SPECTRAL PROPERTIES OF GLUTAMATE DEHYDROGENASE COMPLEXES INVOLVING SUBSTRATE ANALOGUES

J.M. JALLON and M. IWATSUBO
Centre de Génétique Moléculaire du C.N.R.S., F-91 190-Gif-sur-Yvette, France

Received 19 January 1973
Revised version received 29 March 1973

1. Introduction

Topological relations between the various active ligands, substrate, coenzyme and inhibitor on L-glutamate dehydrogenase are still not well defined, although such knowledge would obviously be useful when interpreting the complex kinetic behaviour of this enzyme and its subtile modulation by effectors. The problem of spatial orientation of these ligands is the object of much interest in Fisher's laboratory [1].

Early kinetic studies have shown that different dicarboxylic acids are good competitive inhibitors of L-glutamate [2, 3]. Particularly strong inhibitions have been observed for the reaction product 2-oxoglutarate, the substrate stereoisomer D-glutamate, glutarate and isophthalate. Noteworthy is the fact that all these dicarboxylic acids possess two carboxylates separated by the same distance [3]. Also these substrate analogues increase the affinity of the coenzyme for the enzyme [4, 5].

The purpose of this paper is to study the interactions of the different functional groups within the active site by comparing the optical properties of the reduced nicotinamide chromophore in different ternary complexes of the type enzyme–reduced coenzyme–substrate (or substrate analogue). Saturation conditions with respect to each ligand are chosen and the ternary complexes are formed by adding the substrate (or substrate analogue) to the preformed enzyme–reduced coenzyme complex. Here, we have extended studies which have already been partially described elsewhere [4–14].

2. Material and methods

L-glutamate dehydrogenase, (GDH), was prepared according to Kubo et al. [15]. Its concentration was measured spectrophotometrically on a Cary 15 (E_{279} = 0.97 $cm^2\,mg^{-1}$) using a value of 56,000 for the protomer molecular weight [16]. The nucleotides are from Sigma, and the substrates and substrate analogues from Merck.

The absorption difference spectra were obtained on a Cary 15 using tandem cuvettes; the circular dichroic spectra on a dichrograph Roussel Jouan; the fluorescence spectra on a fluorometer built by one of us (M.I) [17]. All measurements were performed in 0.1 M Tris-HCl buffer pH 7.5 containing 0.5 mM EDTA.

3. Results

3.1. *Comparison of the absorbance spectra*

Earlier studies have shown that, while 2-oxoglutarate provokes a blue-shift, L-glutamate as well as glutarate induces a red-shift of the absorption spectrum of free NADPH [6–8]. Presented in fig. 1 is the difference spectrum induced by D-glutamate, indicative of a red-shift, very similar to the one observed with L-glutamate or glutarate. It is interesting to note the dissymmetry of the spectrum. The exact cause of this effect is not clearly understood.

3.2. *Comparison of the circular dichroic spectra*

The inversion, induced by L-glutamate, of the

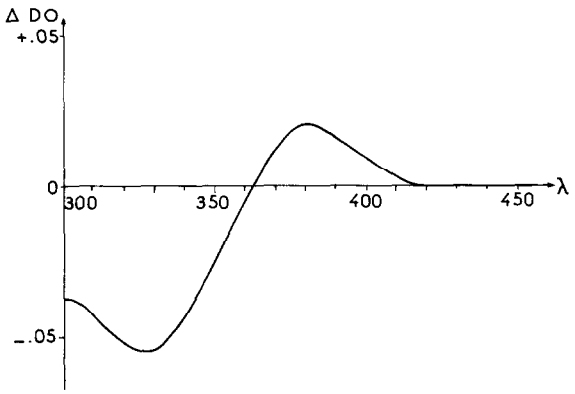

Fig. 1. Absorption difference spectrum GDH–NADPH–D-glutamate/GDH–NADPH+D-glutamate. Conditions: GDH: 86.5 μM; NADPH: 216 μM; D-glutamate: 45.5 mM; spectrum recorded at room temp. on a Cary 15, using tandem cuvettes of 0.85 cm optical pathlength.

