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1-Anilinonaphthalene-8-sulphonate, a Fluorescent Conformational Probe for Glutamate Dehydrogenase

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1. The interaction of 1-anilinonaphthalene-8-sulphonate with ox liver glutamate dehydrogenase was examined. 2. The fluorescence of the dye is enhanced 100-fold on binding. 3. A further enhancement is observed when NADH and GTP are added to the enzyme. 4. By using this property of the dye to measure conformational equilibria in the enzyme the effects of coenzyme, inhibitors, enzyme concentration, ionic strength and pH on the allosteric transitions were studied. 5. GTP and NADH interact with the enzyme in a heterotropic manner. 6. The rate of the structural transition brought about by GTP and NADH is biphasic with half-lives of 34 and 200msec. 7. The relation of these observations to regulatory mechanisms is discussed.

The interaction of purine nucleotides with ox liver GDH* has been intensively studied as a model for allosteric control of enzyme activity (Frieden, 1963; Yielding, Tomkins, Bitensky & Talal, 1964). Of the numerous reagents that inhibit the enzyme, GTP is very effective and is thought to bind at a site different from the active site (Colman & Frieden, 1966*a*,*b*; di Prisco, 1967). The same nucleotide in the presence of NADH causes a structural transition in the enzyme that has been detected by optical rotatory dispersion (Bayley & Radda, 1966). This transition also leads to dissociation of the enzyme aggregate, present at high enzyme concentrations, to the oligomer. The oligomer is now believed to have a molecular weight of 310000 (Eisenberg & Tomkins, 1968) to 280000 (Sund & Burchard, 1968), and it probably consists of six subunits (Eisenberg & Tomkins, 1968), or possibly four (Minssen & Sund, 1969).

The understanding of allosteric transitions in terms of the several models proposed (Monod, Wyman & Changeux, 1965; Kirtley & Koshland, 1967) requires not only knowledge of the ligand-binding functions but also a measure of the conformational equilibria involved (Haber & Koshland, 1967). Spin-labelling (Ogawa & McConnell, 1967), the chemical reactivity of thiol groups (Gerhart & Schachman, 1968) and dye binding (Ullmann, Vagelos & Monod, 1964) have been used as a measure of the transitions of state in several allosteric enzymes. We have reported that ANS is a suitable fluorescent probe for following transitions of state in GDH caused by GTP and other inhibitors (Dodd & Radda, 1967). The effect of steroid hormones on GDH has also been studied by this method (Thompson & Yielding, 1968). ANS has also proved suitable for following the kinetics of the conformational change in GDH. A preliminary account of this work has been given (Dodd & Radda, 1968).

* Abbreviations: GDH, L-glutamate–NAD(P) oxidoreductase (deaminating) (EC 1.4.1.3); ANS, 1-anilinonaphthalene-8-sulphonate.

MATERIALS AND METHODS

GDH [as an $(NH_4)_2SO_4$ suspension or a glycerol solution], NADH (disodium salt), α-oxoglutaric acid and sodium pyruvate were obtained from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). GTP (type IIS), ADP (type I), NAD^+ and diethylstilboestrol were purchased from Sigma Chemical Co. (St Louis, Mo, U.S.A.). Phenanthridine was supplied by Aldrich Chemical Co. Inc. (Milwaukee, Wis., U.S.A.). L-Glutamic acid and AnalaR EDTA were supplied by British Drug Houses Ltd. (Poole, Dorset). ANS, purchased as the ammonium salt from K & K Laboratories Inc. (New York, N.Y., U.S.A.), was purified by several recrystallizations from saturated $MgCl_2$ solutions, when it gave one spot, with R_F 0·61, on t.l.c. on silica with butan-1-ol–ethanol–aq. NH_3 (sp.gr. 0·88)–water (200:47:3:88, by vol.). The yellow–green needles had an extinction coefficient of 6×10^4 l.mole^{-1} cm.$^{-1}$ in 0·1 M-phosphate buffer, pH 7·7, at 350nm. and gave the correct elemental analysis for the magnesium salt. Phosphate buffer was made up by using the AnalaR sodium salts (British Drug Houses Ltd.).

Twice-glass-distilled water was used as a routine.

The enzyme preparation was freed from $(NH_4)_2SO_4$ (or glycerol) by dialysis against 0·1 M-phosphate buffer, pH 7·7, for 24hr. at 4° before use. The resultant solution was

centrifuged to remove any denatured protein, and the enzyme concentrations were estimated by the extinction at 280nm. by using the published value for the extinction coefficient (Olson & Anfinsen, 1952). The homogeneity of the enzyme preparation was established as described by Bayley & Radda (1966).

NADH solutions were made up in tris–HCl buffer, pH 9·0, and concentrations were determined by the extinction at 340nm. The concentrations of GTP and ADP solutions were measured spectrophotometrically at 260nm.

Enzyme activities were measured by following the decrease in extinction of NADH at 340nm. in a Hilger–Gilford kinetic spectrophotometer or a Cary 14 spectrophotometer equipped with a 0–0·1 slide-wire. The sample compartments were thermostatically controlled at 25°. Under the conditions employed the trace was linear over at least the first minute, giving a full-scale deflexion of 0·1 E_{340} units. The concentrations in the assay mixture were: α-oxoglutarate, 5mM; NH_4Cl, 50mM; NADH, 0·1mM; EDTA, 0·05mM. The reaction was started by adding the enzyme solution (0·1ml.) to a solution of the substrates (2·9ml.) in the cuvette. Alanine dehydrogenase activity was assayed by the method of Anderson, Anderson & Churchich (1966) at pH 9·0 in 10mM-tris–HCl buffer containing 0·1M-NaCl.

