Conformational Changes and the Regulation of Glutamate-Dehydrogenase Activity

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1. The effect of NADH and the non-competitive inhibitor GTP on the optical-rotatory-dispersion properties of glutamate dehydrogenase has been studied. 2. Analysis of the data in terms of the a_0 and b_0 parameters of the Moffitt–Yang equation indicates that a conformational change is induced either by NADH or by GTP in the presence of small amounts of NADH. 3. Sedimentation measurements under comparable conditions showed that the enzyme reversibly dissociates into sub-units but that this dissociation is only secondary to the conformational changes. 4. Fluorescence measurements showed that the binding constant of NADH and the number of binding sites on the enzyme increased in the presence of GTP. 5. This is confirmed by studies of fluorescence polarization, which in addition showed that the movement of NADH on the enzyme surface is more restricted in the presence of GTP. 6. The relation of these results to possible regulatory mechanisms is discussed.

The activity of ox-liver glutamate dehydrogenase [L-glutamate–NADP oxidoreductase (deaminating), EC 1.4.1.3] is inhibited by NADH, GTP (Frieden, 1963) and a variety of other substances such as steroid hormones (Yielding & Tomkins, 1960) and phenanthroline (Yielding & Tomkins, 1962). The inhibition by GTP was shown to be of the non-competitive type (Frieden, 1963) and it was therefore suggested that this may be due to some conformational change in the protein. NADH has also been shown to be bound at sites other than the active centre with similar effects. Frieden argued (Frieden, 1963, and references therein), mainly from fluorescence measurements, that such conformational changes do occur, whereas Tomkins and his co-workers maintained that the changes in activity brought about by small molecules are associated with the dissociation of the enzyme into sub-units (Yielding, Tomkins, Bitensky & Talal, 1964). More recently Tomkins and co-workers have accepted the view that regulation of glutamate-dehydrogenase activity is achieved by inducing conformational changes in the enzyme and that dissociation is only a secondary consequence of these changes (Bitensky, Yielding & Tomkins, 1965*a,b*).

Information about the secondary and tertiary structure of proteins and polypeptides in solution may be gained from measurements of the wavelength-dependence of optical rotation (optical

rotatory dispersion) (Urnes & Doty, 1961). The present paper reports our optical-rotatory-dispersion studies on glutamate dehydrogenase in the absence and presence of coenzyme and the non-competitive inhibitor GTP, and their correlation with fluorescence, polarization of fluorescence and sedimentation studies. A brief account of this work has already appeared (Bayley & Radda, 1965). A similar paper dealing with the optical rotatory dispersion of this system has been published by Magar (1965).

MATERIALS

Glutamate dehydrogenase was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, as an ammonium sulphate suspension or a 50% glycerol solution. The latter was employed for most experiments, but there was no essential difference between the two preparations. The enzyme was freed from glycerol or ammonium sulphate by gel filtration on a Sephadex G-25 column at 0° by using the appropriate buffer solution. Experiments were carried out in 0.1M-phosphate buffer, pH 7.7, unless otherwise stated. The protein concentration after gel filtration was determined from the extinction at 280 m μ , by using the published value 0.97 for $E_{1\text{cm}}^{1\%}$ (Olson & Anfinsen, 1952). The homogeneity of fully active enzyme preparation from C. F. Boehringer und Soehne G.m.b.H. has been demonstrated by several workers (Sund, 1961; Frieden, 1963). The different batches used by us were also fully active and homogeneous in the ultracentrifuge.

NAD⁺ and NADH were purchased from C. F. Boehringer

und Soehne G.m.b.H., GTP from Sigma Chemical Co., St Louis, Mo., U.S.A., and L-glutamic acid and AnalR EDTA from British Drug Houses Ltd., Poole, Dorset. Butan-2-ol was freshly distilled before use (b.p. 99.5–100.5°).