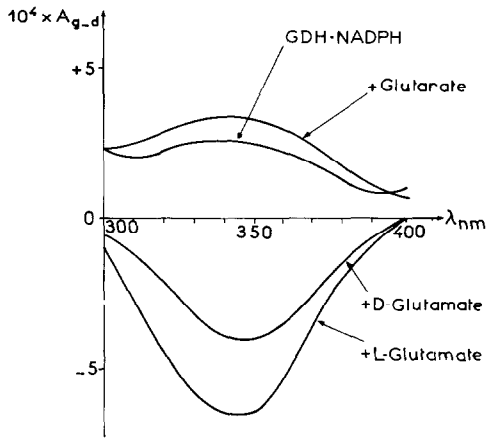

Fig. 2. Circular dichroic spectra of NADPH in various ternary complexes. The spectra are obtained by adding the substrate (or substrate analogue) to the binary GDH–NADPH complex. Conditions: GDH: 82 μM; NADPH: 200 μM; L-glutamate: 50 mM; D-glutamate: 49 mM; glutarate; 89 nM; 0.1 M Tris-HCl buffer containing 0.5 mM EDTA. Spectra recorded at room temp. using a cuvette of 1 cm optical pathlength.

circular dichroic band of NADPH bound to GDH has been shown by several authors [8–11].

As seen in fig. 2, D-glutamate provokes the same effect, but of weaker amplitude (~ 10%), as L-glutamate. Glutarate, on the other hand, provokes an effect similar to the one observed for 2-oxoglutarate, since its binding enhances the amplitude of the positive band observed for the binary E-NADPH-complex. Unlike 2-oxoglutarate however a red-shift is not observed [8, 11]. Isophthalate (89 mM) has no significant effect with respect to the binary complex. Negative results were also obtained with norvaline (50 mM). No detectable shift of the maximum wavelength can be noticed in the dichroic bands of the various spectra presented which are very broad (λ_{max} ~ 345 nm).

3.3. *Comparison of the NADPH fluorescence spectra*

When excited at 340 nm, the GDH bound NADPH fluorescence spectrum is shifted and enhanced by the presence of L-glutamate, an effect that has already been thoroughly investigated [12, 13]. A remarkable fact is that the substrate analogues used all decrease, more or less, the intensity observed for the binary E-NADPH complex (fig. 3). It is already known that 2-oxoglutarate almost completely quenches the fluorescence [8, 13, 14]. The quenching observed here is ~ 15% with glutarate and ~ 30% with D-glutamate and isophthalate. Moreover it appears that D-glutamate as well as isophthalate shift the fluorescence

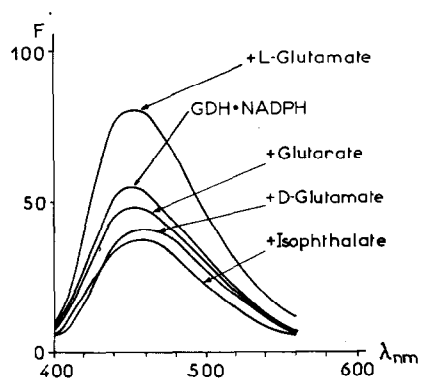

Fig. 3. Fluorescence emission spectra of NADPH in various ternary complexes. The fluorescence spectra are obtained by excitation at 350 nm. The fluorescence intensity is expressed in arbitrary units. The spectra are designated by the name of the second ligand (substrate or substrate analogue) added to the binary GDH–NADPH complex. The spectra have been corrected for the absorption due to both ligands. The free coenzyme has a maximum amplitude of 23 units. Conditions: GDH: 79.5 μM; NADPH: 107 μM; L-glutamate: 26 mM; D-glutamate: 25 mM; glutarate: 49.5 mM; isophthalate: 49.5 mM; 0.1 M Tris-HCl buffer containing 0.1 mM EDTA; t = 10°.

emission maximum towards a longer wavelength (~5 nm).

