The Allosteric Mechanism of Bovine Liver Glutamate Dehydrogenase

EVIDENCE FROM CIRCULAR-DICHROISM STUDIES FOR A CONFORMATIONAL CHANGE IN THE TERNARY COMPLEX ENZYME-(OXIDIZED NICOTINAMIDE-ADENINE DINUCLEOTIDE)-GLUTARATE

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1. Computer averaging of multiple scans was used to refine the circular dichroism spectrum of bovine liver glutamate dehydrogenase, revealing well-defined structure in the aromatic region. 2. The circular dichroism of NAD^+ bound to glutamate dehydrogenase is strongly negative at 260 nm, probably owing to immobilization of the adenosine moiety. Loss of the characteristic adenine–nicotinamide interaction suggests that the coenzyme is bound in an unstacked conformation. 3. Glutarate and succinate, substrate analogues that are both inhibitors competitive with glutamate, do not significantly perturb the circular-dichroism spectrum of the enzyme in the absence of NAD^+ . 4. In the presence of NAD^+ , 150 mM-succinate decreases the negative circular dichroism corresponding to bound coenzyme, but does not affect the protein circular dichroism. However, 150 mM-glutarate causes profound alterations of the circular-dichroism spectra of the bound NAD^+ and of the enzyme, indicative of a protein conformational change. This direct evidence of conformational change specifically promoted by C_5 dicarboxylates confirms the previous inference from protection studies. 5. The conformational change is discussed in relation to the allosteric mechanism of glutamate dehydrogenase.

Bovine liver glutamate dehydrogenase (EC 1.4.1.3) catalyses the reversible oxidative deamination of L-glutamate by either NAD^+ or NADP^+ (Olson & Anfinsen, 1953; Strecker, 1953). A wide variety of other amino acids serve as poorer substrates for the enzyme (Struck & Sizer, 1960; Fisher & McGregor, 1961), and activity with these tends to be enhanced by compounds that inhibit activity with glutamate. Conversely, various compounds which activate the oxidation of glutamate suppress activity with other amino acids. This reciprocity has led to the suggestion that the enzyme can exist in two conformational states, one of which is specific for the dicarboxylate substrate glutamate (Bitensky *et al.*, 1965).

Further evidence of an intimate relationship between dicarboxylate binding and an allosteric transition comes from detailed steady-state initial-rate studies (Engel & Dalziel, 1969). With glutamate as substrate, the Lineweaver–Burk plots of $1/v$ against $1/[\text{NAD(P)}^+]$ show several abrupt transitions in slope, which have been attributed (Dalziel & Engel, 1968) to negative co-operativity in coenzyme binding; subsequent analysis (Engel & Ferdinand, 1973) suggested a more complex pattern of intersite interaction affecting catalytic rate constants as well as binding constants. With norvaline, the best of the

monocarboxylate substrates, the corresponding Lineweaver–Burk plots are perfectly linear (Engel & Dalziel, 1969).

Studies of NAD(P)^+ binding showed that, whereas formation of the enzyme–coenzyme binary complex could be adequately described by a single dissociation constant, the binding of NAD(P)^+ in the presence of glutarate (the deamino analogue of glutamate) showed negative co-operativity (Dalziel & Egan, 1972). The specificity for the dicarboxylate was not investigated in these studies. However, equilibrium-protection studies (Chen & Engel, 1974, 1975) of the reversible inactivation of glutamate dehydrogenase by pyridoxal 5'-phosphate have provided indirect evidence that there is a high degree of specificity in the promotion of conformational change by dicarboxylates. Unlike NADH, the oxidized coenzyme does not protect glutamate dehydrogenase against inactivation by pyridoxal 5'-phosphate (Piszkiwicz & Smith, 1971; Chen & Engel, 1974). NAD^+ becomes an effective protecting agent, however, in the presence of 2-oxo-glutarate or glutarate (Chen & Engel, 1974). Neither adipate nor succinate promotes protection by NAD^+ in this way, nor does the monocarboxylate pentanoate which is an analogue of norvaline.