METHODS

Optical rotatory dispersion was measured with a Bendix-Ericsson automatic recording spectropolarimeter (model Polarmatic 62). Measurements were performed at 25° with fixed constant slit widths (0.5 mm. at the entrance slit and 0.4 mm. at the exit). The instrument was frequently calibrated with a standard sucrose solution. The extinction was kept below 0.8 to eliminate possible 'spurious' effects due to scattered light, although the instrument could measure rotations fairly accurately even when the extinction was as high as 1.5. Cylindrical silica cells of 1.00 cm. path length were used.

Fluorescence measurements were done on two instruments. The Zeiss spectrophotofluorimeter and the Aminco-Bowman instruments gave essentially identical results. The slit widths used were never more than 0.5 mm. on the Zeiss instrument. The combination of 4 mm. and 5 mm. diaphragms was used on the Aminco-Bowman instrument.

Polarization of fluorescence was initially measured on the Aminco-Bowman instrument equipped with two Glan-Thompson prisms. Corrections for the inherent polarization in the monochromators was carried out by the method described by Weill & Calvin (1963). The t_2 value defined by Weill & Calvin (1963) was not more than 1.2. Some of the measurements were later repeated on the Zeiss spectrophotofluorimeter modified to accommodate two Foucault prisms of 12 mm. aperture (the prisms were supplied by Bellingham and Stanley Ltd., London). The results on this instrument agreed to within 5% of those obtained on the Aminco-Bowman instrument. The corrections for polarization on the monochromators were considerably less (t_2 not more than 1.05) on the Zeiss instrument than on the Aminco-Bowman instrument, presumably since the prism-type monochromators in the former produce less polarization of the incident beam than the grating-type monochromators of the latter instrument. By convention 'vertical' polarization is defined as being perpendicular to the plane of propagation of light through the instrument. This plane is horizontal in the Aminco-Bowman instrument and vertical in the Zeiss instrument. In both the angle of observation is 90° to the incident direction. In the Aminco-Bowman instrument fluorescence is observed through the side of the cell; in the Zeiss instrument it is viewed through the bottom.

Sedimentation coefficients were determined by using a Spinco model E analytical ultracentrifuge with schlieren optics.

Enzyme reaction rates were measured as described by Hellerman, Schellenberg & Reiss (1958). The activity of the enzyme preparations was frequently determined after the physical measurements, particularly after sedimentation studies. In most cases there was no difference in the activities before and after the runs. In a few instances we found that denaturation had occurred and these results were rejected. Some inactivation occurred in the spectrophotofluorimeter if the sample was left in the light beam (cf. Chen, 1964) for $\frac{1}{2}$ hr. or longer. This would normally be accompanied by a fall in the fluorescence intensity of the

coenzymes. Measurements were therefore carried out with minimum time of illumination of the sample in the incident beam.

RESULTS

Optical rotatory dispersion. Optical-rotatory-dispersion curves were recorded between 550 and 260 m μ . The initial measurements were done in 20 mM-tris-hydrochloric acid buffer, pH 7.7, containing potassium chloride (0.1 M). Although reasonable curves could be obtained, the solution tended to become cloudy during the measurements owing to denaturation. The solutions were found to be considerably more stable in 0.1 M-phosphate buffer, pH 7.7, and all later measurements were done in phosphate buffers. Generally, the enzyme solutions gave rise to some light-scattering at concentrations above 3 mg./ml. The most convenient concentration region for recording the optical rotations was between 0.8 and 2 mg. of glutamate dehydrogenase/ml. Fig. 1 shows the optical-rotatory-dispersion curves for the enzyme in the absence and presence of 0.1 mM-NADH and in the presence of 6 M-urea. The results are in agreement with Jirgensons's (1961) findings that glutamate dehydrogenase has a positive rotation in the visible region. The addition of NADH, or GTP in the presence of NADH, causes a negative shift in the rotation of the molecule. Denaturation by urea results in a further shift in the negative direction. In contrast with liver alcohol dehydrogenase (Ulmer & Vallee, 1961), binding of NADH to the protein does not produce anomalous dispersion in the region of NADH absorption.