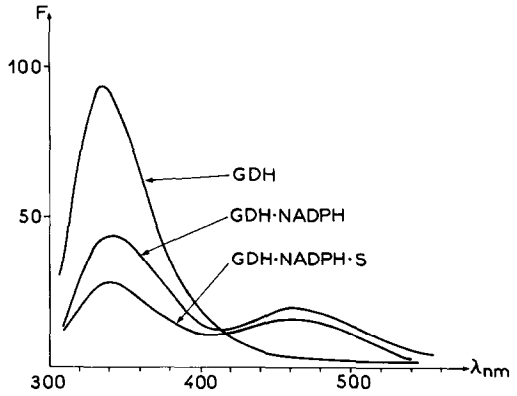

Fig. 4. Protein fluorescence emission spectra in two complexes GDH–NADPH–S (S = L- or D-glutamate). The fluorescence spectra are obtained by excitation at 295 nm. The spectra involving both stereoisomers of glutamate cannot be distinguished. The spectra have been corrected for the absorption of NADPH using a tryptophan solution showing the same fluorescence intensity. Conditions: GDH: 8.1 μM; NADPH: 47.5 μM; L-glutamate: 44 mM; D-glutamate: 22 mM; 0.1 M Tris-HCl buffer containing 0.5 mM EDTA; t = 10°.

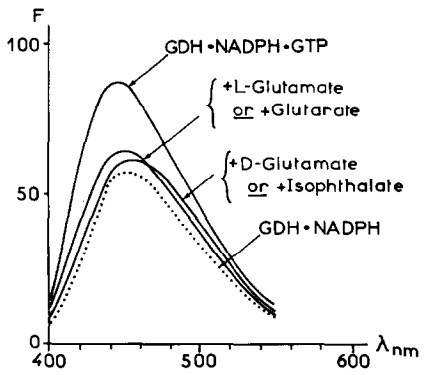

Fig. 5. Fluorescence emission spectra of NADPH in various quaternary complexes. The fluorescence spectra are obtained by excitation at 295 nm. The spectra are designated by the name of the third ligand (substrate or substrate analogue) added to the ternary E–NADPH–GTP complex. The spectra have been corrected for the absorption of the ligands. Conditions: GDH: 79.5 μM; NADPH: 107 μM; L-glutamate: 26 mM; D-glutamate: 25 mM; glutarate; 49.5 mM; isophthalate: 49.5 mM; GTP: 1 mM; 0.1 M Tris-HCl buffer containing 0.5 mM EDTA; 10°.

3.4. *The intrinsic protein fluorescence,* which is known to be quenched when NADPH is added [18], is quenched even further by the addition of either stereoisomer of glutamate. The fact that the effects produced are identical suggests that the protein–coenzyme interactions are very similar in both ternary complexes.

3.5. *Spectral properties of the quaternary complexes involving GTP*

The reduced nicotinamide ellipticity in the various quaternary complexes is slightly decreased in the presence of GTP, as has been shown for the E–NADPH–L-glutamate complex [10].

Fig. 5 compares the fluorescence emission spectra of the quaternary complexes involving GTP and each substrate analogue with both the binary E–NADPH and the ternary E–NADPH–GTP spectra. A similar blue-shift is also observed with isophthalate and D-glutamate as in the ternary complexes. Each quaternary complex is markedly different from the corresponding ternary complex in the absence of GTP (fig. 3). A decrease of the intensity is observed with L-glutamate while an increase is observed with the substrate analogues. However one does not observe any significant increase of fluorescence when one adds GTP to the E–NADPH–2-oxoglutarate complex.

4. Conclusions

We have presented above different optical properties of ternary complexes involving the enzyme GDH, its reduced coenzyme and a substrate or substrate analogue. Taking into account structural differences of each substrate or substrate analogue, our experimental data suggest that:

i) Only D- and L-glutamate provoke an inversion of the ellipticity of the bound reduced nicotinamide which is positive in all the other complexes. Thus the charge borne by NH_3^+ may be directly responsible for this inversion. It is known for many organic compounds that the vicinity of a charge can provoke an important modification of the ellipticity of a given chromophore [19]. The smaller effect observed with D-glutamate might be due to the difference in positioning of the charge in this stereoisomer. An alternative or complementary explanation is related to the fact that D- and L-glutamate are the only compounds with an asymmetric carbon which thus may alter the local symmetry.