The pH of solutions was determined with an E.I.L. direct-reading pH-meter.

Optical rotatory dispersion was measured with a Bendix–Ericsson Polarmatic 62 recording spectropolarimeter. Measurements were performed at 25° with constant slit widths (0·5mm. at the entrance slit and 0·4mm. at the exit). Sedimentation coefficients were determined by using a Spinco model E analytical ultracentrifuge with schlieren optics.

Fluorescence measurements were mainly made on a Zeiss spectrofluorimeter. The emission spectra are uncorrected for photomultiplier response. All fluorescence readings were taken against a known fluorescence standard so that variations in the intensity of the exciting radiation were eliminated. The monochromator slit widths were never more than 1mm. and in all cases light-scattering by the protein was separately studied. In most cases it was negligible compared with the fluorescence signal. At very high enzyme concentrations it was necessary to apply a correction, which never amounted to more than 20%. The extinctions of the solutions at the exciting wavelength were kept below 0·3 to ensure a linear response with concentration. All fluorescence measurements were performed with cells of 1cm. light-path that were placed in the thermostatically controlled sample compartment. Unless otherwise stated the temperature was 25°. When increased sensitivity was desirable fluorescence measurements were carried out in a spectrofluorimeter, constructed in this laboratory, that was equipped with a phase-sensitive amplifier tuned to a mechanical chopper normally operating at 800 cyc./sec.

Fluorescence polarizations were also measured on the Zeiss instrument as described by Bayley & Radda (1966), care being taken to make the appropriate corrections for the inherent polarizations in the monochromators.

The stopped-flow apparatus was constructed in this laboratory and essentially followed the design of Gibson & Milnes (1964). With the exception of the driving syringes, the stopping syringe and the observation tube, the apparatus was constructed of stainless steel. The syringes were 2ml. Everett glass syringes with polythene plungers. The mixing chamber and observation tube were built as a single unit and consisted of 2mm.-bore tubes, and produce a turbulence that brings about rapid and efficient mixing. The instrument was calibrated in a series of experiments with three different reactions: the enzymic reduction of NAD^+ catalysed by GDH, reduction of FMN by $Na_2S_2O_4$ and the reaction of $AgNO_3$ with riboflavin. From these experiments the dead time of the apparatus was shown to be less than 4msec. The reactions were followed by fluorescence by using a high-pressure mercury lamp (Quartzenlampe St75) for excitation. The excitation wavelengths (at 366 or 405nm.) were isolated by a Bausch and Lomb grating monochromator. Emission was observed (through suitable glass filters) perpendicularly to the direction of excitation by an EMI 6097B photomultiplier coupled to a Telequipment S45 oscilloscope. The traces were photographed with a Thompson Land polaroid camera. For runs with ANS a filter cutting off light below 500nm. was used. To increase sensitivity we have used an a.c.-operated mercury lamp (or sometimes a chopped d.c. xenon arc); although this resulted in a trace that contained only a limited number of points this was not the main limitation of our measurements. The main disadvantage of fluorescence measurements still lies in the short-term instabilities of high-powered light sources. At present this limits the accuracy of our rate measurements for reactions (such as we are reporting) where the total fluorescence is relatively small. These difficulties were not present in the reactions used for the calibration of the instrument.

RESULTS

Interaction of ANS with GDH

ANS interacts with GDH and this results in a large enhancement of its fluorescence and a blue shift of 40nm. in its emission spectrum. A further enhancement is observed in the presence of NADH (0·1mM) and GTP (0·1mM) (Fig. 1). The fluorescence-polarization spectrum of bound ANS shows the same transitions in the 320–380nm. region as does that of ANS bound to apomyoglobin (Stryer, 1965). The value of polarization is 0·39 when excitation is at 400nm. and emission observed at 500nm. The dye–enzyme interaction is reversible. This was shown by incubating GDH (2mg./ml.) with ANS (0·1mM) for 30min. and then passing the solution through a Sephadex G-25 column, when no dye (shown by spectra and fluorescence) appeared in the enzyme fraction.

ANS weakly inhibited both the glutamate dehydrogenase and the alanine dehydrogenase activities of the enzyme. Although no detailed kinetic investigation of this inhibition was undertaken, with NADH as the limiting substrate non-competitive inhibition was found with both alanine dehydrogenase and glutamate dehydrogenase activities with K_i values of 0·38mM and 0·14mM respectively.

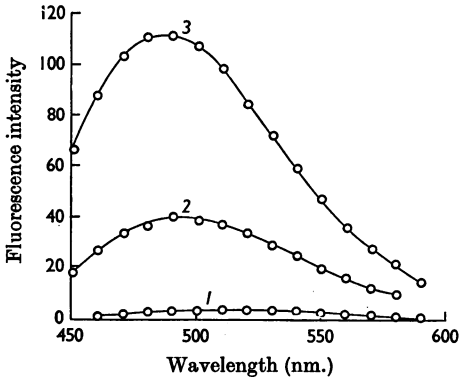

Fig. 1. Fluorescence-emission spectrum of ANS in the presence and absence of GDH. Curve *1*, 20μM-ANS in 0·1M-phosphate buffer, pH7·7; curve *2*, 20μM-ANS+1mg. of GDH/ml.; curve *3*, 20μM-ANS+1mg. of GDH/ml.+0·1mM-NADH+0·1mM-GTP. The curve was corrected for NADH fluorescence by leaving ANS out of the mixture. The excitation wavelength was 410nm.