We have therefore now looked for direct evidence

of conformational change in enzyme complexes with dicarboxylates closely related in structure to the allosteric substrate glutamate. We have studied the circular dichroism of glutamate dehydrogenase and its complexes with NAD^+ , from 250 nm upwards, in the presence of glutarate and succinate, by using automated data collection and processing to handle the complex and overlapping spectra of enzyme and coenzyme. The results of previous circular-dichroism experiments (Bell & Dalziel, 1973) are shown to be in error. By distinguishing effects on the circular dichroism of the protein and the nucleotide, we show that a specific conformational change occurs when the ternary complex enzyme- NAD^+ -glutarate is formed. This change is not seen in the formation of the binary enzyme- NAD^+ or enzyme-dicarboxylate complexes, nor in the ternary complex with succinate. The conformational change is discussed in relation to the allosteric mechanism of glutamate dehydrogenase.

Experimental

Bovine liver glutamate dehydrogenase [$(\text{NH}_4)_2\text{SO}_4$] suspension, and NAD^+ were obtained from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K. Sodium succinate was from BDH Chemicals Ltd., Poole, Dorset, U.K. Glutaric acid, supplied by Koch-Light Laboratories, Ltd., Colnbrook, Bucks., U.K., was recrystallized from ethanol. The enzyme and reagents were prepared as described previously (Chen & Engel, 1974). All experiments were carried out with 0.1 M-potassium phosphate, pH 7.0.

Enzyme solutions for spectroscopy (concn. 9.0 mg/ml) were passed through Millipore filters (pore diam. 5 μm). Dicarboxylates and NAD^+ were added from concentrated stock solutions in 10 μl portions.

Circular-dichroism spectra were recorded on a Jouan Dichrographe IIB, fitted with a 150 W xenon arc. Solutions were thermostatically maintained at 20°C in cells of path length 1.0 mm. The A_{280} of the enzyme solution was kept less than 1.0, and, even in the presence of NAD^+ , the A_{280} of the sample was always less than 1.5. These conditions were rigorously observed to avoid artifacts that may occur at higher absorbance values. The operating voltage varied between 560 and 760 V.

The experimental results were recorded simultaneously on the chart recorder and in digital form via a BCD interface. Points were taken at 0.25 nm intervals, the spectra being scanned at 3.75 nm/min with a nominal instrumental time constant of 10 s. Multiple scans (generally five) were taken for each spectrum. Processing involved averaging the spectra, subtracting the instrumental baseline, normalizing to a standard enzyme concentration of 9 mg/ml to correct for small dilution effects, computation of individual difference spectra, smoothing by serial application of a 30-point third-order polynomial

algorithm, and plotting via Calcomp routines. These operations were programmed on the interactive-graphics system (Tektronix 4010) in the Hewlett-Packard 3000 Computing Laboratory of the National Institute for Medical Research.

Statistical analysis of the digitized data was performed with a matrix-rank-analysis technique. This treats a set of data as a matrix, reducing its rank to the number of significant components, with significance evaluated against the observed standard deviation of the measurements (P. M. Bayley & M. T. Flanagan, unpublished work).

Results

Fig. 1 shows the circular-dichroism spectrum of glutamate dehydrogenase. The high reproducibility of individual spectra and the improvement in detail with summation are evident. The spectrum is positive from 330 to 250 nm and exhibits well defined structure at 288, 280, 265 and 259 nm, characteristic of the aromatic amino acids. This spectrum corrects the limited spectrum for bovine glutamate dehydrogenase previously reported (Bell & Dalziel, 1973), which showed a single positive peak at 297 nm.

The effect of additions of the dicarboxylates is shown in the summed spectra of Fig. 2. Addition of succinate (Fig. 2b) or glutarate (Fig. 2c) produces only a slight perturbation, barely greater than noise level, in the region 285–250 nm (Fig. 2d). There is therefore little or no effect of dicarboxylate alone. This result was tested with four separate enzyme samples prepared identically. In one case, variability of the enzyme spectrum alone was noted between scans during the long scanning time (25 min per scan), and this experiment was discarded. The individual spectra of Fig. 2 were obtained by alternate scanning of enzyme and enzyme plus dicarboxylate, and a fully consistent set of spectra was obtained.