The rotatory-dispersion curves can be analysed in terms of the Moffitt & Yang (1956) phenomenological equation:

$$[m'] = \frac{a_0 \lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0 \lambda_0^4}{(\lambda^2 - \lambda_0^2)^2}$$

In this $[m']$ is the reduced mean residue rotation at

Fig. 1. Optical-rotatory-dispersion curves: □, apoenzyme; ●, apoenzyme + NADH (95 μ M); ■, apoenzyme in 6 M-urea.

wavelength λ and is calculated from the measured specific rotation by the equation:

$$[m'] = \left(\frac{3}{n^2 + 2} \right) \times \frac{\text{MRW}}{100} \times [\alpha]_\lambda$$

In this equation n is the refractive index of the solvent (taken to be that of water) and MRW the mean residue weight of the protein, for which we used the value 115, commonly employed for proteins (Urnés & Doty, 1961). No correction was made for the dispersion of n .

In the calculations of the Moffitt parameters the experimental value 212 was used for the value of the constant λ_0 (Moffitt & Yang, 1956). A test for the correctness of this value lies in the good linearity of the Moffitt plots (Fig. 2). The parameters a_0 and b_0 can be calculated from the intercept on the ordinate and the slope of the lines respectively of the plots shown in Fig. 2. The results of the measurements under a variety of conditions are set out in Table 1, which also contains the results of the sedimentation studies made under conditions comparable with those used in the optical-rotatory-dispersion measurements.

Fluorescence measurements. The emission spectra of $50\mu\text{M}$ -NADH alone and in the presence of glutamate dehydrogenase (1mg./ml.) and glutamate dehydrogenase (1mg./ml.) with GTP (1mM) are recorded in Fig. 3. Light at $340\text{m}\mu$ was used for excitation. The increase in intensity when NADH is bound to enzyme has been noted by Schwert & Winer (1958). It has also been observed that the addition of GTP to the system causes a further increase in the intensity of the emitted light, although there is disagreement about the reasons for it. According to Tomkins, Yielding & Curran (1962), the further enhancement of NADPH fluorescence is due to an increase in the number of binding sites on the enzyme as a result of dissocia-

Fig. 2. Moffitt-Yang plots for glutamate dehydrogenase: $\square$, apoenzyme; $\bullet$, apoenzyme + NADH ($95\mu\text{M}$); $\circ$, apoenzyme + NADH ($86\mu\text{M}$) + GTP (1mM).

Table 1. *Moffitt parameters and sedimentation coefficients for glutamate dehydrogenase*

The enzyme concentration was 2mg./ml. The rotations reported by Bayley & Radda (1965) were expressed as those in water. The present data are corrected for the refractive index of the solvent.

Concn. of NADH (μM)	Concn. of GTP (mM)	b_0	a_0	$S_{20,w}$ (s)
0	0	-190	104	21.3
16	0	-220	104	21.3
26	0	-200	102	21.5
95	0	-250	112	23.5
133	0	-250	118	23.5
190	0	-260	66	—
200	0	—	—	13.0
95	0	-210	100	21.0
(+0.25mM-EDTA)				
0	1	-210	120	21.0
21	1	-250	130	21.0 and 11
32	1	-260	110	12.0
86	1	-270	50	12.0
133	1	-260	35	12.0
16	0.1	-200	95	—
15	0.6	-230	115	20 (small peak at 11.5)
30	0.6	-250	90	11.8 (small peak at 15.7)
Apoenzyme in buffer + 10% (v/v) butan-2-ol		-190	35	10.5

Fig. 3. Fluorescence spectra of NADH in free and bound form: $\square$, NADH ($50\mu\text{M}$); $\bullet$, NADH ($50\mu\text{M}$) + glutamate dehydrogenase (1mg./ml.); $\circ$, NADH ($50\mu\text{M}$) + glutamate dehydrogenase (1mg./ml.) + GTP (1mM).

tion; however, Frieden (1963) showed from quenching experiments that it is a result of a decrease in NADPH-enzyme dissociation constant, which gives rise to binding of more coenzyme at low enzyme concentrations. We have approached the problem from two directions. When a given

Fig. 4. Increase in fluorescence intensity of $15\mu\text{M}$ -NADH on titration with apoprotein in the absence (●) and presence (○) of GTP (1mM).