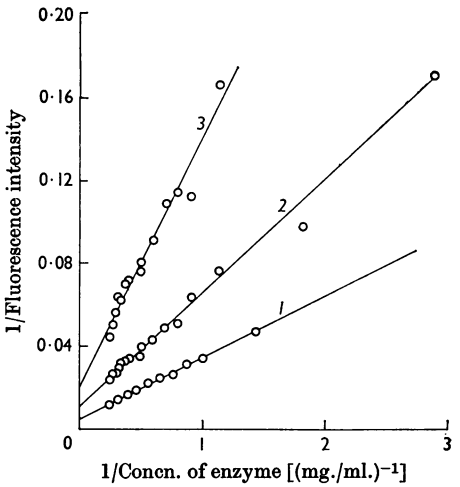

Fig. 2. Double-reciprocal plots of ANS fluorescence against GDH concentration. Curve *1*, 20μM-ANS; curve *2*, 10μM-ANS; curve *3*, 5μM-ANS.

Because the binding is weak it was impossible to determine the fluorescence of completely bound ANS even when 1–5μM-ANS was titrated with up to 10mg. of GDH/ml. These titrations were carried out by diluting a solution containing GDH (10mg./ml.) and ANS at three different concentrations (5, 10 and 20μM) with buffer containing the same concentration of ANS as the initial mixture. The results are presented on the double-reciprocal plots (Fig. 2), from which the enhancement of

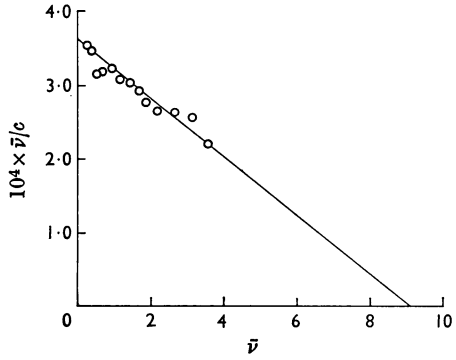

Fig. 3. Scatchard plot for the binding of ANS to GDH. GDH (1mg./ml.) was titrated with increasing amounts of ANS (0–500μM). The excitation wavelength was 410nm. and emission was observed at 490nm.

ANS fluorescence at infinite protein concentration can be obtained from the extrapolated intercept on the abscissa. The maximum enhancement of ANS fluorescence is 100($\pm$20)-fold. The inaccuracy of this value is caused by the very small fluorescence intensity for the free ANS. This value of the enhancement can now be used to analyse the ANS–GDH interaction by the method of Scatchard (1949). Titrations of GDH (1mg./ml.) with a concentrated solution of ANS were carried out so that dilution effects were negligible. From these the plot shown in Fig. 3 was derived, which gave 9 ± 1 ANS-binding sites/oligomer (mol.wt. 310000) with an average K_D of 0·22mM. The error in the intercept mainly arises from the inaccuracy in the derivation of the enhancement value of 100-fold. Extension of the Scatchard plot beyond the last point shown led to deviation from linearity, indicating further sets of even weaker binding sites.

Only high concentrations of ANS (2mM) affected the polymer–oligomer dissociation of the enzyme, but at low dye concentrations (1–200μM) ANS had no effect on the sedimentation, optical-rotatory-dispersion and catalytic properties of the enzyme, although dye binding is still easily detected by fluorescence enhancement.

ANS as a conformational probe

When a solution containing GDH (1mg/ml.), ANS (20μM) and NADH is titrated with GTP the fluorescence enhancement of ANS can be followed (Fig. 4). In all ANS experiments the ANS fluorescence was excited at 410nm. and emission observed at 550nm., so that contributions from NADH fluorescence were eliminated. GTP in the absence of NADH had no effect on the GDH–ANS fluorescence. Similar transitions were observed when the

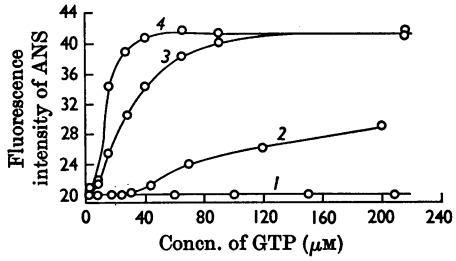

Fig. 4. Fluorescence increase of ANS on titrating GDH with GTP in the presence of various concentrations of NADH. GDH was present at 1mg./ml. and ANS at 2μM. Curve *1*, no NADH present; curve *2*, 10μM-NADH; curve *3*, 50μM-NADH; curve *4*, 500μM-NADH. The excitation and emission wavelengths were 410 and 550nm. respectively.

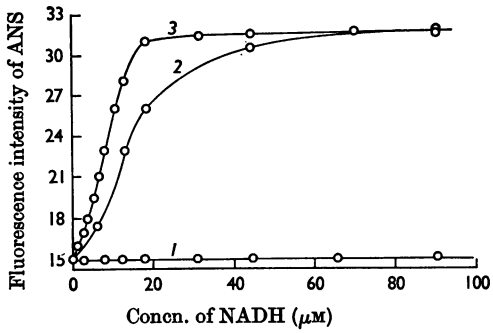

Fig. 5. Fluorescence increase of ANS on titrating GDH with NADH in the presence of various concentrations of GTP. GDH was present at 1mg./ml. and ANS at 20μM. Curve *1*, no GTP; curve *2*, 50μM-GTP; curve *3*, 500μM-GTP. The excitation and emission wavelengths were 410 and 550nm. respectively.

solutions were titrated with NADH in the presence of GTP (Fig. 5). A well-defined final value was reached in the titrations that was not affected by further additions of both ligands. At very low concentrations of one of the ligands (e.g. 10μM-NADH in Fig. 4) the limit could not be observed. The transitions detected by the ANS method occur in the same ligand concentration range as was observed by optical rotatory dispersion (Bayley & Radda, 1966). The same transition can also be observed by following the enhancement of NADH fluorescence by GTP in the presence of GDH (1mg./ml.) and low concentrations of NADH (4μM). Fig. 6 shows that both methods follow a similar titration curve, but the NADH enhancement can only be observed in a very limited range of coenzyme concentration. High concentrations of NADH (Bayley & Radda, 1966) or 5% (v/v)

