Biochimica et Biophysica Acta, 309 (1973) 237–242

BBA Report

BBA 61274

A conformational transition of the oligomer of glutamate dehydrogenase induced by half-saturation with NAD^+ or $NADP^+$

JOHN E. BELL and KEITH DALZIEL

Department of Biochemistry, University of Oxford, Oxford (Great Britain)

(Received March 5th, 1973)

SUMMARY

Changes of the conformation of glutamate dehydrogenase accompanying the binding of NAD^+ and $NADP^+$ as ternary complexes with glutarate have been demonstrated by effects upon the circular dichroism of the enzyme, and by a new technique in which NADH is used as a "tracer probe" of dehydrogenase conformation. Both methods indicate a conformational transition of the oligomer at half-saturation with oxidised coenzyme to which the negative interactions in coenzyme binding are attributed.

Ox liver glutamate dehydrogenase (L-glutamate:$NAD(P)^+$ oxidoreductase (deaminating, EC 1.4.1.3) forms ternary complexes with the substrate analogue glutarate and NAD^+ or $NADP^+$, in which the oxidised coenzymes are much more firmly bound than in their binary complexes with enzyme alone, and the binding cannot be described by a single dissociation constant[1,2]. These findings confirmed the conclusion from earlier kinetic studies[3,4] that either the six subunits of the enzyme molecule are not identical and equivalent, or there are strong negative interactions between them in the binding of oxidised coenzymes in the ternary complexes. The binding studies by equilibrium dialysis were not sufficiently precise to justify detailed quantitative analysis, but it was pointed out that they were consistent with coenzyme binding to two sets of three subunits with different affinities[2]. These and similar results obtained in studies of NADH binding in an abortive ternary complex with glutamate[5] suggested that the hexamer may consist of two functionally distinct trimers, which differ in intrinsic affinity for coenzyme, or between which there are negative interactions. In the latter case, half-saturation of the hexamer presumably induces a conformation change that decrease the affinity of the remaining, unliganded trimer for enzyme.

Evidence for such a conformational transition was sought by studies of the effects

of binding NAD^+ or $NADP^+$ on the circular dichroism of the enzyme, and on the fluorescence quantum yield of enzyme-bound NADH. The enzyme, glutaric acid, coenzymes and nucleotides were purchased and prepared as previously described[2]. All the experiments were carried out at 23 °C in 0.11 M sodium phosphate buffer, pH 7.0, containing 10 μM EDTA. The protein concentration was calculated from absorbance measurements at 280 nm and the extinction coefficient[1] of 0.93 $cm^2 \cdot mg^{-1}$. Fluorescence measurements were made with a Farrand Mark I fluorimeter, with excitation at 340 nm and emission at 465 nm, and nominal band widths of 5 nm. Measurements of circular dichroism were made in quartz cells, light path 1 cm, with a Roussel–Jouan dichrograph II: ellipticities, θ, are expressed as degrees$\cdot cm^2 \cdot dmole^{-1}$.

The fluorescence quantum yield of NAD(P)H bound to glutamate dehydrogenase is greater than that of the free coenzyme[6], and is altered by additional ligands such as glutamate, glutarate, GTP and ADP, as well as by changes of pH[7]. The likeliest explanation of these diverse effects of other ligands is a change in the environment of the enzyme-bound coenzyme caused by a change of enzyme conformation. On this basis, NADH was used as a "natural" probe of the dehydrogenase conformation by measurements of the fluorescence of a small amount of NADH, totally bound by a large molar excess of glutamate dehydrogenase, as a function of the fractional saturation of the enzyme with NAD^+ or $NADP^+$.