The experimental (unsummed) spectra (e.g. those shown in Fig. 1) were subjected to rank analysis in an attempt to establish a significance for the small variations between individual spectra. A set of ten spectra (five enzyme; five enzyme+dicarboxylate), each with 150 points covering the range 330–255 nm, was reduced by standard methods with the corresponding error matrix set at 1 or 2 units ($\times 10^{-5}$, as in Fig. 1), equivalent to the observed instrumental noise level. The diagonal elements indicated one major component only for both the sets (enzyme, enzyme+succinate) and (enzyme, enzyme+glutarate). The second diagonal element was less than 5% significant in each case, indicating that the system contains only one spectroscopic component, namely that of the native enzyme, with a certainty of 95%.

The spectra of binary and ternary complexes containing the coenzyme were recorded with a 1.5-fold

Fig. 1. Circular dichroism of bovine glutamate dehydrogenase

Circular-dichroism spectra were recorded for bovine glutamate dehydrogenase (9.0 mg/ml) in 0.1 M-phosphate buffer, pH 7.0. The path length was 1 mm. The five upper curves (●, ○, ■, □ and ▲) are experimental spectra successively displaced vertically for clarity; the lowest curve (△) is the averaged spectrum. The symbols do not in fact indicate experimental points; they are merely superimposed as markers to make it easier to distinguish the individual spectra.

molar excess of NAD^+ over enzyme subunits (based on a subunit mol.wt. of 55 393; Moon & Smith, 1973). The circular dichroism of enzyme plus NAD^+ (Fig. 3b) exhibits a strong negative perturbation relative to enzyme alone (Fig. 3a), presumably owing to the circular dichroism of the bound coenzyme. The same effect is shown, but to a lesser extent, in the presence of succinate (Fig. 3c). The spectrum in the presence of glutarate, however, is clearly different, being considerably more negative than that of enzyme plus NAD^+ above 270 nm, and more positive below 270 nm.

These differences are more clearly seen in the sub-

tracted, smoothed spectra (Fig. 4). All of the differences are relatively intense compared with the circular dichroism of free NAD^+ (Fig. 4a). The difference spectrum of (enzyme+ NAD^+)—enzyme (Fig. 4b) shows a minor contribution from free NAD^+ superimposed on a strong negative band (Fig. 4b). The less intense difference spectrum in the presence of succinate (Fig. 4c) is similar in form to that of bound NAD^+ (Fig. 4b). In the presence of glutarate, an extensive perturbation from 310 nm downwards is observed (Fig. 4d). A further difference spectrum of 4(d) minus 4(b) (Fig. 4e) is derived for comparative purposes.

Fig. 2. Effect of dicarboxylates on the circular dichroism of bovine glutamate dehydrogenase

Conditions were as for Fig. 1: (a) enzyme alone; (b) enzyme plus 0.15 M-succinate; (c) enzyme plus 0.15 M-glutarate; Curve (d) is the difference (c) - (a). Curves (a) and (b) are displaced +4 and +2 units respectively for clarity. See note on Fig. 1 regarding significance of symbols.

Fig. 3. Effect of dicarboxylates on the circular dichroism of glutamate dehydrogenase in the presence of NAD^+

Conditions were as for Fig. 1: (a) enzyme alone; (b) enzyme plus NAD^+ (250 μM); (c) enzyme plus NAD^+ plus 0.15 M-succinate; (d) enzyme plus NAD^+ plus 0.15 M-glutarate. All curves are the average of five scans. See note on Fig. 1 regarding significance of symbols.

Discussion

The most prominent feature of the circular-dichroism spectrum of bovine glutamate dehydrogenase is the sharply defined positive peak at 288 nm. The apparent peak at 297 nm recorded by Bell & Dalziel (1973) is probably an artifact. Their experimental conditions represent a tenfold greater optical absorbance than that used here. The general effect of excessive absorbance is for the circular-dichroism signal to fall to an artificial zero, thereby creating an apparent peak. When additional absorbance is introduced (e.g. from NAD^+), the quantitative results must be suspect.