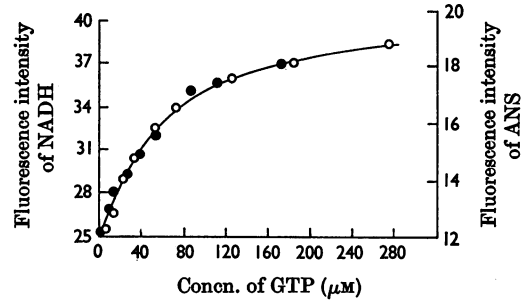

Fig. 6. Titration of GDH with GTP in the presence of NADH. GDH was present at 1mg./ml. and NADH at 4μM. ○, no ANS, excitation at 355nm., emission observed at 470nm.; ●, 10μM-ANS, excitation at 410nm., emission observed at 550nm.

dioxan (Churchich & Wold, 1963) cause dissociation of the enzyme aggregate to the oligomer. Neither of these reagents, however, lead to an enhancement of the ANS–GDH fluorescence. 'Fully active non-associating' GDH prepared by the method of Colman & Frieden (1966*a*,*b*), on the other hand, also gives the normal titration curve by GTP and NADH when observed by the ANS method.

Analysis of the results. It is convenient at this stage to introduce the nomenclature that is used in the discussion and presentation of these and subsequent experiments. The enzyme conformation predominating before the action of NADH and GTP is called the R form and the final conformation is called the T form. The $\bar{R}$ function (Monod *et al.* 1965) was calculated on the assumption that the initial ANS fluorescence intensity in the titration represented all the protein existing in the R form and the final value represented all the enzyme existing in the T form. [This assumption is inherent in other methods for following the conformational equilibria (cf. Gerhart & Schachman, 1968).] At any intermediate fluorescence value the $\bar{T}=1-\bar{R}$ function (the proportion of enzyme in the T form) is calculated on the assumption that:

$$\text{ANS fluorescence} = f_R R + f_T T$$

where f_R is the intrinsic fluorescence intensity of ANS bound to the R form and f_T that for ANS bound to the T form, and R and T are the concentrations of the two forms. f_R and f_T are calculated from the initial and final values of fluorescence and the known concentration of the enzyme. The assumption that the ANS fluorescence is proportional to the enzyme concentration in the range studied is borne out by the observations shown in Fig. 7 and by the fact that the shape of the titration curves is independent of the amount of ANS used (Table 1).

The steepness and symmetry of the titration curves were evaluated by calculating the parameters:

$$R_s = \frac{s_{0.9}}{s_{0.1}} \text{ and } R_a = \frac{(s_{0.1})(s_{0.9})}{(s_{0.5})^2}$$

where $s_{0.1}$, $s_{0.5}$ and $s_{0.9}$ are the ligand concentrations at 10%, 50% and 90% of the change in ANS fluorescence intensity during the titration. This nomenclature was introduced by Koshland, Némethy & Filmer (1966) for expressing the steepness (R_s) and symmetry (R_a) of the ligand-binding curves.

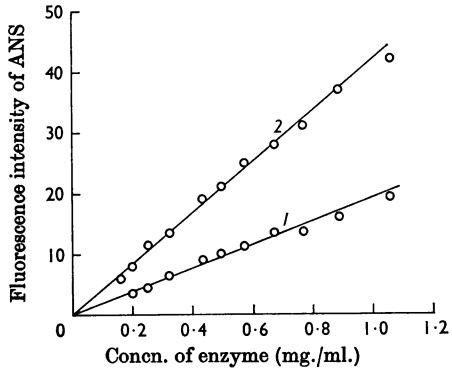

Fig. 7. Increase of ANS fluorescence with GDH concentration. ANS was present at 10 μM. Curve *1*, only GDH present; curve *2*, NADH (0·2 mM) and GTP (0·4 mM) added.

Variation of titration parameters with ANS concentration. The titration parameters were independent of the ANS concentrations used (Table 1). In all titrations the lowest concentrations of ANS giving a satisfactory fluorescence intensity and not interfering with any of the enzyme properties were used. This varied from 2 μM- to 20 μM-dye.

Effect of enzyme concentration on titration curves. These results are shown in Table 2. It is clear that $s_{0.5}$ increases with increasing enzyme concentration whereas the values for fluorescence enhancement, R_s and R_a, remain constant throughout the concentration range studied. It is important to note that enzyme at concentrations as low as 0·22 mg./ml. gives curves of similar shapes to those obtained with enzyme at 2·2 mg./ml. The changes are still clearly observable at 0·11 mg. of enzyme/ml. (enhancement 1·9-fold), although because of the small fluorescence intensities measured the shape of these curves could not be accurately obtained.

Effect of NADH concentration on the GTP titration curves. When GDH (1 mg./ml.) is titrated by GTP

Table 1. *Variation of the $\bar{T}$ function with ANS concentration*

GDH was present at 0·2 mg./ml. and GTP at 0·26 mM. NADH was the titrating ligand.