The fluorescence enhancement, Q, defined as the ratio of the specific fluorescence of enzyme-bound NADH to that of free NADH, was measured by a modification of the method, described previously[7], of titrating a small concentration of coenzyme with enzyme to a constant fluorescence level. The fluorescence of enzyme-bound NADH was measured in a solution containing a large concentration of enzyme (approx. 10 mg/ml = 183 μM protomers), 0.2 M glutarate and 1–2 μM NADH, corrected for the fluorescence of enzyme and glutarate alone. The protein concentration was then decreased by diluting aliquots of the solution with a solution containing coenzyme and glutarate at the same concentration, and the fluorescence measurements were repeated. The fluorescence enhancement remained the same over an appreciable range of smaller protein contentations (Fig. 1), showing that at the higher protein concentration all the NADH was bound to the enzyme. The fluorescence of free coenzyme at the same concentration was measured separately. In this way, Q was determined in the presence of various concentrations of NAD^+ and $NADP^+$. Some of the results with NAD^+ are illustrated in Fig. 1, which shows that with 100 μM NAD^+ there is no change in the fluorescence of the bound NADH, but there is a decrease with higher NAD^+ concentrations which is not due to displacement of the bound NADH, since Q still reaches a constant value with increase of enzyme concentration.

The variation of Q with increasing concentrations of NAD^+ and $NADP^+$ is shown in more detail in the titration curves of Fig. 2. Up to 100 μM oxidised coenzyme there is no change of Q, but above this level there is a sudden and marked change to a new constant value at about 250 μM NAD^+ or $NADP^+$. We attribute this to a change in the environment of the enzyme-bound NADH brought about by a conformation change induced by $NAD(P)^+$ binding to other subunits in the oligomer. Also shown in Fig. 2 is the per cent

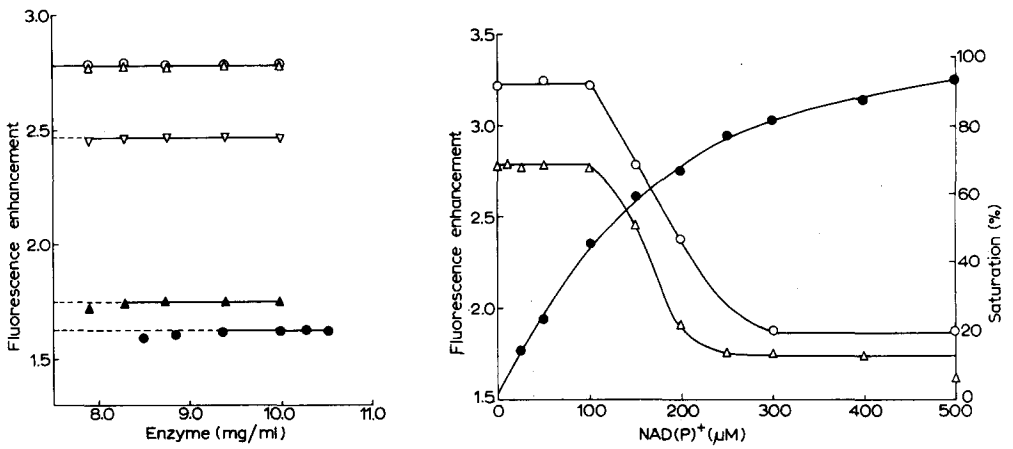

Fig. 1. Determination of the fluorescence enhancement, Q, for NADH bound to glutamate dehydrogenase in the presence of 0.2 M glutarate, by titration of 2 μM NADH with enzyme (○——○). The effects of several concentrations of NAD^+ on the value of Q obtained are shown: 100 μM (△——△), 150 μM (▽—— ▽) 250 μM (▲——▲) and 500 μM (●——●). 0.11 M phosphate buffer, pH 7.0.

Fig.2. The effects of NAD^+ (△——△) and $NADP^+$ (O——O) on the fluorescence enhancement, Q, of NADH bound to glutamate dehydrogenase in the presence of 0.2 M glutarate. The NADH concentration was 2 μM, the enzyme concentration 10 mg/ml, in 0.11 M phosphate buffer, pH 7.0. The saturation curve for NAD^+ binding is also shown (●——●).

saturation of the enzyme binding sites with NAD^+ over the range of total NAD^+ concentration studied, calculated from the coenzyme concentrations and the binding curves obtained by Dalziel and Egan[2] under the same conditions. The conformation change indicated by the fluorescence of the NADH "tracer" label begins at about 40% saturation of the binding sites with oxidised coenzyme and is complete at about 70% saturation.

