CIRCULAR DICHROISM STUDIES ON THE COMPLEX BETWEEN BEEF LIVER GLUTAMATE DEHYDROGENASE AND NADH*

R. KOBERSTEIN and H. SUND
Fachbereich Biologie der Universität Konstanz, BRD-775 Konstanz
P.O. Box 733, West-Germany

Received 24 September 1971

1. Introduction

Glutamate dehydrogenase (GluDH, EC 1.4.1.3) shows allosteric properties. The association-dissociation equilibrium as well as the kinetic properties are affected by a variety of small molecules [2, 3]. Among these the coenzyme and GTP are of particular interest because of their role in the regulation of the reaction catalyzed by GluDH. At high NADH concentration inhibition by the coenzyme occurs [4], leading to the assumption of a second, regulatory binding site for NADH within the polypeptide chain. From dissociation kinetics [5] and fluorescence titration [6] further indirect evidence for a second NADH binding site was obtained. GTP also inhibits the enzymatic activity and causes dissociation into the oligomer in the presence of NADH [5].

In the present paper circular dichroism (CD) measurements are reported which 1) give *direct* evidence for the existence of the second NADH binding site and 2) show that, using CD techniques, it is possible to visualize the binding of GTP and its influence on the different enzyme–NADH–substrate complexes.

2. Experimental

Beef liver GluDH, NADH, GTP, and ADP were purchased from C.F. Boehringer and Soehne GmbH (Mannheim, Germany). All other chemicals were of reagent grade from commercially available sources. The GluDH concentration was determined using the absorbance factor $A_{280} = 0.97$ [$cm^2\,mg^{-1}$]. The concentrations of GTP, ADP, and NADH were measured spectrophotometrically at 260 nm and 340 nm, respectively. CD measurements were performed in 0.67 M sodium phosphate buffer, pH 8.0, at $20 \pm 0.5^\circ$ using a Cary 60 spectropolarimeter equipped with a Cary 6002 CD accessory. For CD titrations μl amounts of concentrated solutions of the nucleotides were added to the protein, thus keeping changes in enzyme concentration negligibly small. Quartz cells (Hellma, Mühlheim, Germany) with 1 cm pathlength were used in all experiments.

3. Results and discussion

The CD spectrum of NADH changes to positive ellipticities upon binding to GluDH (fig. 1). A further enhancement of the NADH ellipticity accompanied by a blue shift of 10 nm is observed after addition of 2-oxoglutarate, probably due to a tighter fixation of the dihydronicotinamide moiety of the coenzyme in the ternary complex. Compared to this, the abortive ternary complex GluDH– NADH–*L*-glutamate exhibits an inversed NADH ellipticity with a red shift of the minimum to 345 nm. This inversion of the CD spectrum may be caused either by a close lying perturbing charge [7] (probably the $-NH_3^+$ group of the substrate molecule) or by a conformational change in the environment of the chromophore.

* Studies on Glutamate dehydrogenase, part VIII, for part VII see [1].

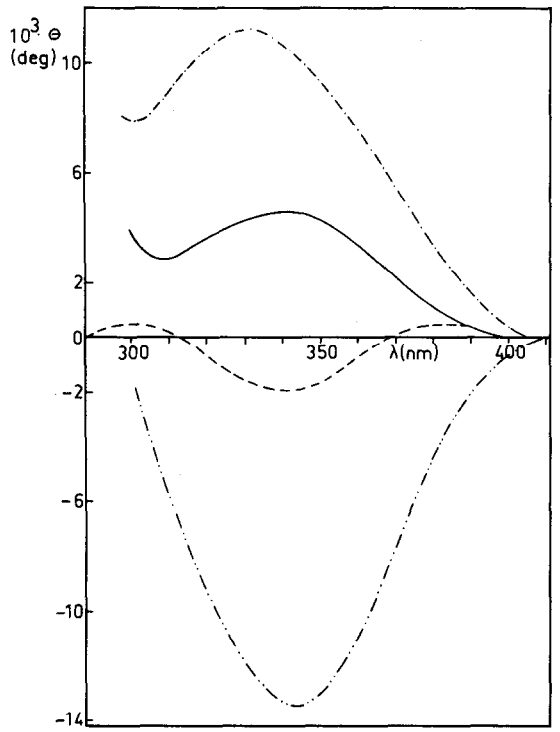

Fig. 1. CD spectra of GluDH–NADH–substrate complexes. – – – NADH, —— GluDH + NADH, — · — · — GluDH + NADH + 2-oxoglutarate, — · · — GluDH + NADH + *L*-glutamate. The concentrations were: for NADH 314 μM in the absence and 100 μM in the presence of GluDH, for GluDH 3.14 mg/ml, for *L*-glutamate and 2-oxoglutarate 10 mM. The ordinate represents measured ellipticities.