amount of NADH is titrated with increasing concentrations of protein the curves approach a limiting value that is characteristic of the completely bound coenzyme (Fig. 4). The limiting fluorescence values for the bound coenzyme depend on the presence or absence of GTP, indicating an increase in the binding constant (see the Discussion section). The second method involves titrating a constant amount of protein with increasing concentrations of the coenzyme (Fig. 5). This is limited to concentration ranges of the coenzyme where the absorption of light at $340\text{m}\mu$ is not too great. From these curves the increase in fluorescence on binding can be derived from the intercept of the initial and final slopes of the curve and by comparing this value for the fluorescence with that of the free NADH at the same concentration. The ratios of intensities of bound to free coenzyme are 2.8 and 3.8 in the absence and presence of GTP respectively. These values agree well with the values 2.9 and 3.9 that can be derived from the first method in Fig. 4. From the limiting values of fluorescence for the bound coenzyme the fraction of NADH bound can be calculated and the data in Fig. 5 can be analysed by the method described by Scatchard, Coleman & Shen (1957). $\bar{\nu}$, i.e. mol. of NADH bound/mol. of enzyme, is calculated and $\bar{\nu}/C$ (where C is the concentration of free NADH) is plotted against $\bar{\nu}$. The slope of the line then is $-K_{\text{assoc.}}$ (the association constant for the binding) and the intercept on the abscissa gives the number of binding sites/mol. These results are summarized in Table 2.

Polarization of fluorescence. The change in the fluorescence polarization of NADH as a result of complex-formation can again be measured by the two different methods described for fluorescence above. These results are shown in Figs. 6 and 7. The difference in the polarization at the limiting value in Fig. 6 is experimentally significant and the experimental points are derived from a large

Fig. 5. Changes in fluorescence intensity on titration of glutamate dehydrogenase (1.7mg./ml.) with NADH: Δ , NADH alone; ●, NADH + enzyme; ○, NADH + enzyme + GTP (1mM).

Table 2. *Binding constants and number of sites for NADH on glutamate dehydrogenase*

	From fluorescence		From polarization of fluorescence	
	$10^6 K_{\text{assoc.}}$ (M^{-1})	No. of sites	$10^6 K_{\text{assoc.}}$ (M^{-1})	No. of sites
Without GTP	0.5	21	0.4	19
With 1 mM-GTP	0.8	25	0.8	25

Fig. 6. Polarization of fluorescence of $15\mu\text{M}$ -NADH in the presence of glutamate dehydrogenase in the absence (●) and presence (○) of GTP (1mM).

Fig. 7. Polarization of fluorescence on titration of glutamate dehydrogenase (2mg./ml.) with NADH: ●, NADH+enzyme; ○, NADH+enzyme+GTP (1mM).

number of measurements. The polarization spectrum of bound NADH shows a wavelength-independence of the exciting light throughout the $340\text{m}\mu$ absorption band of the coenzyme.

The polarization of NADH fluorescence was measured with polarized incident light. The polarization is then given by the relation:

$$p = \frac{V_v - H_v}{V_v + H_v}$$

where the capital letter refers to the direction of the emitted radiation and the subscript to that of the incident light, V meaning vertical polarization and H horizontal polarization. The measured polarization for the NADH-glutamate-dehydrogenase system is the sum of the contributions by free and bound forms of the coenzyme; α , the fraction of coenzyme bound to the protein, is not a direct function of p (the value of polarization), however, since the total intensity of the V_v and H_v components also depends on the relative specific fluorescence intensities of the free and bound forms of the coenzyme. If I_v (free) and I_v (bound) represent the V_v components of the specific fluorescence intensities of the free and bound coenzyme respectively, and I_h (free) and I_h (bound) the H_v components, then the polarization is related to α as follows:

$$p = \frac{[(1-\alpha)I_v(\text{free}) + \alpha I_v(\text{bound})] - [(1-\alpha)I_h(\text{free}) + \alpha I_h(\text{bound})]}{[(1-\alpha)I_v(\text{free}) + \alpha I_v(\text{bound})] + [(1-\alpha)I_h(\text{free}) + \alpha I_h(\text{bound})]}$$

The four specific intensity components can be

obtained experimentally during the measurements by comparing I_v and I_h components in the limiting case at high enzyme concentration (all coenzyme bound) and NADH without the protein present (NADH free), i.e. the components used in deriving Fig. 6. Then α can be calculated for each point in Fig. 7, and the data analysed in a manner similar to that described for the fluorescence measurements above. The binding constants and the number of binding sites calculated this way agree well with those derived from unpolarized fluorescence measurements (see Table 2).