Concn. of ANS (μM)	$s_{0.1}$ (μM-NADH)	$s_{0.5}$ (μM-NADH)	$s_{0.9}$ (μM-NADH)	$s_{1.0}$ (μM-NADH)	R_s	R_a	Final ANS fluorescence/ initial ANS fluorescence
2	2·3	8·0	16	33	7·0	0·58	1·7
4	2·0	6·0	~ 29	42·5	—	—	1·7
10	1·6	4·8	9·3	15	5·8	0·65	1·6
20	1·5	5·8	12·8	21	8·5	0·56	2·0
52	2·0	4·5	11·5	22	5·8	1·14	1·6
100	1·6	5·3	13	22·5	8·1	0·74	1·6

Table 2. *Variation of the $\bar{T}$ function with GDH concentration: symmetry of the titration curves*

GTP was present at 0·21 mM and ANS at 20 μM. NADH was the titrating ligand.

Concn. of GDH (mg./ml.)	$s_{0.1}$ (μM-NADH)	$s_{0.5}$ (μM-NADH)	$s_{0.9}$ (μM-NADH)	$s_{1.0}$ (μM-NADH)	R_s	R_a	Final ANS fluorescence/ initial ANS fluorescence
0·11	0·55	2·8	11	22	20	0·73	1·9
0·22	1·3	4·0	9·5	20	7·3	0·77	2·5
0·43	1·9	5·8	15	28	7·9	0·86	2·03
0·65	2·8	7·3	17·5	35	6·2	0·95	1·96
0·87	3·3	9·8	21	42	6·4	0·73	2·05
1·5	4·5	10	29	51	6·4	1·3	1·96
2·2	6·5	19·8	42·5	53	6·5	0·71	1·75

Table 3. *Variation of the $\bar{T}$ function with NADH concentration*

GDH was present at 1 mg./ml. and ANS at 20 μM. GTP was the titrating ligand.

Concn. of NADH (μM)	$s_{0.1}$ (μM-GTP)	$s_{0.5}$ (μM-GTP)	$s_{0.9}$ (μM-GTP)	$s_{1.0}$ (μM-GTP)	R_s	R_a	Final ANS fluorescence/ initial ANS fluorescence
0	0	0	0	0	0	0	0
5	33	82	121	130	0·37	0·59	1·26
10	36	110	350	600	0·97	1·04	1·53
50	11	28·5	98	155	8·9	1·33	1·84
100	4·5	17·5	46·5	65	10·3	3·02	1·80
500	8·0 (?)	12·0	32	65	4·0	1·78	1·66
1000	2·63	8·5	23	25–50	9·2	0·8	1·59

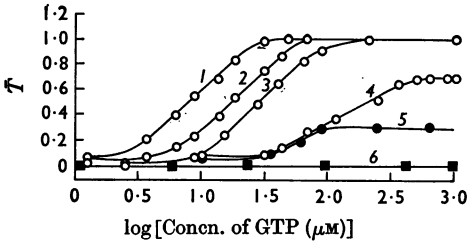

Fig. 8. $\bar{T}$ function derived from ANS fluorescence in the titration of GDH with GTP. GDH was present at 1 mg./ml. and ANS at 20 μM. Curve *1*, 1 mM-NADH; curve *2*, 0·1 mM-NADH; curve *3*, 50 μM-NADH; curve *4*, 10 μM-NADH; curve *5*, 5 μM-NADH; curve *6*, no NADH.

in the presence of different amounts of NADH (Table 3) a series of titration curves is obtained in which the $s_{0.5}$ value decreases with increasing NADH concentrations (Fig. 8). In all cases (except at 5 μM- and 10 μM-NADH) the same enhancement is observed and the curves have approximately the same steepness and symmetry, as shown by the R_s and R_a values. This is more clearly shown in plotting log[GTP] against $\bar{T}$, when the curves (Fig. 8) are parallel. The two curves at low NADH concentrations (5 μM- and 10 μM-NADH) can be further enhanced by addition of more NADH at the end of the titration curves, whereas in the other cases this does not happen.

Variation of titration parameters with ionic strength. The half-transition points ($s_{0.5}$) are gradually shifted to higher ligand concentrations with increasing ionic strength, although the shape of the titration curves (R_s and R_a) is not significantly affected. (Sodium chloride was used to change the ionic strength.) The final fluorescence enhancement value decreases somewhat with increasing ionic strength (Table 4).

Effect of pH *on the transitions.* The effect of pH on the allosteric behaviour of the enzyme was investigated by using a buffer consisting of (final concentrations) 50 mM-sodium dihydrogen phosphate, 20 mM-trisodium citrate, 20 mM-glycine and 20 mM-tris–hydrochloric acid. The pH of the solutions was adjusted to the required value with either concentrated hydrochloric acid or potassium hydroxide. Enzyme prepared in this buffer at pH 7·7 had the same activity as in 0·1 M-potassium phosphate at the same pH and the activity remained constant over a 150 min. period. The effect of pH on the fluorescence of ANS bound to the enzyme in the absence of ligands is shown in Fig. 9. The variation in the parameters for the titration of GDH (1 mg./ml.) and GTP in the presence of NADH (0·2 mM) with pH is summarized in Table 5.

Effect of other ligands on ANS fluorescence. The effect of a variety of ligands and ligand combinations on the fluorescence of ANS (20 μM) bound to GDH (1 mg./ml.) was studied, and the following ligands and combinations of ligands had no effect at final concentrations of at least 1 mM for each ligand: NAD^+; NAD^+–D-glutamate; NAD^+–NADH; NADH–D-glutamate; NADH–D-glutamate–ammonium chloride; GTP–α-oxoglutarate; GTP–NAD^+; ATP; ATP–NADH; guanosine; guanosine–NADH.

The NADH–GTP effect on the fluorescence of ANS bound to GDH could be reversed by activators of the enzyme (Table 6). ADP by itself decreased the fluorescence of bound ANS, probably by displacing some of the ANS molecules from the enzyme. Thus at high ANS concentrations (40 μM) there was a 43% decrease in ANS fluorescence on changing the ADP concentration from 0·3 to 0·7 mM.

