Biochem. J. (1972) **128**, 933–940
Printed in Great Britain

Protein Fluorescence of Nicotinamide Nucleotide-Dependent Dehydrogenases

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(*Received* 21 *February* 1972)

1. The decrease in the protein fluorescence (F) of *Neurospora crassa* glutamate dehydrogenase is linearly related to the increase in the fraction of the coenzyme sites occupied by NADPH (α) at pH6.35. Under these conditions NADPH causes this enzyme to dissociate to monomers. 2. There is a non-linear relationship of F to α for NADH binding to give the alcohol dehydrogenase–NADH–isobutyramide complex, the L-glycerol 3-phosphate dehydrogenase–NADH complex and the bovine glutamate dehydrogenase–NADH–glutamate complex. The non-linearity is accurately represented by $F=[1-\alpha(1-x)]^n$ where n is the number of NADH-binding sites per protein molecule. 3. The co-operative binding of GTP to bovine glutamate dehydrogenase in the presence of NADH gives a linear relationship between F and α. 4. The prediction from the equation $F=[1-\alpha(1-x)]^n$ that initial tangents to non-linear protein-fluorescence-quenching curves will intercept the fluorescence when $\alpha=1$ at a value of total ligand concentration less than the sum of the concentration of binding sites in the solution plus the dissociation constant of ligand is quantitatively fulfilled. 5. Non-linear protein-fluorescence titrations may be used to obtain information about the distribution of ligand among the protein molecules in solution.

In the preceding paper (Holbrook, 1972) it was shown that the change in the protein fluorescence of lactate dehydrogenase (F) was non-linearly related to the fraction (α) of the coenzyme-binding sites occupied with NADH. The change was represented by the equation $F=[1-\alpha(1-x)]^n$. It was suggested that this equation would also describe the changed protein fluorescence when NADH binds to other dehydrogenases when the n coenzyme-binding sites are intrinsically identical, indistinguishable and do not interact (Holbrook, 1972). For the situation where $n=1$, or when there is complete positive or negative co-operativity in NAD(P)H binding, it was suggested that F would be linearly related to α. The present paper examines these suggestions for a number of dehydrogenases and demonstrates the usefulness of protein fluorescence as a probe for the state of aggregation of the NADPH complex of *Neurospora crassa* glutamate dehydrogenase.

Experimental and Results

The changed protein fluorescence as a function of the fraction of coenzyme-binding sites occupied by NAD(P)H was measured by using the titrating split-beam differential fluorimeter. The measured protein fluorescence (excited at 305nm, emission through a Kodak–Wratten no. 18A filter) and the measured difference in NAD(P)H fluorescence (excited at 320nm, emission through a Kodak–Wratten no. 98 filter) were corrected for decreases in the intensity of the exciting radiation and for the emitted fluorescence by the added NAD(P)H. The apparatus, correction factors and methods of handling experimental results were identical with those described in the previous paper (Holbrook, 1972). All buffers were prepared by adjusting solutions of the acid, equivalent to the anion mentioned, with 5M-NaOH to the required pH.

Horse liver alcohol dehydrogenase

This was obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. The crystals were centrifuged down and were redissolved in 67mM-phosphate buffer, pH7.2, and were dialysed for 24h against changes of this buffer containing charcoal (1mg/ml) at 0°C. The solvent for the titrations was the phosphate buffer containing 10mM-isobutyramide; the final protein concentration was 84μg/ml. NADH (12mM) was added at 5μl·min^{-1}. The uncorrected difference in NADH fluorescence was visually estimated from

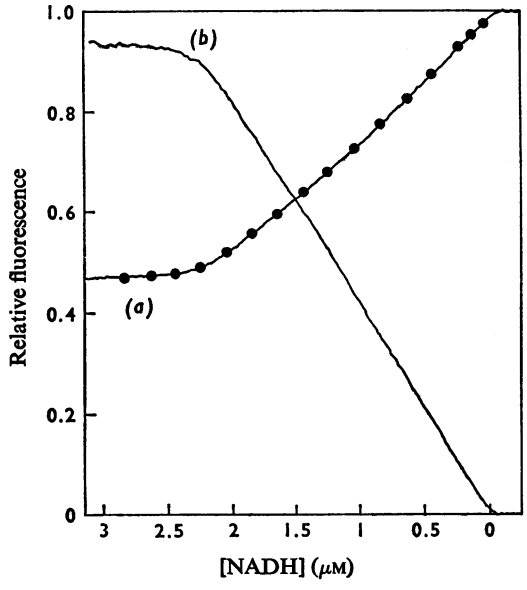

Fig. 1. *Comparison of the decrease in protein fluorescence and increase in NADH fluorescence on formation of the alcohol dehydrogenase–NADH–isobutyramide complex*

