ADENINE BINDING TO GLUTAMATE DEHYDROGENASE: NATURAL AND MAGNETIC CIRCULAR DICHROISM STUDIES

J.M. JALLON and Y. RISLER
Centre de Génétique Moléculaire du C.N.R.S., F-91190-Gif-sur-Yvette, France

and

C. SCHNEIDER and J.M. THIERY
Groupe de Biophysique Moléculaire, C.E.N. Saclay, B.P. No. 2, F-91190-Gif-sur-Yvette, France

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1. Introduction

In the study of a nicotinamide adenine dinucleotide dependent dehydrogenase, one meets, among others, the following problems: are both aromatic rings of the nucleotide necessary for the binding to the enzyme and for the catalysis; which is their respective role; how are they interacting with the protein and with one another? In the case of lactate dehydrogenase, crystallographic data are available showing that both the adenine and nicotinamide parts are bound to the protein [1].

For glutamate dehydrogenase, only indirect information is deduced from kinetic studies and from studies of spectral perturbations associated with ligand binding. Kinetic studies have shown that apart from NAD(H)* and NADP(H), a certain number of analogues are active as coenzymes, although with different efficiencies [2]. For example, NMNH binds, but with a much higher Michaelis constant. ADP is an activator of the catalytic reaction as seems to be NAD at high concentrations, but not NADP [3]. To interpret these latter kinetic data, Frieden postulated that NAD may bind, on each protomer, to two different sites: the active site and the regulatory site [3].

Certain data [2, 4, 5] are available which shed light on the nature and specificity of these two sites. In particular, Cross and Fisher [2] have shown that ADP or NADH binding to GDH produces in the ultraviolet (250–300 nm) similar absorbance spectral modifications which they interpreted, despite their complexity, as perturbations of the adenine and aromatic protein chromophores, suggesting that adenine is implied in the NADH binding. The adenine perturbation is very weak with NADPH, while NADH and NADPH produce the same spectral effects at the level of the nicotinamide band (~ 340 nm) [2, 5]. On the other hand, circular dichroism studies have clearly shown that NADPH has only one and NADH two nicotinamide binding sites [6, 7].

It was thus tempting to study both the natural and the magnetic optical activity of the adenine chromophore in the different bound species in order to try to understand better the binding of the adenine part, and to clarify the involvement of protein aromatic residues.

* *Abbreviations:*
ADP: adenosine 5′-diphosphate, GDH: glutamate dehydrogenase, N: any one of the three nucleotides studied in this letter, NAD(H): nicotinamide-adenine dinucleotide, NADP(H): nicotinamide-adenine dinucleotide phosphate, NMN(H): nicotinamide mononucleotide; CD (resp., MCD): natural (resp., magnetic) circular dichroism, ΔA (resp., ΔA_M): natural (resp., magnetic) experimental circular dichroism (expressed as an absorbance difference), ΔE (resp., ΔE_M): natural (resp., magnetic) molar circular dichroism (expressed as a molar extinction coefficient difference), A_{279}: specific absorptivity at 279 nm.

2. Material and methods

GDH was prepared according to Kubo et al. [8]. Its concentration was measured spectrophotometrically on a Cary 15 (A_{279} = 0.97 cm^2mg^{-1}) using a value of 56,000 for the protomer molecular weight [9]. Solutions of the nucleotides (ADP, GTP, NADH, NADPH, (Sigma)) were freshly prepared before each experiment. All the optical measurements were carreid out in 0.1 M Tris-HCl buffer, pH 7.5, containing 0.5 mM EDTA, at room temp. in a quartz cuvette of 1 mm optical pathlength.

All dichroic spectra were recorded on a modified Roussel Jouan CD II Dichrograph, equipped with a specially made superconducting coil giving a magnetic field equal to 6.7 Tesla [10]. Data were converted to digital form every 0.5 nm and then corrected with a Digital Equipment PDP 12 computer. In this paper, magnetic circular dichroism (MCD, ΔA_M, or $\Delta\epsilon_M$) always refers to the magnetically induced optical activity obtained after correction for the natural circular dichroism (CD, ΔA, or $\Delta\epsilon$). The respective contributions of the free added ligand and enzyme were subtracted from the experimental spectra of the enzyme–ligand complexes after the adequate dilution corrections. For the difference spectra, the signal to noise ratio was of the order of 2 for NADH and NADPH, and 4 for ADP. For this reason, and as the bands are rather large, the uncertainty on the maxima is around 2 nm.