In contrast with GTP, other allosteric inhibitors of GDH such as diethylstilboestrol (Fig. 10), and phenanthridine gave an enhancement of ANS fluorescence in the absence of NADH although NADH did affect both titration curves. The diethylstilboestrol-induced fluorescence enhancement was not reversible by ADP.

Table 4. *Variation of the $\bar{T}$ function with ionic strength*

GDH was present at 0·23 mg./ml. and ANS at 20 μM. NADH was the titrating ligand.

Concn. of NaCl added (M)	$s_{0.1}$ (μM-NADH)	$s_{0.5}$ (μM-NADH)	$s_{0.9}$ (μM-NADH)	$s_{1.0}$ (μM-NADH)	R_s	R_a	Final ANS fluorescence/ initial ANS fluorescence
—	1·0	3·7	8·5	19	8·5	0·62	2·4
0·1	1·75	6·5	20	30–45	14·3	0·84	2·2
0·2	3·3	9·5	25	40–50	7·6	0·91	2·16
0·3	7·0	15	44·5	~90	6·4	0·98	2·15
0·4	8·5	25·5	75	~140	8·8	0·98	2·04
0·5	22	86	214	~390	9·7	0·64	1·92

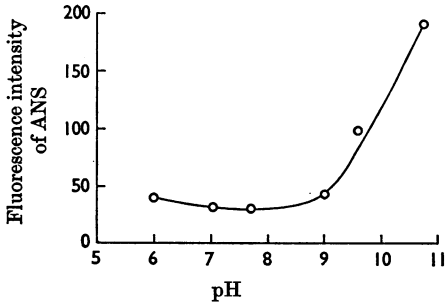

Fig. 9. Effect of pH on ANS fluorescence in the presence of GDH. GDH was present at 1 mg./ml. and ANS at 20 μM. The fluorescence of free dye is constant over this pH range.

Rate of conformational change. The rates of change of state were measured with the stopped-flow apparatus by employing fluorescence as the method of detection. The rate could be followed either when GTP (25–400 μM) was rapidly mixed with NADH (25–200 μM), GDH (0·5–2 mg./ml.) and ANS (20 μM) or when NADH was mixed with GTP, GDH and ANS. (The concentration ranges refer to the final concentrations obtained after mixing.) Under these conditions two distinct rate processes are observed (Fig. 11), which are clearly shown when the data are plotted on a semi-logarithmic scale. The good linearity of these plots shows that both rates are of first-order. We are confident that the two-phase curve is real, since we have observed it in about 50 successful experiments; no change was observed in any of the blanks (at least 30, constituting various combinations of the reactants). Thus when ANS (20 μM) was mixed with solutions of GDH alone, or with GDH (1 mg./ml.) and NADH, (100 μM) or with GDH, NADH and GTP (100 μM), all the enhancement was over by the time observation began (i.e. in less than 4 msec.). Similarly when NADH or GTP alone was mixed rapidly with a solution containing GDH and ANS in no case did we observe any changes. The fluorescence of NADH could be easily excluded by using an emission filter that cut out all light below 500 nm. so that the changes detected were those associated with ANS fluorescence. The mean deviations in our rate measurements were 30% in both the slow and the fast phases. Thus when GDH (1 mg./ml.), NADH (100 μM) and ANS (20 μM) were mixed with GTP (75 μM) the half-lives obtained were: 34 ± 13 msec. and 200 ± 60 msec. for the fast and slow phases respectively in an average of ten measurements. The rates were the same within experimental accuracy when GTP was mixed with the remaining components or when NADH was mixed with the other components. ANS could also be placed into either of the mixing syringes without affecting the rates.

We were only able to follow the rates when the change in fluorescence was sufficiently large (i.e. when the change corresponded to the full enhancement value obtained in the titrations shown in Figs. 4 and 5). This limitation arose because we always observed a change above a background fluorescence. Also, correction had to be made for scattering by the sample chamber. This limited the concentration range of the ligands that we could use in our rate measurements to those given above. We were also limited in the available concentration range in protein (0·5–2 mg./ml.), since at low protein concentrations our method of detection was too insensitive to be accurate, and at higher protein concentrations we had problems from background scattering.

Within these restrictions we found no significant variations in rate with either GTP or NADH concentrations, and both phases were observed and occurred at the same rate at 0·5 mg. of enzyme/ml. as at 2 mg./ml. Thus the average half-life for the fast phase in 27 measurements, which included variations in GDH, NADH and GTP concentrations, was 34 ± 12 msec. and for the slow phase in 30 measurements 240 ± 60 msec., i.e. all the variations observed were within the experimental error.

Table 5. *Variation of the $\bar{T}$ function with* pH

GDH was present at 1 mg./ml., ANS at 40 μM and NADH at 0·2 mM. GTP was the titrating ligand.

pH	$s_{0\cdot1}$ (μM-GTP)	$s_{0\cdot5}$ (μM-GTP)	$s_{0\cdot9}$ (μM-GTP)	$s_{1\cdot0}$ (μM-GTP)	R_s	R_a	Final ANS fluorescence/ initial ANS fluorescence
6·0	19	90	315	590	16·6	0·74	1·7
7·2	24	69	120	210	5·0	0·61	1·8
7·4	16	39	90	140	5·6	0·95	1·9
7·8	10	22	44	75	4·4	0·91	2·02
8·0	3	16·5	38·3	50	12·4	0·42	2·02
9·0	1·5	13·5	48	30	32	0·40	1·6
9·6	1–2	19	46·5	50	23–47	0·13–0·26	—
10·0	2	8	32	50	11–16	1·0–1·5	1·2

Table 6. *Reversal of the NADH–GTP-induced conformational change in GDH as indicated by ANS fluorescence intensity*