DISCUSSION

The importance of conformational changes in the regulation of enzymic activity has been discussed in detail by Monod, Changeux & Jacob (1963). The term 'allosteric effect' defines a phenomenon in which a conformational change is induced in the enzyme (which then may lead to either activation or inhibition) by a small molecule bound at a site other than the active centre. Glutamate dehydrogenase has been assumed to be an enzyme in this class (Monod *et al.* 1963). GTP is a non-competitive inhibitor of glutamate dehydrogenase (Frieden, 1963) and both NADH and GTP are bound at a second site. They are also known to cause dissociation of the enzyme into sub-units, but it is thought that they may act differently in that the effect of NADH alone can be reversed by substances that chelate Zn^{2+} (e.g. EDTA), whereas GTP requires NADH and does not require Zn^{2+} to be effective.

The results of optical-rotatory-dispersion measurements (see Table 1) combined with the sedimentation data clearly show that NADH at concentrations higher than 0.1mM induces some change in the protein that is reflected in the b_0 value of the Moffitt equation, yet does not affect the a_0 parameter or the sedimentation coefficient, $S_{90,w}$, below 0.2mM concentration. This effect can be reversed by EDTA. Since b_0 is related to the effective helical content of proteins we may conclude that changes that affect it are due to an alteration in the secondary structure of the protein. In the absence of measurements of rotations further down in the u.v. region (which are at present limited by the high absorption by the coenzyme in this region) we cannot say what the precise nature of this change is.

We are also aware that possible contributions to b_0 from excitations other than those of the helical peptide groups may arise when the inhibitor or coenzyme is bound to the protein. We consider that this is, however, unlikely on the following grounds: (1) There is no significant contribution to the rotation by the $340\text{m}\mu$ electronic transition of the bound coenzyme, as shown by the lack of

Cotton effect in this region. One might expect this transition to be optically active if other transitions further down in the u.v. region were active. (2) It is shown below that, according to the fluorescence data, 10 mol. of NADH can be bound/mol. of enzyme without affecting the value of b_0 . (3) Even when NADH exhibits a large Cotton effect in the visible region on being bound (as with liver alcohol dehydrogenase), this does not lead to a change in b_0 (Rosenberg, Theorell & Yonetani, 1964). (4) Further, even if the rotatory strength of the coenzyme transitions were not low they are significantly outnumbered by the optically active transitions of the peptide chain itself, just as the aromatic side-chain transitions are insignificant for most proteins (Schellman & Schellman, 1956). (5) Beychok & Blout (1961) have shown that with myoglobin the haem chromophore makes no significant contribution to the b_0 value of the apoprotein in spite of the fact that it has a strong anomalous dispersion (Beychok & Blout, 1961). (6) The linearity of the Moffitt plots in the presence of the coenzyme and GTP with the same λ_0 value, namely 212, as used for the apoenzyme is further support for the insignificance of the contributions from the coenzyme transitions.

The effect of 1 mM-GTP in the presence of concentrations of NADH (e.g. 21 μ M) that are alone too low to cause any change in the optical-rotatory properties of the enzyme is similar to the effect of NADH since it produces a similar change in b_0 . However, under these conditions a_0 also changes (it decreases from 150 to about 50). This latter change is paralleled by a change in $S_{20,w}$, which indicates a dissociation into sub-units. In such a reversible dissociating system the value of $S_{20,w}$ cannot be taken as the final criterion of the state of aggregation. No interpretation has been made of $S_{20,w}$ values between 21.0s for the apoenzyme and 12.0s for the dissociated form characterized by light-scattering (Yielding *et al.* 1964). At lower concentrations of GTP (e.g. 0.6 mM) it is possible to choose NADH concentrations that will lead to a change in b_0 but not affect a_0 , and only cause some dissociation as shown by the appearance of two peaks of different mobilities (with $S_{20,w}$ values 20 and 11.5s). GTP alone does not affect the rotatory properties of the enzyme and the combined effects of GTP and NADH cannot be reversed by EDTA.