Protein fluorescence (*a*) was excited at 305nm and measured through a Kodak–Wratten no. 18A filter. The difference in NADH fluorescence (*b*) between solutions with and without enzyme was excited at 320nm and measured through a Kodak–Wratten no. 98 filter. NADH was continuously added to a stirred solution of horse liver alcohol dehydrogenase (84μg/ml) in a solution of isobutyramide (10mM) in 67mM-sodium phosphate buffer, pH7.2. The points (●) were calculated from $F=[1-\alpha(1-0.69)]^2$ and were slightly decreased in value to simulate the reabsorption of the protein fluorescence by added NADH.

Fig. 1 to be 0.94 and the corrected value was 0.95. The non-linear relation between F and α is given in Table 1.

L-*Glycerol 3-phosphate dehydrogenase*

This was prepared according to Telegdi (1964), except that, as a final purification, a solution of enzyme which had been crystallized was filtered through a column of Sephadex G-200 in a buffer of 10mM-EDTA and 0.3M-NaCl adjusted to pH6.0 with 5M-NaOH. The second of the three major protein peaks eluted was the active enzyme. After storage, the enzyme was carefully refractionated with $(NH_4)_2SO_4$ until the equivalent weight per NADH-binding site was decreased to 36000±5%, and the protein fluorescence of the enzyme–NADH complex

Table 1. *Variation of the protein fluorescence (F) with the degree of saturation (α) of the NADH-binding sites of dehydrogenases*

Protein fluorescence in the absence of added NADH ($\alpha = 0$) was 1000. Other details are in the Experimental and Results section of the text.

Horse liver alcohol dehydrogenase–NADH–isobutyramide complex													
F	930.12	876.10	826.97	777.72	733.39	685.92	643.38	603.77	563.05	526.28	496.51	483.92	
α	0.1128	0.2036	0.2903	0.3772	0.4642	0.5556	0.6344	0.7187	0.8083	0.8865	0.9520	0.9815	
Rabbit muscle L-glycerol 3-phosphate dehydrogenase–NADH complex													
F	884.33	750.25	635.86	535.20	445.33	370.32	308.22	259.09	231.10	216.22	207.39	204.65	
α	0.1305	0.2419	0.3506	0.4737	0.5899	0.6941	0.7996	0.8819	0.9337	0.9591	0.9703	0.9795	
Bovine liver glutamate dehydrogenase–glutamate–NADH complex													
F	889.2	848.6	792.7	848.7	713.7	680.5	652.3	629.1	607.9	568.2	534.3	514.6	493.8	477.9	450.0	432.3
α	0.125	0.152	0.222	0.270	0.314	0.357	0.398	0.432	0.460	0.516	0.571	0.608	0.640	0.657	0.726	0.755

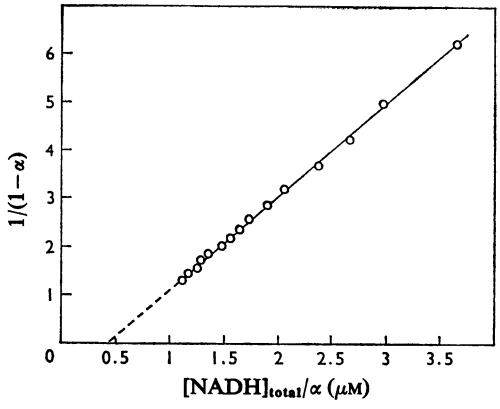

Fig. 2. *Test of the independence of the two NADH-binding sites of* L-*glycerol 3-phosphate dehydrogenase*

Analysis of the fraction (α) of the total NADH-binding sites (E_0) occupied during the titration of the enzyme (14.4μg/ml) in 67mM-sodium phosphate buffer, pH7.2, with NADH according to the equation $K_d/(1-\alpha) = [NADH]_{total}/\alpha - [E_0]$.