Ligand	Percentage reversal	Concn. of ligand at full reversal (mM)	Concn. of ligand at half reversal (mM)
ADP	120	0·7	0·1
L-Leucine	113	11	1·2
L-Methionine	90	30	13

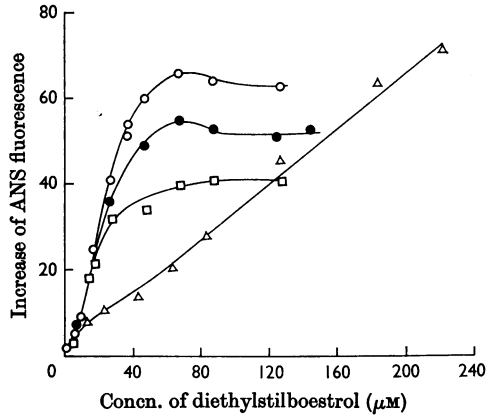

Fig. 10. Titration of GDH with diethylstilboestrol in the absence and presence of NADH. GDH was present at 1 mg./ml. and ANS at 20 μM. ○, 63 μM-NADH; ●, 630 μM-NADH; □, 1·3 mM-NADH; △, no NADH.

By changing the observation filter to a yellow filter (Chance Brothers OY 8) it is possible to follow NADH fluorescence, provided that ANS is not included in the reaction mixtures. When NADH (0·1 mM) is mixed with GDH (1 mg./ml.) enhancement of NADH fluorescence occurs before observation begins, whereas in the presence of GTP (75 μM) the rate of enhancement can be observed. In this case we can only detect the fast phase (with approximately the same rate constant as the ANS change above). Should there be, however, a slow change consisting of less than 10% of the total change we would not have been able to detect it.

DISCUSSION

The interaction of ANS with GDH is weak although it is accompanied by a large increase in the fluorescence quantum yield of the dye and by a blue shift in its emission maximum. These changes indicate that the 9 binding sites/oligomer are weakly hydrophobic. The number of binding sites found by Thompson & Yielding (1968) is considerably different from ours. We have no immediate explanation for this discrepancy. Clearly this number is very sensitive to the value of enhancement derived by extrapolation for the fluorescence of bound ANS and it is essential that this extrapolation should be carried out at a series of protein concentrations.

The fact that GTP, NADH and NAD^+ do not compete with ANS binding shows that the ANS-binding sites do not overlap with the binding sites of these ligands. ANS inhibits the enzyme only at high concentrations and has no effect on its optical-rotatory-dispersion properties at the concentrations used in the fluorescence experiments. The effect of the dye at high concentrations on the activity and sedimentation properties of the enzyme is probably

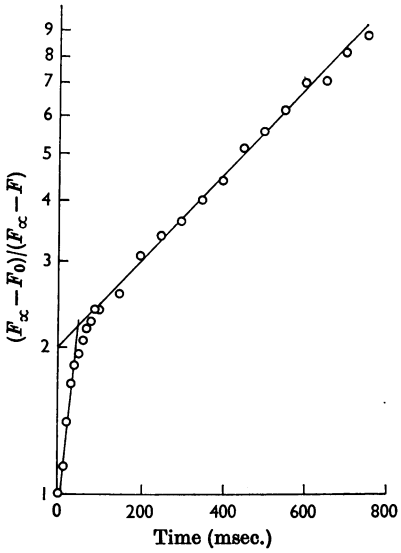

Fig. 11. Semi-logarithmic plot of oscilloscope trace of ANS fluorescence in stopped flow. The final concentrations in the reaction mixture were: GDH, 1 mg./ml.; ANS, 200 μM; NADH, 0·1 mM; GTP, 75 μM. A solution containing GDH, ANS and NADH was mixed with an equal volume of a solution containing GTP.

similar to that of a detergent, involving many weak interactions. This is supported by the fact that when GDH is titrated with ANS deviations from linearity occur in the Scatchard plots at very high dye concentrations.

In contrast ADP competes with some (though not all) ANS-binding sites, providing further support for the suggestion that the ADP-binding and GTP-binding sites of GDH are not identical (Colman & Frieden, 1966*a*,*b*; Dodd & Radda, 1967).

ANS reflects the structural transitions in the protein brought about by the combined action of GTP and NADH. This is supported by two observations: (1) the changes detected by ANS take place over the same ligand concentration range as those detected by optical rotatory dispersion (Bayley & Radda, 1966); (2) under some conditions (4 μM-NADH) the conformational changes can be detected by the fluorescence of NADH; if now ANS is added to the system the change in ANS fluorescence closely parallels that in NADH (Fig. 6).

Our results are at variance with those of Thompson & Yielding (1968), who were unable to detect any changes in ANS fluorescence in the presence of GTP and NADH.

The changes in ANS fluorescence caused by the NADH–GTP heterotrophic interaction are not dependent on the state of further aggregation of GDH into units larger than the 310000-mol.wt. oligomer (Eisenberg & Tomkins, 1968). This is supported by the following observations: (1) the transition is clearly observed at protein concentrations in the range 0·1–2 mg./ml. and the value of enhancement of ANS fluorescence remains constant in this range; (2) ANS titration curves are normal even with the 'fully active non-associating' acetylated enzyme, prepared by the method of Colman & Frieden (1966*a*,*b*); (3) when dissociation is brought about by high concentrations of NADH (Bayley & Radda, 1966) or by 5% dioxan (Churchich & Wold, 1963) no enhancement of ANS fluorescence is observed.