3. Results

Fig. 1 shows an example of CD spectra of free and GDH-bound nucleotide (GDH-N), in the case of ADP. Fig. 2 shows the CD difference spectra between the GDH-N complexes and their free constituents, calculated from spectra similar to those presented in fig. 1, for ADP, NADH, and NADPH, respectively. From the difference spectra, it is apparent that there is a broad negative minimum for ADP and a positive maximum for NADPH, both centered around 262 nm. Moreover these spectra show a broad shoulder or a trough of small intensity around 285 nm; for NADH, the observed effect seems more complex: a trough centered around 270 nm with several marked irregularities (fig. 2).

The spectral perturbations visible around 285 nm

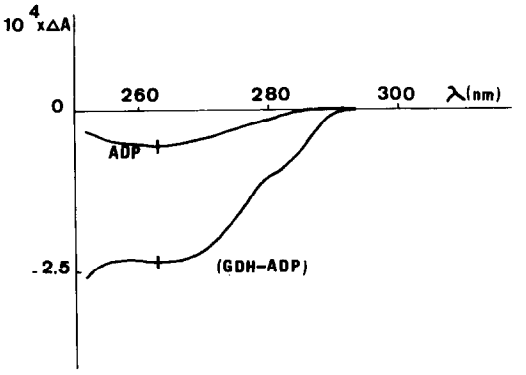

Fig. 1. CD spectra of free and GDH bound ADP (ΔA). Conditions: GDH: 79 μM; ADP: 340 μM; 0.1 M Tris-HCl buffer, pH 7.5, containing 0.5 mM EDTA; room temp.; 1 mm optical pathlength. In all figures the noise level is represented by vertical bars.

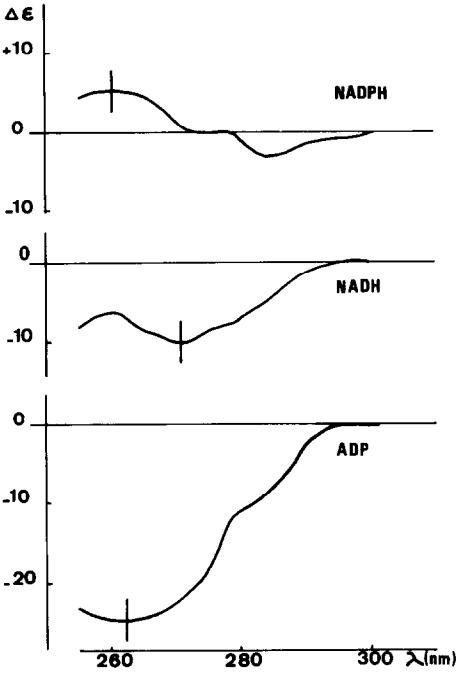

Fig. 2. CD difference spectra of GDH bound nucleotides: Δε (GDH + nucleotide) − (Δε (GDH) + Δε (nucleotide)). For the Δε calculation, the complex concentration is supposed to be equal to the enzyme concentration, the ligand concentration being saturating. Conditions: GDH: 79 μM; ADP: 30 μM; NADH: 425 μM; NADPH: 417 μM; 0.1 M Tris-HCl buffer, pH 7.5, containing 0.5 mM EDTA; room temp.; 1 mm optical pathlength.