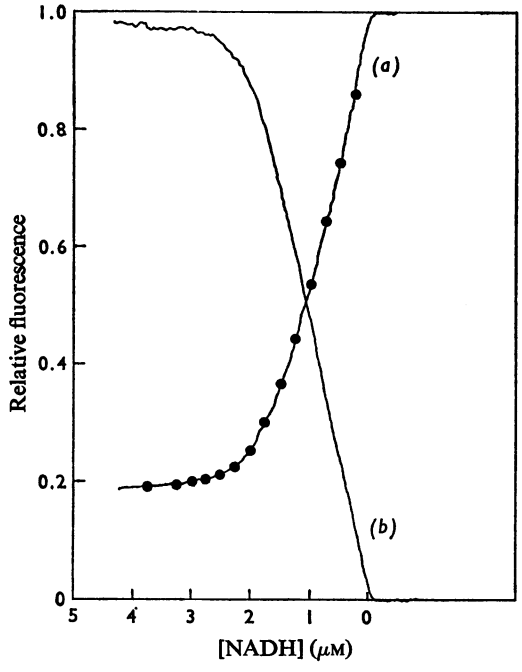

Fig. 3. *Non-linear decrease in the protein fluorescence of* L-*glycerol 3-phosphate dehydrogenase on forming a complex with NADH*

The curves are from two experiments in which (*a*) the protein fluorescence and (*b*) the difference in NADH fluorescence was measured during the titration of the enzyme (75μg/ml) with NADH in 5mM-EDTA, pH7.5. The points (●) were calculated from $F = [1-\alpha(1-0.438)]^2$ and were slightly decreased in value to simulate the reabsorption of the protein fluorescence by the added NADH.

was 0.19 of that of the free enzyme. Protein concentration was determined by using $E^{1\%}_{280} = 5.7$.

In an experiment to test the independence of the NADH-binding sites, a solution of the enzyme (14.4μg/ml) in 67mM-phosphate buffer, pH 7.2, was titrated with NADH and the difference in NADH fluorescence was measured. A plot of $1/(1-\alpha)$ against $[NADH]_{total}/\alpha$ was linear (Fig. 2). The dissociation constant, given by the reciprocal slope, was 0.5μM. The concentration of NADH-binding sites, given by the extrapolated intercept of the graph with the $[NADH]_{total}/\alpha$ axis, was 0.4μM.

The non-linear relation between F and α was obtained from titrations of enzyme (75μg/ml) with 2mM-NADH at 5μl·min^{-1} in a buffer of low ionic strength: 5mM-EDTA at pH7.5 (Fig. 3). The uncorrected maximum difference in NADH fluorescence was 0.98 and the corrected value was 0.99. In the low-ionic-strength buffer the affinity of the enzyme for NADH was increased and the dissociation constant is approximately 30nM. The non-linear relation between F and α is given in Table 1.

Ox liver glutamate dehydrogenase

This was a suspension in $(NH_4)_2SO_4$ solution prepared by a method depending on ion-exchange chromatography developed in collaboration with Whatman Biochemicals, Maidstone, Kent, U.K.). The enzyme was prepared for experiments by finally dialysing a concentrated solution against 30mM-phosphate buffer, pH6, containing charcoal (1mg/ml). Reproducible S-shaped curves were only obtained with GTP if particular care was taken to remove, and not to recontaminate the enzyme with, nucleotides.

The change in protein and nucleotide fluorescence during titration of enzyme (0.16mg/ml) and 0.15mM-NADH in 6.7mM-phosphate buffer, pH7.2, with GTP (1mg/ml) added at 5μl·min^{-1} is shown in Fig. 4. Enzyme was omitted from the reference cuvette for both titrations. The NADH-fluorescence increased and the protein fluorescence decreased on addition of GTP. No corrections were made to the recorded results, since GTP is colourless at 320–400nm. The linear relation between F and α was demonstrated because the ratio (nucleotide fluorescence)/(1-protein fluorescence) was constant within experimental error.