The fine-structure details of the enzyme spectrum represent the overlapping effects of all the aromatic amino acids. Since these may contribute components of opposite sign, the interpretation of individual peaks and troughs is not necessarily definitive. Even so, the dichroic absorption at 295 nm and above may be assigned to red-shifted $^1\text{L}_a$ electronic transitions of tryptophan presumably corresponding to buried

residues. The peak at 288 nm (and possibly the subsidiary peak at 280 nm) could derive from the typical $^1\text{L}_b$ electronic transitions of tryptophan, as judged from the spectra of model compounds (Strickland, 1974). This transition is not very environmentally sensitive (288–292 nm). The correlation of the peaks at 265 and 259 nm with resolved fine structure of phenylalanine derivatives (Strickland, 1974) may be noted. Tyrosine residues may be producing a net negative contribution, giving rise to the troughs at 283 and 276 nm as also found for model compounds (Strickland, 1974). Undoubtedly the effects of different residues lead to a cancelling of overall intensity. The spectral maximum $\Delta\epsilon_{265} = 14 \times 10^{-5}$ corresponds to $\Delta\epsilon_M = +8.8 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$, based on subunit molarity, where each subunit contains 3 tryptophan, 18 tyrosine and 26 phenylalanine residues (Moon *et al.*, 1972). Thus the intensification or elimination of the contribution of individual residues as a result of conformational change may show up as a relatively large percentage change of the total aromatic spectrum of the enzyme.

Fig. 4. Calculated difference circular-dichroism spectra for the effect of dicarboxylates on bovine glutamate dehydrogenase in the presence of NAD^+

Curve (a) shows the circular-dichroism spectrum of $250 \mu\text{M-NAD}^+$ in 0.1M-phosphate buffer. Curves (b)–(e) are difference spectra derived from Fig. 3 as follows: (b) (enzyme+ NAD^+) minus (enzyme alone) ($3b-3a$); (c) (enzyme+ NAD^+ +succinate) minus (enzyme alone) ($3c-3a$); (d) (enzyme+ NAD^+ +glutarate) minus (enzyme alone) ($3d-3a$); (e) (enzyme+ NAD^+ +glutarate) minus (enzyme+ NAD^+) ($3d-3b$). Curves (a) and (e) are separated from the rest for clarity. All curves are numerically smoothed (see the text). See note on Fig. 1 regarding significance of symbols.

This spectrum for bovine liver glutamate dehydrogenase may be compared with that recorded at low resolution for the enzyme from pig liver (Dessen & Pantaloni, 1969). This shows similar broad positive features extending to 300nm, with a maximum close to 260nm, although the fine structure was not resolved.

In the absence of NAD^+ the effect of either succinate or glutarate on the circular-dichroism spectrum of the enzyme is minimal, although at the concentrations used the dicarboxylate compounds should saturate the enzyme (Chen & Engel, 1974). Fig. 2 shows no significant effect of these compounds on any of the components of the aromatic fine structure. The statistical analysis established this with 95% probability. On a qualitative basis the spectra appear to differ very slightly in the 260nm region.

The essential constancy of the circular-dichroism spectra seen in Fig. 2 may be contrasted with the observed effects of glutamate, 2-oxoglutarate or

glutarate on the absorption spectrum of glutamate dehydrogenase (Prough *et al.*, 1972; Cross *et al.*, 1972). The very small observed perturbations of the absorption spectrum would be consistent with a change in the local environment of individual tyrosine or tryptophan residues. We conclude that these dicarboxylate ligands do not affect the overall conformation of glutamate dehydrogenase when binary enzyme–dicarboxylate complexes are formed.

In the experiments involving NAD^+ , only a small molar excess of the coenzyme was used. Hence the quantitative evaluation of the contributions of various species to the observed spectrum requires a knowledge of the stoichiometry of NAD^+ binding and the values of the relevant dissociation constants. The binding process is complex, however, and, in the formation of the ternary complex with glutarate, a single value for the dissociation constant for NAD^+ is insufficient (Dalziel & Egan, 1972). We have therefore taken limiting upper values of $100 \mu\text{M}$ for the dissociation constant of the enzyme– NAD^+ binary complex, and $10 \mu\text{M}$ for the corresponding constant governing dissociation of NAD^+ from the ternary complex enzyme– NAD^+ –glutarate. With these values, the degree of saturation for a single site under the experimental conditions used here would be 61% in the absence of glutarate and 90% in its presence. This allows us at least to assign lower limits to the intensities of the circular-dichroism spectra of the binary and ternary complexes on a molar basis. Obviously it would be desirable to increase the degree of saturation with NAD^+ , but the results of kinetic (Engel & Dalziel, 1969) and binding (Dalziel & Egan, 1972) experiments indicate that concentrations of NAD^+ in excess of 2mM would be required to approach saturation. In spectroscopic measurements, the absorption of the free coenzyme ultimately interferes, and presents a major difficulty in characterizing the properties of individual complexes. However, the results presented here indicate the feasibility of determining the stoichiometry of NAD^+ binding by circular-dichroism titration.