The shape of the titration curves for a given protein concentration is independent of ANS concentration in the range 2 μM–2 mM (Table 1). In the range of protein concentrations employed in our studies the fluorescence of bound ANS is proportional to GDH concentration both in the presence and absence of NADH and GTP (Fig. 7). These sets of observations enable us to use ANS fluorescence as a quantitative measure of the fraction of protein in the active (R) and inhibited (T) conformations. The $\bar{T} = 1 - \bar{R}$ functions (Monod *et al.* 1965; Rubin & Changeux, 1966) derived in this way involve several assumptions, and it is important to emphasize these as they are inherent in other experimental methods as well (Gerhart & Schachman, 1968). These are that the limit reached in the titrations represents the enzyme predominantly in the T form, that the initial fluorescence value represents the enzyme existing predominantly in the R form and that the enhancement of ANS fluorescence is proportional to the number of subunits in the T form in one oligomer if there are intermediate stages (Koshland *et al.* 1966).

The first assumption is justified by the observations that no further increase in fluorescence beyond the limit can be obtained by adding GTP and NADH at very high concentrations. It has not been possible to test the second assumption experimentally, although the conclusions will be valid even if as much as 10% of the enzyme is initially in the inactive form. The third assumption would be valid if the model of Monod *et al.* (1965) applied to our system. If the conformational changes were sequential (Koshland *et al.* 1966) the assumption would be valid only if the intrinsic fluorescence of ANS bound to the intermediate conformations truly reflected the number of subunits that have changed. In any case the qualitative conclusions that can be drawn do not depend on the validity of these three assumptions. The $\bar{T}$ function can be simply considered as a method for presenting the experimental results.

The variation of the titration curves with NADH

and GTP concentrations confirms the suggestions of Bayley & Radda (1966) in that the conformation change occurs over a narrow concentration range of the titrating ligand and in that the half-points in the transitions depend on the combined effects of NADH and GTP, showing that the interaction is heterotropic (Figs. 5 and 6 and Table 3).

The effect of pH on the allosteric transitions of GDH cannot be interpreted in detail, since the changes observed may reflect the pH-dependence of NADH or GTP binding or that of the protein conformational equilibrium. But it is important that the system responds to the ligands most sharply (i.e. with the lowest value for R_s) at pH 7·8 (Table 5), which is close to the pH optimum of the enzyme. ANS binding and the fluorescence of bound ANS is constant in the range pH 5·5–9·0 (Fig. 9) and is not the cause of the observed effect. The increase in ANS fluorescence between pH 9·0 and 11·0 is due to the dissociation of the enzyme into peptide subunits (Fisher, McGregor & Power, 1962).

The allosteric response of the enzyme decreases with increasing ionic strength of the solutions (Table 4). This decreased response is a result of the change in the position of the transitions (measured by $s_{0.5}$) and not that of a change in the co-operativity of the transition (measured by R_a). Similar effects are observed on chemical modifications of the GTP-binding sites (Price & Radda, 1969). The effect of several anions on the allosteric response of dogfish GDH is similar (Corman & Kaplan, 1967). Salts also increase the stability of GDH (di Prisco & Strecker, 1966), suggesting that they interact with the enzyme.

The effect of other inhibitors (diethylstilboestrol, phenanthridine) is different from that of GTP in two respects: it occurs in the absence of coenzyme and does not reach a distinct limit of fluorescence enhancement of ANS. The effect of NADH on these transitions is more complex than with GTP and suggests that the conformational changes brought about by GTP and NADH may be different from those taking place with other inhibitors.

Since ANS fluorescence follows the conformational transition, the rate of this change is directly observable, in this case by stopped-flow fluorescence. The biphasic exponential curves measure a biphasic structural change. This conclusion is based on the following observations: (1) the rates of NADH and ANS binding under the conditions of our experiments are too fast to be measurable; (2) GTP binding is equally likely to be fast, particularly since the same rates are observed when GTP is added to the enzyme before mixing or when GTP is in the other mixing syringe; (3) the fluorescence we were observing was that of ANS alone; (4) in no single case did we observe fast or slow changes in our stopped-flow apparatus in the control experiments; (5) the biphasic curves were observed very consistently (in at least 50 experiments); (6) the time-constant of our instrument has been established by several independent methods. We are emphasizing these points because we are fully aware of the possible artifacts that may arise in fast-reaction measurements.

One possible explanation for the two rates is that the fast phase represents a conformational change in the subunits brought about by the binding of the ligands. This is then followed by a slower rearrangement of the subunits into a different geometry of the oligomer. An alternative explanation assumes that the dissociation of the aggregate (undoubtedly present at 1 mg. of enzyme/ml.) is rate-limiting. But because ANS is intrinsically incapable of detecting this dissociation our observation would require that only the dissociated form (i.e. the oligomer) is capable of undergoing the conformational change. From our limited studies on the effect of protein concentrations on the rates we prefer the first hypothesis.

Using the published values for the binding constants of GTP (Colman & Frieden, 1966*a*,*b*) and NADH (Bayley & Radda, 1966) to the R form and the T form of the enzyme one can generate theoretical $\bar{T}$ functions by the method of Rubin & Changeux (1966). Our initial calculations suggest that the changes in GDH cannot be accommodated by the simple model. Because there is some uncertainty in the experimental binding constants and some doubt as to whether they represent the microscopic constants we consider that a more detailed presentation of these calculations is not justified at present. Our conclusions, however, are supported by some other studies (Malcolm & Radda, 1968).

In recent years a great deal of emphasis has been placed on the importance of co-operativity in enzyme regulation. Our results show that external variables, such as ionic strength and pH, significantly alter the response of an enzyme to inhibitors, merely by changing the position at which the transitions occur. We consider that these effects may provide an additional important mechanism for regulation, particularly in a less aqueous environment such as exists inside a cell or a membrane.

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