Independent evidence of conformation changes accompanying the "negatively cooperative" binding of NAD^+ and $NADP^+$ was sought in measurements of the circular dichroism of the enzyme. The CD spectra in the presence of various ligands are shown in Fig. 3. There is a marked change of ellipticity at 297 nm when the ternary complex with NAD^+ and glutarate is formed (but not with the binary enzyme–NAD^+ complex), and CD titrations of the enzyme with oxidised coenzymes, in the presence of glutarate, were made at this wavelength (Fig. 4). With both coenzymes, the ellipticity does not change significantly until the coenzyme concentration exceeds 80 μM, and the major change is complete at 150 μM coenzyme. The saturation curve for NAD^+ is also reproduced in Fig. 4 and shows that the conformational transition indicated by the change of enzyme circular dichroism occurs over a narrow range of fractional saturation with coenzyme around 50%, as in the fluorescence enhancement experiments.

Negative cooperativity cannot be explained by the concerted symmetry model for allosteric proteins[8,3]. Moreover, the fact that the perturbation indicated by two quite independent methods is not proportional to the fractional saturation of the

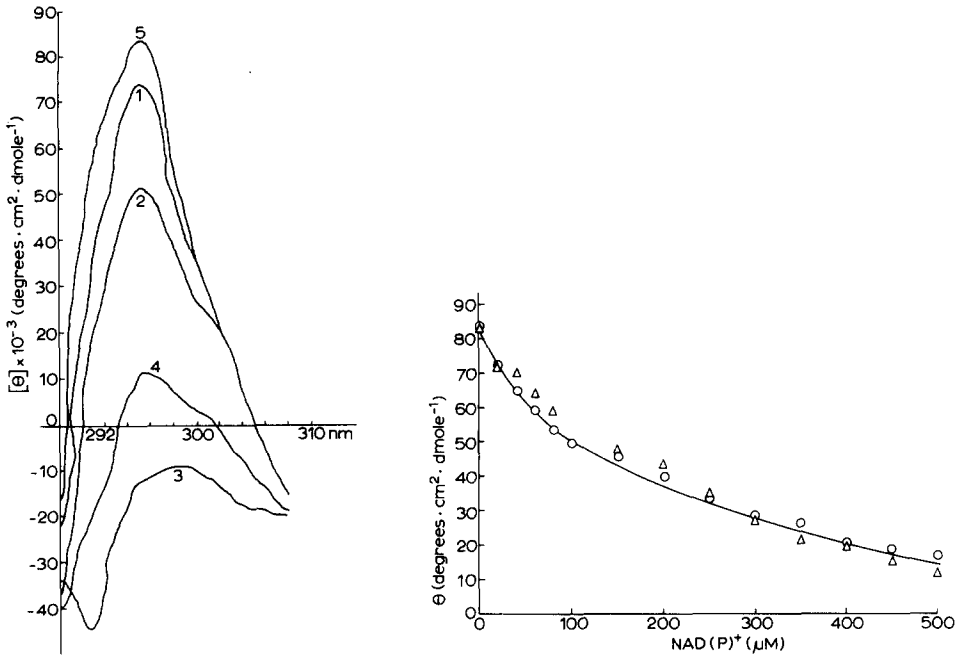

Fig. 3. Circular dichroic spectra of glutamate dehydrogenase in 0.11 M phosphate buffer, pH 7.0 (Curve 1), and with the addition of 0.2 M glutarate (Curve 2), 0.2 M glutarate and 500 μM NAD^+ (Curve 3), 0.2 M glutarate, 500 μM NAD^+ and 1 mM ADP (Curve 4), and 0.2 M glutarate and 1 mM ADP (Curve 5).

Fig. 4. CD titrations of glutamate dehydrogenase (10 mg/ml) with NAD^+ (△ — △) and $NADP^+$ (O——O), The ellipticity at 297 nm is plotted against the total concentration of coenzyme added. The saturation curve for NAD^+ binding is also shown (•——•). The experiments were made in 0.11 M phosphate buffer, pH 7.0, containing 0.2 M glutarate.

coenzyme binding sites shows that it does not represent a conformation change imposed on each subunit by combination with NAD^+ or $NADP^+$, as in the simplest sequential induced-fit models for allosteric proteins[9]. Indeed, the change of quantum yield of enzyme-bound NADH must reflect a conformation change of the subunit to which NADH is bound induced by binding of NAD^+ or $NADP^+$ at another subunit in the oligomer. Thus, a more complex sequential model is demanded for glutamate dehydrogenase, and the method used here should be of general utility for distinguishing between types of sequential models for allosteric dehydrogenases.