These results suggest that the changes in a_0 are related to the dissociation of the enzyme. This is reasonable, since a_0 should depend on the degree of interaction between helices and to some extent also on interactions with the solvent. Indeed, when dissociation is brought about by a non-specific reagent such as butan-2-ol, b_0 remains constant, yet a_0 again decreases. Similar observations were made

by Herskovits, Townend & Timasheff (1964), who showed that a_0 is quantitatively related to the intermolecular association of β -lactoglobulin.

The results from the fluorescence measurements show that the binding constant towards NADH increases in the presence of GTP. This results in an increased fluorescence not only at low protein concentration where only a fraction of the coenzyme is bound but also at the limiting value that the curves approximate in Fig. 4. The specific fluorescence intensities of bound NADH are therefore different under the two conditions. The increased enhancement of fluorescence in the presence of GTP is consistent with a tighter binding of the coenzyme, since the enhancement is probably due to the lower ability of bound NADH to lose its energy from the excited state through non-radiative deactivation by the solvent molecules. This is in accord with Frieden's (1963) observations for NADPH, and the numerical values of the binding constants are of similar magnitude for the two systems. At the same time we find, unlike Frieden (1963), that the number of binding sites increases (cf. Tomkins *et al.* 1962), possibly as a result of dissociation. Sund (1961) found that dissociation brought about by dilution of the enzyme also results in an increase of the number of binding sites but that this non-specific dissociation leads to a diminution in the binding constant of NADH.

Our conclusions based on fluorescence measurements receive further support from the study of fluorescence polarization. The degree of polarization is a measure of the rotational motion of the bound chromophore during the life-time of the excited state. This motion is a composite of two rotations: the overall rotation of the macromolecule and the 'free' rotation of the chromophore on the surface of the macromolecule. The former should be larger for the complex of the coenzyme with the dissociated enzyme than for the complex with the aggregate. Dissociation thus should lead to a decrease in the fluorescence polarization. The observed increase therefore can be attributed to the smaller freedom of the coenzyme on the protein in the presence of GTP.

Tomkins and his collaborators have shown that the physical changes (e.g. dissociation) observable at high enzyme concentrations are related to the mechanism of non-competitive inhibition of enzymic activity under assay conditions (Yielding *et al.* 1964). Thus the conformational changes we observed are probably responsible for the inhibitions. This enzyme thus may serve as a suitable model for studying the physical basis of control of enzymic activity. It is therefore noteworthy that the conformational changes induced by NADH appear fairly sharply over a narrow range as the coenzyme concentration is increased. For example, with

26 μM -NADH there is no effect on the b_0 value of the enzyme (enzyme concentration 2 mg./ml.), yet with 95 μM -coenzyme there is a change that remains the same at higher coenzyme concentration (e.g. 133 μM). From the fluorescence-polarization data we can calculate the amount of coenzyme bound under the above conditions. Thus, when the total coenzyme concentration is 26 μM , 22 μmoles of NADH are bound/2 μmoles of glutamate dehydrogenase, whereas with 95 μM -NADH, 32 μmoles of NADH are bound at the same enzyme concentration. Therefore approx. 4–5 mol. of NADH/mol. of protein of mol.wt. 10^6 is required to induce the conformational change. The measurements do not enable us to make a more precise estimate of the NADH allosteric sites, but it appears that approx. 1 site/mol. of mol.wt. 250 000 (which is the mol.wt. of the sub-unit after reversible dissociation) may be involved in the regulation of the enzymic activity.

It is tempting to speculate that whereas the regulation by NADH is of the on-off type (giving rise to the well-known sigmoid-shaped curve for allosteric inhibition) the role of GTP is to shift the threshold value at which NADH is capable of inducing the conformational change. Our results with GTP are consistent with this hypothesis.

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