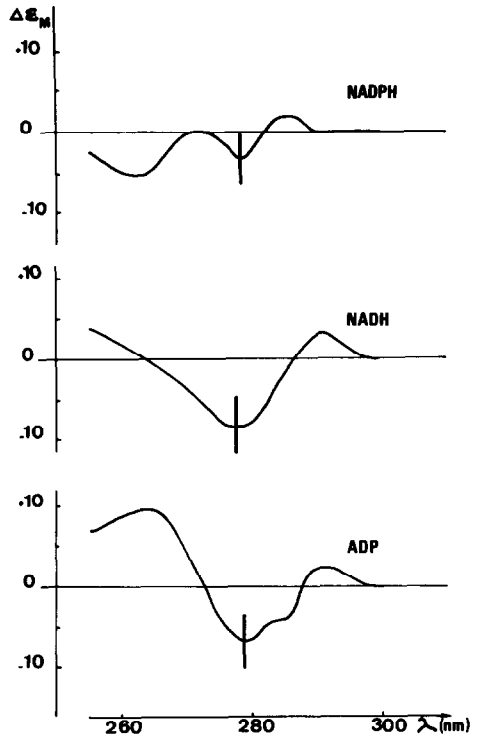

Fig. 3. MCD difference spectra of GDH bound nucleotides: $\Delta\epsilon_M$ (GDH + nucleotide) − ($\Delta\epsilon_M$ (GDH) + $\Delta\epsilon_M$ (nucleotide)). $\Delta\epsilon_M$ is reported for a 6.7 Tesla magnetic field. Same experimental conditions as in fig. 2.

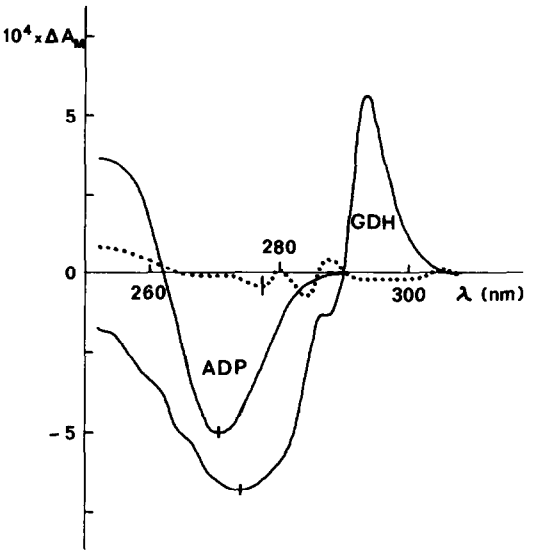

Fig. 4. MCD spectra of free ADP and GDH. ΔA_M is reported for a 6.7 Tesla magnetic field. The dotted line represents the deviation curve for the GDH MCD fitting, i.e. the difference spectrum between the GDH MCD and the sum of the tyrosine and tryptophan contributions, 4 nm shifted to longer wavelengths (see text). Below 260 nm, this deviation curve shows a non negligible positive peak which may be due to other chromophores (phenylalanines and cysteines) (see Discussion). Conditions: GDH: 79 μM; ADP: 340 μM; 0.1 M Tris-HCl buffer, pH 7.5 containing 0.5 mM EDTA; room temp.; 1 mm optical pathlength.

are clearly associated with perturbations of the GDH aromatic residues. At lower wavelengths, free ADP has a negative dichroism band centered around 262 nm (fig. 1). The observed difference spectrum for bound ADP has the same aspect, thus corresponds to an enhancement of the dichroic band intensity without any important shift of the extremum wavelength. For NADPH, the extremum wavelength seems also unchanged but the adenosine binding results in a decreased band intensity. In the case of NADH, the apparent shift of the maximum wavelength might be due to the superposition of two bands having similar intensities (centered around 262 and 285 nm, and associated with adenine and aromatic amino acid perturbations). However, one cannot exclude that it is more complex and results from two differently bound adenines, as will be discussed later.

Fig. 3 shows the MCD difference spectra for ADP, NADH and NADPH. All three spectra show peaks of small amplitudes around 285–290 nm, a trough centered at 278 nm and another extremum at a shorter wavelength; there ADP has a positive maximum and NADPH a negative minimum, both centered around 262–265 nm. For NADH the difference spectrum is again different and tends towards a positive maximum at a shorter wavelength.

The exact assignment is more difficult as the free constituents have complex MCD spectra in this spectral region, reflecting several well resolved transitions (fig. 4). The free nucleotide spectrum is in good agreement with those published for ADP derivatives [11] showing a trough at 271 nm and a peak at 252 nm, associated with the two transitions of the adenine moiety. Although tryptophan and tyrosine have different MCD spectra (roughly a S shape curve for tryptophan and a unique trough of small intensity for tyrosine). both spectra present a minimum at 266 and 276 nm, respectively [12].