ii) Among the substrate analogues studied, all dicarboxylic acids, except L-glutamate quench the bound coenzyme fluorescence. The maximum quenching is observed with 2-oxoglutarate, where a carbonyl group is conjugated with a carboxylate. There are many examples of organic molecules where charged groups like carboxylates, or polarizable groups like carbonyls provoke an intramolecular quenching of the fluorescence of a fluorophore [20]. Consequently a close contact between the substrate (or substrate analogue) and the reduced coenzyme in the GDH active site would provide a simple explanation for the very diverse and important spectral effects observed. It is probable that the protein is largely responsible for the establishment of this interaction. A direct interaction between substrate and coenzyme at the active site of dehydrogenases was proposed a number of years ago by Theorell and co-workers [21]. This hypothesis has long suffered from the popularity of allosterism but has been recently revived by Cross and Fisher studying L-glutamate dehydrogenase [1].

We have also reported the effect of GTP on the ternary complexes optical properties. The fluorescence data show that the quaternary complexes spectra are very different from the various ternary complexes. This fact may reflect the result of a conformational change induced by GTP or a competition between GTP and the substrate, as suggested by Fisher and Cross [1]. Preliminary binding data exclude the second hypothesis [22]. It is most probable that GTP induces a conformational change at the active site, a slow phenomenon the kinetics of which we have described recently [23].

Acknowledgements

Stimulating discussions with Drs. F. Labeyrie and A. di Franco are greatly acknowledged.

References

[1] D.G. Cross and H.F. Fisher, J. Biol. Chem. 245 (1970) 2612.
[2] M. Iwatsubo, M. Ishibashi, T. Takashina, K. Ibaraki and H. Kashida, Symposium Enzyme Chem., Japan 10 (1954) 135.
[3] W.S. Caughey, J.D. Smiley and L. Hellerman, J. Biol. Chem. 225 (1957) 163.
[4] M. Iwatsubo, B. Lecuyer, A. di Franco and D. Pantaloni, Compt. Rend. 263 (1966) 558.
[5] K. Dalziel and R.R. Egan, Biochem. J. 126 (1972) 875.
[6] R.R. Egan and K. Dalziel, Biochim. Biophys. Acta 250 (1971) 47.
[7] D.G. Cross, J. Biol. Chem. 247 (1972).
[8] A. di Franco and M. Iwatsubo, European J. Biochem. 30 (1972) 517.
[9] B. Lecuyer, Thèse de 3 ème Cycle, University of Paris Sud, France (1968).
[10] J.M. Jallon and M. Iwatsubo, Biochem. Biophys. Res. Commun. 45 (1971) 964.
[11] R. Koberstein and H. Sund, FEBS Letters 19 (1971) 149.
[12] A.D. Winer and G.W. Schwert, J. Biol. Chem. 231 (1958) 1065.
[13] H.F. Fisher, J. Biol. Chem. 235 (1960) 1830.
[14] J.R. Brocklehurst, G.H. Dodd, R.B. Freedman, A.D.B. Malcolm, N.C. Price and G.K. Radda, in: Pyridine nucleotide dependent dehydrogenases, ed. Sund (New York, 1970).
[15] H. Kubo, M. Iwatsubo, H. Watari and T. Soyama, J. Biochem. (Japan) 46 (1959) 1.
[16] E.L. Smith, M. Landon, D. Piszkiewicz, W.J. Bratten, T.J. Langley and M.D. Melamed, Proc. Natl. Acad. Sci. U.S. 67 (1970) 724.
[17] M. Iwatsubo and C. Capeillère, Biochim. Biophys. Acta 146 (1967) 349.
[18] A. Di Franco, Thèsis, University of Paris Sud, France (1971).
[19] W.B. Gratzer and D.A. Cowburn, Nature 222 (1969) 426.
[20] G.G. Guilbault, Fluorescence (Marcel Dekker, New York, 1967).
[21] H. Sund and H. Theorell, in: The enzymes, Vol. 7, eds. P.D. Boyer, H. Lardy and K. Myrback (New York, 1963) p. 25, and references therein.
[22] J.M. Jallon (1972) unpublished results.
[23] M. Iwatsubo, J.M. Jallon and A. di Franco, in: Dynamical aspects of conformation in biological macromolecules, ed. C. Sadron (1973) in press.