The coenzyme-binding capacity of the enzyme was measured by the method of Hummel & Dreyer (1962). A bed (22cm×0.9cm diam.) of Sephadex G-50 (fine

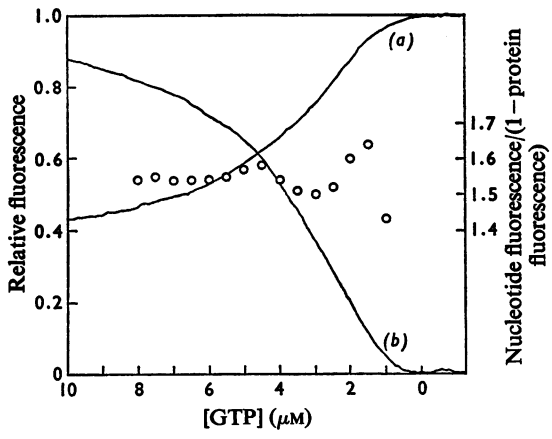

Fig. 4. *Demonstration of the linear relationship between (a) the decrease in protein fluorescence and (b) the increase in NADH fluorescence as GTP combines with bovine liver glutamate dehydrogenase*

The enzyme (0.16mg/ml) was continuously titrated with GTP in 6.7mM-sodium phosphate buffer, pH7.2, containing 0.15mM-NADH. The points (o) are the ratios of (nucleotide fluorescence)/(1−protein fluorescence).

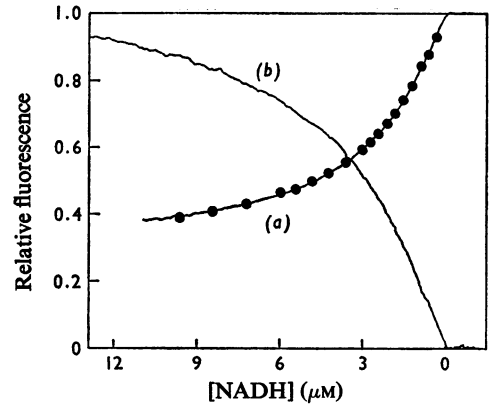

Fig. 5. *Non-linear decrease in the protein fluorescence with the formation of the bovine glutamate dehydrogenase–NADH–glutamate complex*

The bovine liver enzyme (0.16mg/ml) was continuously titrated with NADH in 30mM-H_3PO_4–0.1M-glutamic acid adjusted to pH6.0 with 5M-NaOH. In two separate experiments the reaction was followed by (*a*) decreased protein fluorescence and (*b*) increased NADH fluorescence. The points (•) were calculated as $[1-\alpha(1-0.827)]^6$ and their values were decreased to simulate the protein fluorescence reabsorbed by the added NADH.

grade) was equilibrated with a buffer (30mM-H_3PO_4–0.1M-glutamic acid, adjusted to pH6 with 5M-NaOH) containing 24.6μM-NADH. The enzyme (1.01mg in 1.0ml of the buffer–NADH solution) was applied to the column. The extinction at 340nm of the eluate was recorded directly with 0.2 extinction full-scale on a recorder by using a flow cuvette of 4mm light-path. A Unicam SP.500 spectrophotometer was modified by inclusion of a beam splitter after the monochromator, two photomultipliers and analogue electronics, which gave the logarithm of the ratio of the two photomultiplier currents. Only 1.05 mol of NADH was bound per 56000g of enzyme under these conditions. The relation between F and α was determined by titrating enzyme (0.16mg/ml) in 3ml of buffer (30mM-H_3PO_4–0.1M-glutamic acid adjusted to pH6 with 5M-NaOH) with 3.6mM-NADH added at 5μl·min^{-1} (Fig. 5). The maximum difference in NADH fluorescence was initially estimated to be 1.05. By trial and error this value was then adjusted until a plot of $1/(1-\alpha)$ against $[NADH]_{total}/\alpha$ was linear ($\Delta F_{max.} = 1.125$). This plot corresponded to $K_{E,NADH} = 2\mu M$ and 2.7μM-NADH-binding sites. The non-linear relation between F and α is given in Table 1.

