cannot be explained by the difference in the buffers used. It may be noticed that the positive increase in ellipticity in the first portion of the curve (curve 2) recalls the low positive ellipticity of the binary E-NADH complex (curve 1). This correlation suggests that the first effect, at low NADH concentration, is associated with one bound nicotinamide per protomer

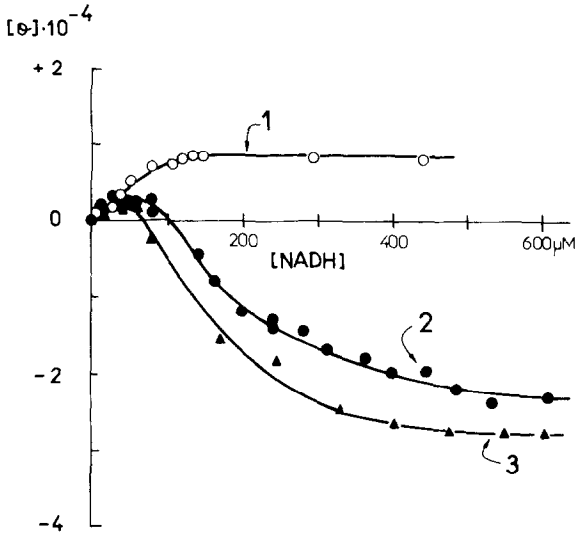


Fig. 2 : Variations of the ellipticity at 340 nm as a function of the NADH concentration in the following complexes.

Curve 1 : E-NADH ; curve 2 : E-GTP-NADH ; curve 3 : E-$Zn^{2+}$-NADH.
Conditions : E 80 μM ; GTP 470 μM ; $ZnCl_2$ 140 μM ; room temperature.

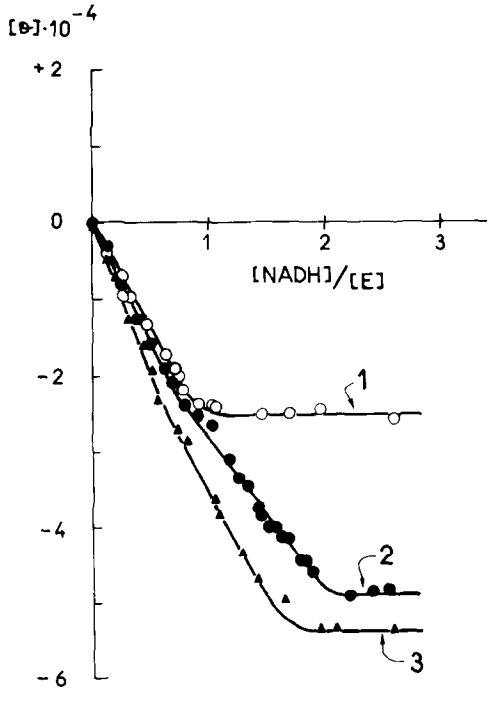


Fig. 3 : Variations of the ellipticity at 340 nm as a function of the NADH concentration per enzyme chain in the following complexes.

Curve 1 : E-Lglutamate-NADH ; curve 2 : E-Lglutamate-GTP-NADH;
curve 3 : E-Lglutamate-$Zn^{2+}$-NADH.
Conditions : E 78 μM ; L-glutamate 20 mM ; GTP 470 μM ; $ZnCl_2$ 140 μM ; room temperature.

with a weak positive circular dichroism and a higher affinity. The negative effect, which appears only when GTP or $Zn^{2+}$ is present, can be associated with a second bound nicotinamide of lower affinity. Our data are thus in agreement with the existence of 2 sites of different but low affinities. Therefore their respective titrations are not possible. From the saturation curve obtained one can roughly estimate a dissociation constant, $K_d$, of about 50 µM for the second site, whichever effector is used.

On the other hand, when the same study is carried out in the presence of L-glutamate, clearcut results are obtained concerning the existence of two distinct sites for the nicotinamide moiety of the reduced coenzyme, NADH. Indeed, titration curves are obtained as shown by the strictly linear relationship between the ellipticity and the amount of added coenzyme. One site, and only one, is titrated with NADH in the E-L-glutamate complex as observed by the variation of the ellipticity at 340 nm (fig. 3). In the presence of GTP or $Zn^{2+}$, two sites are titrated. In such titrations, the slopes measure the molar ellipticities which are very similar for the first and the second site, whichever effector is used. However one may notice not only a small change in slope, but a slight curvature towards the second equivalent point. This can be due to a weaker affinity for this site and/or a lower molar ellipticity.