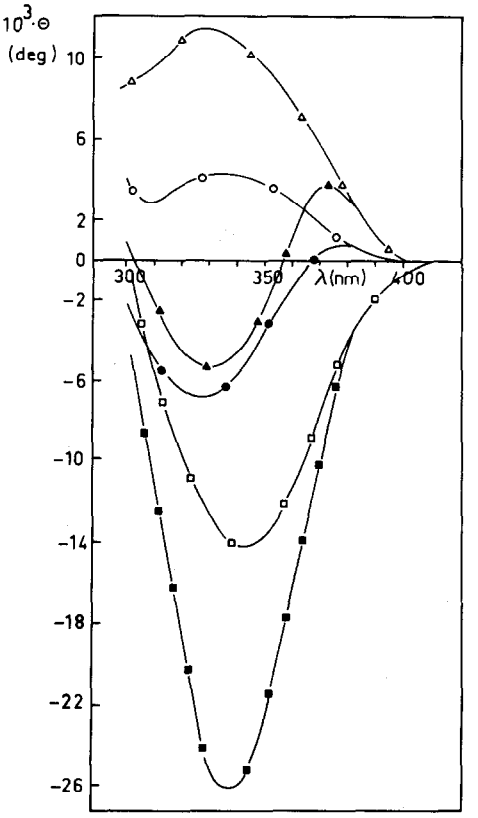

Fig. 2. CD spectra of GluDH–NADH–substrate complexes in the presence of 500 μM GTP. –○–○–, –●–●– GluDH + NADH + GTP, –△–△–, –▲–▲– GluDH + NADH + 2-oxoglutarate, –□–□–, –■–■– GluDH + NADH + *L*-glutamate + GTP. NADH concentrations: 60 μM (open symbols), 165 μM (–●–●–), 200 μM (–■–■), and 280 μM (–▲–▲–). The concentrations of GluDH, *L*-glutamate, and 2-oxoglutarate are the same as in fig. 1.

In the presence of GTP a complex situation is observed. At low NADH concentrations (open symbols in fig. 2) the CD spectra of NADH in the different complexes are quite the same as in the absence of GTP (fig. 1), whereas at high NADH concentrations the presence of GTP causes a strong shift of the NADH ellipticities to negative values in all complexes (filled symbols in fig. 2). The unaltered long wavelength part ($\lambda > 380$ nm) of these spectra indicates that the curves with filled symbols represent the original (open symbols) spectra *plus* an additional blueshifted negative CD spectrum of NADH.

This conclusion is clearly confirmed by CD titrations with NADH of GluDH (fig. 3) and GluDH-2-oxoglutarate (fig. 4) in the absence and presence of GTP. It is evident that, after saturation of the first NADH binding site (56 μM), in the presence of GTP a second binding site with a lower affinity for NADH appears, whereas in the absence of GTP normal titration curves are obtained. The slightly decreasing titration curve of the GluDH-2-oxoglutarate–NADH complex at high NADH concentrations (fig. 4) indicates very weak second NADH binding also in the absence of GTP.

The NADH molecule bound to the second site exhibits a strong negative CD band and thus causes the decrease of the titration curves at high NADH

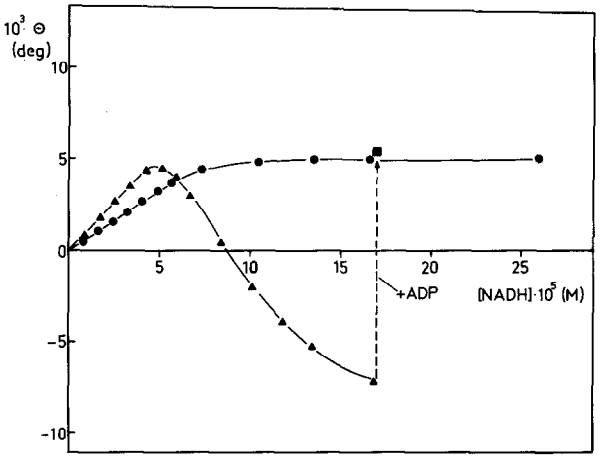

Fig. 3. CD titration of GluDH with NADH at 330 nm in the presence (–▲–▲–) and absence (–●–●–) of GTP (500 μM). The arrow indicates the effect of the addition of ADP (final concentration 1 mM) on the enzyme–NADH–GTP complex. GluDH concentration 3.14 mg/ml.

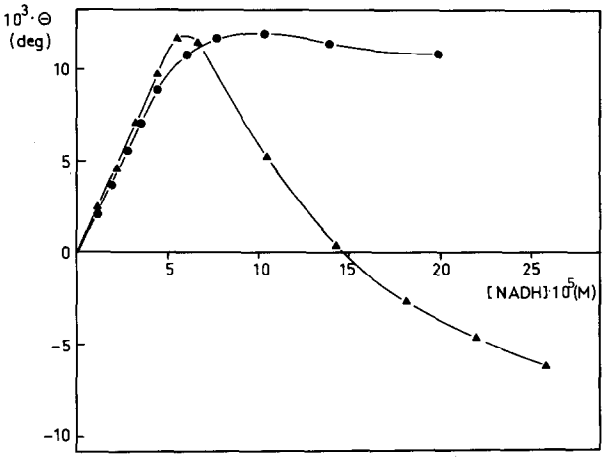

Fig. 4. CD titration of GluDH + 2-oxoglutarate with NADH at 335 nm in the presence (–▲–▲–) and absence (–●–●–) of GTP (500 μM). GluDH concentration 3.14 mg/ml, 2-oxoglutarate 10 mM.

levels in the presence of GTP. Because of the weaker binding of NADH to the second binding site the endpoint of the titration could not be determined so that dissociation constants cannot be given at the present stage. Titrations with NADPH instead of NADH give no indication for a second binding site for this coenzyme, indicating that the additional phosphate group in the NADPH molecule prevents the binding of this coenzyme to the second binding site.

As indicated by the arrow in fig. 3, the addition of ADP to the GluDH–NADH–GTP complex leads to the disappearance of the CD spectrum of the second NADH binding site.

In conclusion, the present study shows that 1) GluDH possesses – at least – two NADH binding sites, which are clearly discernible by CD measurements in the presence of GTP, and 2) that the dihydronicotinamide ring of NADH represents a highly sensitive chromophore which can be used as a "reporter" group for environmental changes in GluDH–coenzyme–substrate complexes.

Acknowledgements

We are grateful to Mr. H. Denzel for his technical assistance. This work was supported by research grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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