The strong negative difference circular-dichroism spectrum of bound NAD^+ (Fig. 4b) extends to 260nm. It shows some positive component at 270nm, consistent with the presence of some free NAD^+ . The intensity at 260nm, where free NAD^+ makes effectively no contribution, is characteristic of adenine mononucleotide spectra intensified by greater asymmetry, caused, for example, by immobilization. It is comparable with the intensity of the circular dichroism of ADP bound to glutamate dehydrogenase (Sund *et al.*, 1971; Jallon *et al.*, 1973). The coenzyme has apparently lost the interaction between adenine and nicotinamide (cf. Bayley & Debenham, 1974), which gives rise to the typical spectrum of NAD^+ containing the 'couplet effect' (Bayley, 1973), and it is presumably unstacked. The difference spectrum in

the presence of succinate (Fig. 4c) shows a similar but weaker negative effect with some additional curvature 270 nm. There are two possible interpretations of this spectrum. If NAD^+ binds much more weakly to the enzyme-succinate complex than to the free enzyme, very little ternary complex may be formed. The difference between Figs. 4(b) and 4(c) would then reflect displacement of some of the bound NAD^+ by succinate. Alternatively it is possible that the ternary complex is formed, but that in this complex the asymmetry of the bound adenine moiety is less pronounced than in the binary complex. Without knowledge of the dissociation constant for NAD^+ binding in the presence of succinate it is not possible to choose between these alternatives.

The effect on the circular-dichroism spectrum produced by addition of glutarate to enzyme plus NAD^+ is evident from Fig. 3(d), and the difference spectrum after subtraction of the spectrum (Fig. 3a) of enzyme alone is shown in Fig. 4(d). This is different in magnitude and spectral shape from the difference spectrum obtained when NAD^+ alone is added to the enzyme (e.g. Fig. 4b). The chromophores responsible for the perturbation out to 310 nm must be those of the protein. The extent and intensity of the effect suggest a significant change in the conformational relationship between the enzyme and coenzyme. In molar terms, the intensity change in the circular dichroism at 280 nm is $-1.5 \text{ M}^{-1} \cdot \text{cm}^{-1}$, comparable with the values from model compounds (-1 to $-2.5 \text{ M}^{-1} \cdot \text{cm}^{-1}$) for a single immobilized tryptophan residue (Strickland, 1974). It is also noteworthy that the interactions responsible for the intensified negative spectrum of bound NAD^+ at 260 nm have apparently been largely abolished in the presence of glutarate. A possible alternative interpretation is illustrated in Fig. 4(e). The difference between Figs. 4(d) and 4(b) now appears as a 'couplet' spectrum, which is frequently diagnostic of the exciton interaction of two chromophores (cf. Bayley, 1973). A stacking interaction between adenine and tryptophan could be responsible for these effects, generating this spectrum in addition to that of the previously bound nucleotide shown in Fig. 4(b). It is noteworthy that several authors have previously suggested that there may be a tryptophan residue at the active site of glutamate dehydrogenase (e.g. Chen, 1964; Fisher & Cross, 1965; Summers & Yielding, 1971).

These results indicate clearly that a change of enzyme conformation in the presence of NAD^+ is induced by glutarate and not by succinate. This direct evidence correlates exactly with the inference drawn from equilibrium-protection studies (Chen & Engel, 1974). We suggest therefore that an analogous change in the active ternary complex enzyme- NAD^+ -glutamate may provide the basis of the allosteric mechanism of glutamate dehydrogenase. It would explain why non-linearity in the Lineweaver-Burk

plots is observed only with glutamate as the amino acid substrate, and also why norvaline and alanine, which lack the second carboxylate group, are much poorer substrates than glutamate. In aspartate, as in succinate, the two carboxylate groups are separated by one less methylene group than in glutamate/glutarate; aspartate shows no substrate activity with glutamate dehydrogenase.

An obvious next step will be to search for evidence of the postulated conformational change in the active ternary complex by rapid-reaction studies.

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