Neurospora crassa glutamate dehydrogenase

This enzyme was prepared and assayed according to Fincham & Coddington (1963). Their preparation was modified in two ways. Nucleic acid was precipitated with protamine sulphate after the first $(NH_4)_2SO_4$ fractionation. Also the calcium phosphate-gel treatment was replaced by filtering the enzyme through a column of Sephadex G-200, equilibrated with 0.02M-H_3PO_4 adjusted to pH7.2 with 5M-NaOH. Based on enzyme activity, the pure enzyme prepared at the University of East Anglia was only 70% pure on arrival in Bristol for the titrations. The relation between F and α was investigated by titrating enzyme (0.135mg/ml) in 50mM-phosphate, pH6.35, with NADPH. The results in Fig. 6 are the corrected values of fluorescence. The relation between F and α was linear, because the ratio (nucleotide fluorescence)/(1−protein fluorescence) was constant and independent of α. A plot of $1/(1-\alpha)$ against $[NADPH]_{total}/\alpha$ was also linear (Fig. 7). The reciprocal slope ($K_{E,NADPH}$) was 0.4μM and the intercept (the concentration of NADPH-binding sites) was 2.32μM. This latter value corresponds to one NADPH-binding site per 58000g of 70%-pure protein. The equivalent weight of pure protein per NADPH-binding site is about 41000. The independence of the NADPH-binding sites could also be deduced, since a plot of $\log[\alpha/(1-\alpha)]$ against $\log([NADPH]_{free})$ was linear, with a slope of 1.0. Results at higher pH values and higher enzyme concentrations were more complex. The enhancement of

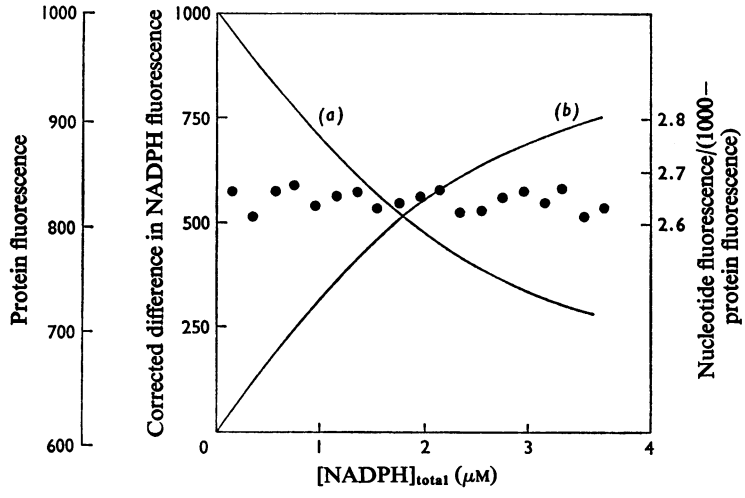

Fig. 6. *Linear decrease in protein fluorescence with the formation of the Neurospora crassa glutamate dehydrogenase–NADPH complex*

The enzyme (0.135mg/ml) was titrated in sodium phosphate buffer at pH6.35 with NADPH. In two experiments the formation of the complex was monitored by (*a*) the decreased protein fluorescence and (*b*) the increased NADPH fluorescence. These results have been corrected for trivial changes in protein fluorescence due to radiation absorbed by the added NADPH. The points (●) are the ratios of (nucleotide fluorescence)/(1000 − protein fluorescence).

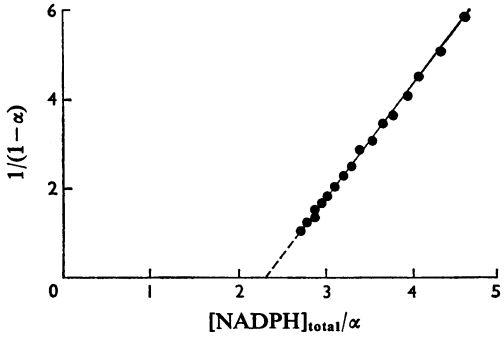

Fig. 7. *Independence of the NADPH-binding sites of Neurospora crassa glutamate dehydrogenase at pH6.35*

The results from Fig. 6 (●) were analysed according to $K_d/(1-\alpha) = [NADPH]_{total}/\alpha - [E_0]$. The line (——) is drawn for $K_d = 0.4\mu M$ and $[E_0] = 2.32\mu M$.

NADPH fluorescence was greater and the affinity of the enzyme for NADPH was less.

Discussion

The results in the present paper show that the relation between the protein fluorescence (*F*) and the fraction of ligand-binding sites occupied (α) is linear for some enzyme–ligand pairs and non-linear for others. These results will now be interpreted in terms of a theory of protein fluorescence given in the preceding paper (Holbrook, 1972).