When NADPH is used instead of NADH, the results indicate (fig. 4)

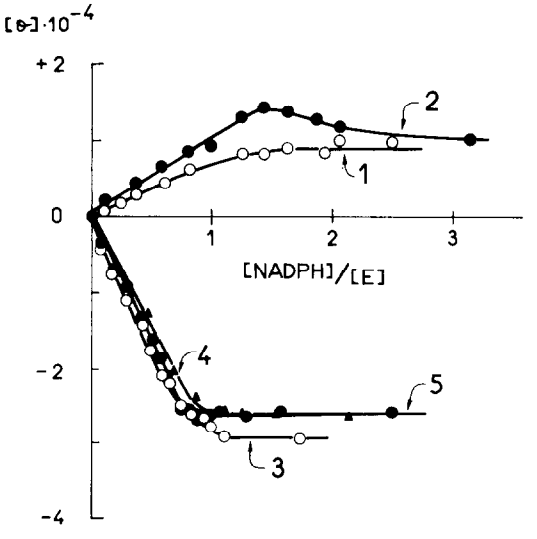


Fig. 4 : Variations of the ellipticity at 340 nm as a function of the NADPH concentration per enzyme chain in the following complexes.

Curve 1 : E-NADPH ; curve 2 : E-GTP-NADPH ; curve 3 : E-Lglutamate-NADPH ; curve 4 : E-Lglutamate-GTP-NADPH ; curve 5 : E-Lglutamate-$Zn^{2+}$-NADPH. Conditions : E 78 µM ; L-glutamate 20 mM ; GTP 470 µM ; $ZnCl_2$ 140 µM ; room temperature.

TABLE 1

Characteristics of the reduced coenzyme binding sites

| Complex | Molar ellipticity / site | | | Binding sites |
|---|---|---|---|---|
| | Total | $N_1$ (active) | $N_2$ (regulatory) | |
| | [degree.cm$^2$/decimole] x $10^4$ | | | n * |
| E - NADH | + 0.9 | + 0.9 | | |
| E - NADPH | + 1.0 | + 1.0 | | |
| E - Lglu - NADH | - 2.5 | - 2.5 | | 0.90 |
| E - Lglu - NADPH | - 2.6 | - 2.6 | | 0.92 |
| E - GTP - NADH | - 2.5 | + | - 2.5 *** | |
| E - $Zn^{2+}$ - NADH | - 2.8 | | - 2.8 *** | |
| E - GTP - NADPH | | + | - | |
| E - $Zn^{2+}$ - NADPH | | | | |
| E - Lglu - GTP - NADH | - 5.0 | - 2.5 ** | - 2.5 | 2.05 |
| E - Lglu - $Zn^{2+}$ - NADH | - 5.4 | - 2.8 ** | - 2.6 | 1.75 |
| E - Lglu - GTP - NADPH | - 2.6 | - 2.6 | | 0.92 |
| E - Lglu - $Zn^{2+}$ - NADPH | - 2.6 | - 2.6 | | 0.92 |

* Mean value from 3 titrationsexperiments ( ± 0.10).
** Estimated values at the slope change.
*** Underestimated because of the additive positive effect.

that about one site is titrated in the presence of L-glutamate, in the presence as well as in the absence of the effector. The difference observed in the maximum ellipticity in the presence of the effector (GTP or $Zn^{2+}$) is not clearly meaningful. Another difference between NADPH and NADH should be noticed on figure 4. As far as the ternary complex E-GTP-NADPH is concerned, the saturation curve shows increasing amplitudes followed by a very slow decrease. This could be interpreted as the binding of NADPH to the second regulatory site with a very poor affinity under our experimental conditions.

While previous papers reported the existence of two bound NADH per protomer with light experimental support (1,2,5), our data clearly and precisely demonstrate the existence of two distinct nicotinamide binding sites for NADH. The nicotinamide involvement is revealed by the modification of the ellipticity in the 340 nm band. Only one, at least with a substantial affinity, exists for NADPH. The results concerning the number of sites and their respective characteristics are summarized in Table I. It is interesting to compare the fact that both bound nicotinamides, at the active center as well as at the regulatory center, have similar molar ellipticities, with the fact that a certain number of identical residues are functionally involved in both sites (11).

The nicotinamide moiety of both NADH and NADPH bound at the active center has an ellipticity which is markedly affected by the presence of L-glutamate (inversion of the ellipticity). This reflects an interaction which may be direct as proposed by Fisher (12), although no experimental evidence is available. In the absence of an effector, GTP or $Zn^{2+}$, the binding of NADH to a regulatory site does not exist with a substantial affinity. Indeed the binding of a second NADH molecule with higher or similar affinity-through its adenine moiety, or through its nicotinamide moiety but without a change in molar ellipticity- would deplete the free NADH concentration such that a titration at one equivalent of the protomer concentration could not be obtained.

In the presence of an allosteric effector there is evidence for a second nicotinamide binding site which exists in the absence of L-glutamate but which can be titrated only in its presence (in addition to the active site). This regulatory nicotinamide binding site seems to be induced by the allosteric effector. This interaction is likely an indirect one, as exactly the same dichroïc effect occurs with both the anionic nucleotide GTP and the divalent cation $Zn^{2+}$. Our interpretation is that the effector provokes a discrete conformational change of the protein demasking a second nicotinamide binding site, which is probably the slow process that we observed previously with GTP and $Zn^{2+}$ (3, 13).

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