The relatively narrow range of fractional saturation of the enzyme with oxidised coenzyme over which the conformation change indicated by both methods occurs is striking. It was earlier[2] suggested that the binding curves for both coenzymes might be described in terms of only two dissociation constants, and that the enzyme oligomer might consist of two sets of three subunits which differ in intrinsic affinity for coenzyme, or between which negative interactions occur. The present results strongly support this latter view. We suggest that the binding of NAD^+ or $NADP^+$, as a ternary complex with glutarate or glutamate, to

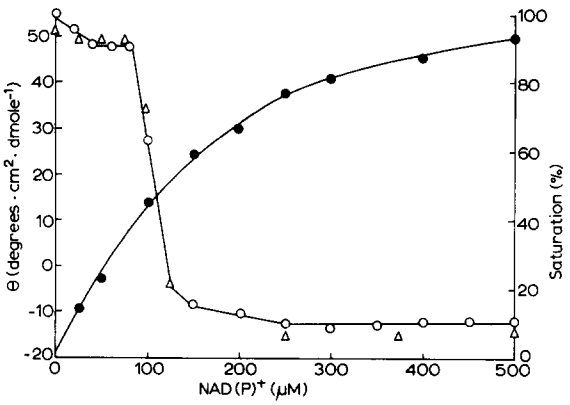

Fig. 5. CD titrations of glutamate dehydrogenase with NAD^+ (△ — △) and $NADP^+$ (O——O) in the presence of glutarate and ADP. The conditions were as in Fig. 4, except that 1 mM ADP was present.

three of the six subunits causes a conformational transition of the oligomer as a result of which the affinities of the remaining three subunits for oxidised coenzyme – and the fluorescence quantum yield of bound NADH – are decreased.

That the conformational transition observed is associated with the negative interactions was confirmed by CD titrations of the enzyme with oxidised coenzymes in the presence of excess ADP, as well as glutarate, shown in Fig. 5. In contrast to the results in absence of ADP (Fig. 4) the ellipticity changes continuously with addition of coenzyme and is approximately proportional to the fractional saturation with oxidised coenzyme. It was show previously[2] that in the presence of ADP the firm binding of coenzyme below half-saturation does not occur, and there is no evidence of negative interactions in either kinetic or equilibrium binding studies under these conditions.

The use of NAD(P)H as a "natural" fluorescence probe of protein conformation may be fairly generally applicable to oligomeric dehydrogenases. It should be emphasized that in the experiments described here there is a 100-fold excess of enzyme subunits over NADH molecules, so that only 1% of the enzyme subunits will be bound to NADH. In the absence[7] of positive interactions in the binding of NADH, the predominant enzyme species will be unliganded enzyme oligomers and oligomers with only one protomer liganded by NADH. It will be interesting to apply this technique to dehydrogenases for which there is evidence from X-ray crystallography of asymmetry of the oligomer in enzyme–coenzyme compounds, such as lactate dehydrogenase[10], liver alcohol dehydrogenase[11], and glyceraldehyde-3-phosphate dehydrogenase[12]. The latter enzyme from rabbit muscle also shows negative interactions in coenzyme binding[13]. It may be remarked that in studies of the binding of NAD^+ to the dimeric liver alcohol dehydrogenase by competition with NADH, using the very large fluorescence enhancement which accompanies NADH binding, Theorell and McKinley-McKee[14] noted a decrease of Q with increasing NAD^+ concentrations, attributed to "quenching". Perhaps a more likely explanation is the formation of "mixed" complexes $E \cdot (NAD)(NADH)$ in which the fluorescence enhancement is smaller than in $E \cdot (NADH)_2$.

This work was supported by the Medical Research Council, London. K.D. is a member of the Oxford Enzyme Group.

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