The free protein spectrum results from the superposition of tyrosine and tryptophan contributions. The best reconstruction of the GDH spectrum from each aromatic amino acid contribution (MCD spectra of free tyrosines and tryptophans recorded at pH 8.3 [13]) is obtained with a 4 nm red shift of these contributions. With such a transformation, commonly used for absorption and MCD spectral analyses [12], we obtain by a least square fitting, a tyrosine to tryptophan molar ratio for GDH equal to 4.2, which is in good agreement with the experimental ratio equal to 4 [9]; the root mean square deviation (above 260 nm) is equal to 7% of the protein MCD spectrum.

The 285–290 nm peaks (fig. 3), where the signal to noise ratio is low, could only be associated with a tryptophan perturbation. An interpretation of the effects at shorter wavelengths is difficult. In the case of ADP, the cross-point is located at 272 nm. Moreover the MCD effects observed around 265 and 278 are of opposite sign and of comparable areas. The ADP difference spectra might result from the superposition of two S shaped curves centered at 272 and 288 nm which could be associated with two band shifts of opposite direction. An interpretation of the small MCD difference spectrum observed with NADPH is two band shifts towards lower wavelengths. The NADH MCD difference spectrum might reflect, as the CD one, two different adenine bindings. Thus our experimental data seem to show a concerted perturbation of adenine and at least one aromatic acid, probably a tryptophan. That only one adenine transition is affected might be due to a wrong orientation of the other one for an efficient interaction.

4. Discussion

The presented data show that upon binding to GDH i) one notices an increase of the adenosine CD contribution with either ADP or NADH, and a decrease with NADPH; ii) one also sees a change in the adenine MCD spectrum; iii) in CD as well as in MCD one observes a perturbation of the aromatic residues of the protein itself, probably a tryptophan (as suggested by MCD). Kägi et al. [14] have also observed an increased CD of ADP bound to phosphocreatine kinase; similar results were obtained by Girault et al. [15] for the interaction of ADP with the photosynthesis coupling factor. Kägi et al., using the oscillator coupling theory, have interpreted their CD data as due to the stacking of adenine with a protein tryptophan [14].

The essential difference between CD and MCD has been discussed many times [11]. CD is linked to an asymmetry of the electric charge distribution around the chromophore (a strong coupling with another oscillator might produce such an effect). In organic chromophores with no degenerate state, MCD is associated with couplings between electronic energy levels, proportional to the magnetic field and less sensitive to the asymmetry around the chromophore. Thus these two spectroscopic methods reflect different molecular parameters, and yet we observe parallel perturbations. The simplest interpretation would be a short distance, rigid and stereospecific interaction between the adenine of ADP, NADH, or NADPH and a a tryptophan residue of GDH.

Moreover this study has shown that the adenosine moiety of NADPH, although bearing an extra bulky phosphate group on the ribose, is able to bind rigidly to GDH. That this interaction is different from the one between ADP and GDH is obvious from the observed spectra. The CD and MCD difference spectra observed with NADH and ADP are also different. Our experimental data suggest that the effects observed with NADH might result from the superposition of two adenine perturbations taking place at two different sites, one similar to the one observed with NADPH (binding at the active site) and one similar to the one observed with ADP (binding at the regulatory site). Quantitative studies are needed to test this hypothesis which would imply the existence of two NADH binding sites per protomer, each consisting of two subsites, an adenine subsite and a nicotinamide subsite [6].

It should be noted that in the case of ADP these MCD difference spectra correspond to about 8% of the MCD effects observed for the ADP–GDH complex. The differential effect (local hypo or hyper-chromism) observed in absorption is of the same order [5]. This analogy between MCD and absorption has been theoretically predicted by R. Harris [16], but, as far as we know, is shown for the first time for the interaction of organic chromophores. Such effects should always be kept in mind when MCD is used as an analytical technique for the precise titration of chromophores. For example, it should be noted that the deviation curve obtained in the determination of the tyrosine to

tryptophan ratio may be partially explained by similar interactions (chromophore–chromophore interactions and chromophore–polarizable group interactions).

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