A linear relation between *F* and α is expected either if there is only one ligand-binding site per protein molecule or if there are *n* completely co-operative sites. The results for NADPH binding to *Neurospora crassa* glutamate dehydrogenase show a linear relation between *F* and α. There is no positive co-operation between the binding sites in solution at pH6.35 at a protein concentration of 0.135mg/ml, because the plot in Fig. 7 is linear. Complete negative co-operation would also give a linear plot if only one of the *n* sites per molecule was saturated. This is excluded because the equivalent weight per NADPH-binding site (41000) shows that all the NADPH-binding sites were saturated with NADPH. There is thus only one NADPH-binding site per protein molecule with a molecular weight of 41000. Thus addition of NADPH under these conditions results in a dissociation of the oligomer (mol.wt. 240000; Barratt & Strickland, 1963). The dissociation has been confirmed by measuring the elution volume of the enzyme from Sephadex G-200 columns equilibrated with NADPH at pH6.35 (Gore *et al.*, 1972). The relative protein fluorescence of an aggregated form of the enzyme–NADPH complex is predicted to be lower than that of the monomeric complex (0.658), because of energy

transfer across subunit interfaces. Thus observation of transient protein fluorescence changes on addition of NADPH to the aggregated protein will give information on the order in which the dissociation occurs. The dissociation is relevant to an understanding of the mechanism of this enzyme, since the activity is inhibited by NADPH and low pH values and activated by 2-oxoglutarate and high pH values (West *et al.*, 1967).

There was also a linear relation between the changed protein and NADH fluorescence when GTP was added to the bovine glutamate dehydrogenase–NADH complex in the presence of excess of NADH. The sigmoidal shape of both curves (Fig. 4) suggests that positive co-operation between the binding sites, rather than dissociation into molecules with only one binding site, is the explanation for the linear relationship. The physical basis of the changed nucleotide and protein fluorescence in the presence of GTP is not clear. It might be due to changed conformation of the enzyme–NADH complex in the presence of GTP (Bayley & Radda, 1966). However, there are recent claims that 2mol of NADH are bound to 56000g of enzyme in the presence of GTP (Jallon & Iwatsubo, 1971; Koberstein & Sund, 1971). This finding suggests the alternative that in the presence of GTP, the increased NADH fluorescence is a result of an increase in the concentration of bound NADH and that the decrease in protein fluorescence is due to resonance transfer from excited tryptophan residues to the increased concentration of bound NADH molecules.

For three systems, the alcohol dehydrogenase–NADH–isobutyramide complex, the glycerol 3-phosphate dehydrogenase–NADH complex and the bovine glutamate dehydrogenase–NADH–glutamate complex, there was a non-linear relation between F and α (Table 1). By using the unrestrained-curve-fitting procedure described by Holbrook (1972) these results were fitted to the equation $F=[1-\alpha(1-x)]^n$. The procedure is shown in Fig. 8. The agreement between the fitted solutions and the experimental results is shown by the calculated points (which have been decreased to account for the reabsorption of the protein fluorescence by the added NADH), which are plotted in Figs. 1, 3 and 5. The excellent agreement between the measured protein fluorescence and that calculated on the basis of geometric quenching was expected for alcohol dehydrogenase from the results of Theorell & Tatemoto (1971), discussed by Holbrook (1972). The agreement between the value of n obtained as a result of curve fitting (2 ± 0.3) and the known dimeric structure of this enzyme supports the concept of geometric quenching.

Geometric quenching would be expected only if the NADH molecules are bound at indistinguishable and non-interacting sites. The results of Kim & Anderson (1969) are preliminary evidence that all the polypeptide chains of L-glycerol 3-phosphate dehydrogenase are identical. These workers concluded, on the basis of fluorimetric titrations at concentrations of NADH-binding sites too high to provide a sensitive test of independence, that the two NADH-binding sites per enzyme molecule were indistinguishable and could be represented by a single dissociation constant. The results in Fig. 2 show no interaction between the sites under more favourable conditions. The value of n obtained by curve fitting (2 ± 0.3) agrees with the dimeric structure of this protein. The protein fluorescence (points), calculated on the basis of geometric quenching with $n=2$ and $x=0.438$, agrees well with the experimental results (curve) in Fig. 3. Thus geometric quenching adequately describes the non-linear relation between F and α for this protein.

The precision with which the non-linear relation of F to α for NADH binding in the bovine glutamate dehydrogenase–NADH–glutamate complex could be established was less than for the other enzymes. Although the presence of glutamate decreases the dissociation constant for NADH (Fisher & McGregor, 1960) to about 2μM, this was not low enough to obtain a linear increase in the NADH fluorescence with increased concentration of NADH over a large proportion of the titration curve. The

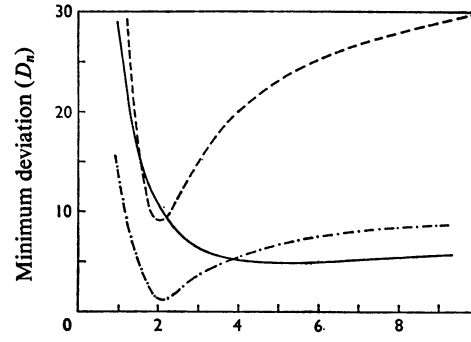

Fig. 8. *Curve-fitting non-linear protein-fluorescence changes to the equation* $F=[1-\alpha(1-x)]^n$

Attempts were made to fit the non-linear change in protein fluorescence (F) with increased fractional saturation with NADH (α) to the equation $FC_J=[1-\alpha_J(1-x)]^n$. Values were taken from Table 1. Alcohol dehydrogenase (–·–·–·–, $J=12$); L-glycerol 3-phosphate dehydrogenase (– – – –, $J=12$); and bovine glutamate dehydrogenase (——, $J=8$). Deviation $=\left(\frac{1}{J-2}\sum(FC_J-F_J)^2\right)^{\frac{1}{2}}$. The unrestrained-curve-fitting method (Holbrook, 1972) was used.

Table 2. *Comparison of the ratio of initial tangents to protein- and nucleotide-fluorescence titrations determined experimentally and calculated on the basis of geometric quenching*

The values of n and x for each enzyme obtained by curve-fitting were used to calculate $(1-x^n)/n(1-x)$. This value is compared with the ratio of initial tangents to protein- and nucleotide-fluorescence titrations found experimentally.

System	Solution of $F=[1-\alpha(1-x)]^n$ by curve-fitting for		Ratio of initial tangents of protein:nucleotide fluorescence	
	n	x	Calculated from n and x as $\frac{1-x^n}{n(1-x)}$	Ratio found experimentally
Alcohol dehydrogenase and isobutyramide+NADH	2	0.690	0.848	0.87
L-Glycerol 3-phosphate dehydrogenase+NADH	2	0.438	0.718	0.73
Bovine glutamate dehydrogenase and glutamate+NADH	6	0.827	0.652	0.66
Bovine glutamate dehydrogenase and NADH+GTP	1	0.400 (0.858)*	Not applicable to the sigmoidal curves	
Neurospora crassa glutamate dehydrogenase+NADPH	1	0.658	1.000	1.0

* This value is $(0.4)^{1/6}$ and is the geometric quenching when only one ligand is bound per hexamer and may be compared with the value in the row above.

precision in α depends on the estimated end-point and is thus lower than usual. The amino acid sequences of the six polypeptide chains, of which the molecule of glutamate dehydrogenase is composed at the protein concentration used, are identical (Smith *et al.*, 1970) and the linear plot of $1/(1-\alpha)$ against $[NADH]_{total}/\alpha$ is evidence that these sites do not interact. Such a system could exhibit geometric quenching. The results of attempts to fit the values to $F=[1-\alpha(1-x)]^n$ are shown in Fig. 8. For values of n less than 3 the fit is less good than for $n=6$. However, the limited accuracy of the original experimental results means that no meaningful distinction could be made between fits with $n=5$–9. Nevertheless, the agreement between the protein fluorescence calculated with $n=6$ and $x=0.827$, which is plotted in Fig. 5, is well within experimental error.

One consequence of geometric quenching is that an initial tangent to a graph of F against $[NADH]_{total}$ will intersect the line corresponding to the protein fluorescence when $\alpha=1$ at a total concentration of NADH equal to $(K_d+[E_0])\cdot(1-x^n)/n\cdot(1-x)$ and not at $K_d+[E_0]$ (where K_d is the dissociation constant of the enzyme–NADH complex and $[E_0]$ is the total concentration of NADH-binding sites in the solution). In Table 2 the values of $(1-x^n)/n\cdot(1-x)$ are compared with the ratios of the concentrations of NADH at which initial tangents to protein- and NADH-fluorescence titrations intersect the values of the fluorescence at $\alpha=1$. There is excellent agreement. This is a complete test that the values of x and x^2 are those predicted by geometric quenching for the dimeric proteins. For the higher polymers it is a test that x and x^n agree with geometric quenching, although it ignores the intermediate values.

The results for alcohol dehydrogenase and glycerol 3-phosphate dehydrogenase are adequately described by geometric quenching. If the total concentration of a dimeric protein is $[P_0]$ and $[PL_j]$ are the individual concentrations of molecules with j ligand-binding sites occupied, then for geometric quenching:

$$F=\frac{[PL_0]+[PL_1]\cdot x+[PL_2]\cdot x^2}{[P_0]}$$

$$\alpha=\frac{[PL_1]+2\cdot[PL_2]}{2\cdot[P_0]}$$

$$[P_0]=[PL_0]+[PL_1]+[PL_2]$$

These three equations may be solved for the relative concentration of monoliganded dimer:

$$[PL_1]/[P_0]=\frac{1-F-\alpha(1-x^2)}{0.5\cdot(1-x)^2}$$

Since x and x^2 may be obtained from the value of F when $\alpha=1$, then without any assumptions about the relative concentrations of PL_j it is possible to calculate the fraction of the protein molecules that are

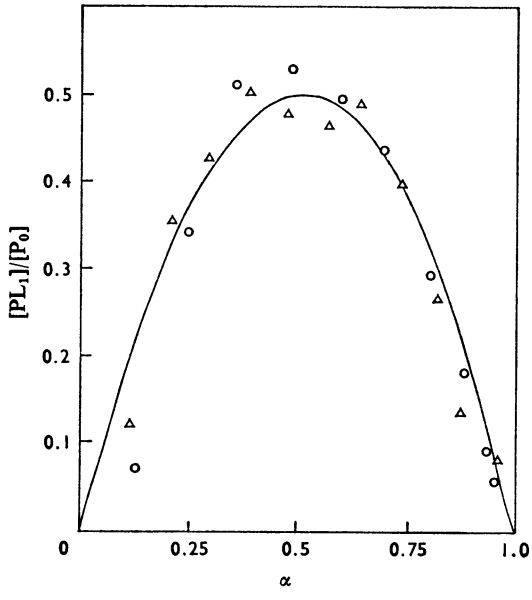

Fig. 9. *Calculation of the fraction of a dimeric protein which is monoliganded* ($[PL_1]/[P_0]$)

The line is calculated as $2\alpha(1-\alpha)$. Values are calculated from the non-linear protein fluorescence by using $[PL_1]/[P_0] = [1-F-\alpha(1-x^2)]/0.5\cdot(1-x)^2$; the values of n and x were taken from Table 2 and those of F and α from Table 1 for alcohol dehydrogenase (△) and L-glycerol 3-phosphate dehydrogenase (○).

monoliganded. All that is required is a set of results relating F to α. This was taken from Table 1 and was used to calculate $[PL_1]/[P_0]$ and this is compared with the function $[PL_1]/[P_0] = 2\alpha(1-\alpha)$, which is that predicted if the affinity of the unliganded protein for NADH is four times that of the monoliganded protein (Fig. 9). The reasonable agreement between the function and values determined by assuming geometric quenching may either be taken as a justification for geometric quenching or as a proof that the affinity of the unliganded dimer for NADH is four times that of the monoliganded dimer.

The non-linear change in protein fluorescence with increasing α thus contains information about the distribution of ligand between the various partially liganded protein molecules. The relation between F and α must be established experimentally with great precision if full use is to be made of the information. Increased precision can best be achieved if a dual-wavelength device is employed, with one wavelength being used to excite protein fluorescence and the other being used to measure an optical property proportional to α in the same solution at the same time. This would largely eliminate time-dependent fluctuations and, in particular, it would ensure that the chemical composition of the solution was identical for both measurements and remove the present uncertainty in aligning titration curves.

The fluorimeter was constructed with a grant-in-aid from The Royal Society (London). We thank Mrs. A. Ingram (supported by the Science Research Council) and Mr. M. Buller (on vacation from Wycliffe College, Stonehouse, U.K.) for making some of the measurements and Whatman Biochemicals Ltd., Maidstone, Kent, U.K., for their support in developing some of the enzyme preparations.

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