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CONFORMATIONAL CHANGES OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE INDUCED BY THE BINDING OF NAD^+

A UNIFIED MODEL FOR POSITIVE AND NEGATIVE COOPERATIVITY

JOHN E. BELL* and KEITH DALZIEL
Department of Biochemistry, University of Oxford, Oxford (U.K.)

Summary

The fluorescence of the natural coenzyme, NADH, is used to monitor the environment of the nicotinamide moiety at the active centre of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12). Changes of the fluorescence quantum yield and polarization of a small amount of NADH, totally bound by an excess of enzyme, show that at half-saturation of the oligomer with NAD^+ a conformational change is induced which affects the active centre regions of the remaining subunits. This conformational transition is not effected by adenosine diphosphoribose, suggesting that the binding of the nicotinamide moiety of NAD^+ to two subunits is essential for the change of tertiary structure of the remaining subunits that causes the observed changes of the fluorescence properties of the NADH "tracer probe". It is suggested that this conformational transition of the oligomer is responsible for the major decrease of affinity for NAD^+ which occurs at half-saturation, and possibly for the activation by NAD^+ of the reductive dephosphorylation reaction catalysed by the enzyme. It is also suggested, by analogy with haemoglobin, that the molecular basis of the negative cooperativity may be the creation of additional intersubunit bonds during the binding of the first two NAD^+ molecules to the tetramer, and a change from a "relaxed" quaternary structure to a "tense" structure at half-saturation.

Introduction

It has long been known that in the binding of NAD^+ or NADH to glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate : NAD^+ oxidoreductase, phosphorylating, EC 1.2.1.12) the apparent dissociation con-

* Present address: Department of Biochemistry, Duke University Medical Center, Durham, N.C., U.S.A.

stant for the coenzyme increases with increasing saturation [1]. This indicates either that the tetrameric enzyme comprises intrinsically non-equivalent subunits [2], or that the binding of successive coenzyme molecules to the tetramer causes the affinity of the remaining binding sites for coenzyme to decrease [3,4]. The latter effect might be caused by direct interaction between ligand molecules in the tetramer, steric or electrostatic, or by conformational changes induced in the oligomer by partial saturation with ligand.

Asymmetry of the tetramer of glyceraldehyde-3-phosphate dehydrogenase has been suggested on the basis of hybridization experiments with the rabbit and the yeast enzymes [5], in which one new molecular species of precisely average electrophoretic mobility was formed, suggesting an $\alpha_2\beta_2$ structure for each enzyme. Also consistent with such a structure is the half-of-the-sites reactivity of the enzymes towards some alkylating and acylating reagents [6—9]. However, recent detailed studies of coenzyme binding to the apoenzyme from rabbit muscle in this laboratory [10] have shown that four dissociation constants are required to describe the binding of NAD^+ or NADH to the tetrameric enzyme, and this cannot be explained simply in terms of an $\alpha_2\beta_2$ symmetry.

A number of previous studies have been concerned with conformational changes induced in glyceraldehyde-3-phosphate dehydrogenase by coenzyme binding. Changes of optical rotatory dispersion [11] and sulphydryl group reactivity [3] suggested that the major conformational change resulted from the binding of the first molecule of NAD to the tetramer. More recently, the same conclusion was drawn from studies of the binding of NAD and its fluorescent analogue ϵ-NAD by a variety of spectroscopic techniques [12]. On the other hand, other studies of the reactivity of sulphydryl groups in the enzyme showed that the profile of the reactivity underwent a marked change when two molecules of NAD^+ were bound to the tetramer [13]. In temperature-jump studies [14], no relaxation effects could be detected for the binding of the first molecule of NAD^+ to the enzyme, but on binding a second molecule of NAD^+ a single relaxation process, independent of coenzyme concentration, was detected, suggesting that the $E\text{-}NAD_2^+$ species underwent a conformational change.

Although these studies clearly reflect various conformational changes in the protein resulting from ligand binding, they do not allow of a clear distinction between a conformational change of the induced-fit imposed on each subunit by its combination with NAD^+, and a conformational change imposed on one subunit, and directly affecting its active centre region, by combination of NAD^+ with another subunit. Such a distinction can be made by the use of the natural reduced coenzyme, NADH, as a "tracer fluorescent probe", as was shown with glutamate dehydrogenase [15]. This hexameric enzyme also exhibits negative cooperativity [16—18], and both the NADH tracer probe technique and measurements of protein circular dichroism showed the phenomenon to be related to a conformational transition of the oligomer at half-saturation with oxidised coenzyme [15]. The main objectives of the present work were to apply the tracer probe technique to rabbit muscle glyceraldehyde-3-phosphate dehydrogenase in order to detect subunit-subunit interactions accompanying NAD^+ binding, and to study the concomitant changes of the protein fluorescence as an additional intrinsic, although less specific, index of conformation changes.

Materials and Methods

Crystalline rabbit muscle glyceraldehyde-3-phosphate dehydrogenase holoenzyme and apoenzyme were prepared as described previously [10]. Protein concentrations were estimated by absorbance measurements using extinction coefficients at 280 nm or 0.98 $cm^2 \cdot mg^{-1}$ for holoenzyme and 0.83 $cm^2 \cdot mg^{-1}$ for apoenzyme [19] and a molecular weight of 140 000 for the tetramer [20].

Fluorescence measurements were made with a Farrand Mark I fluorimeter, using 1-cm light path cuvettes. In general fluorescence emission was measured at right angles to the exciting beam. Fluorescence spectra were recorded with a slit width of 1 nm for the scanning monochromator. All spectra were corrected for the appropriate blanks. Emission spectra were not corrected for photomultiplier response because such corrections are in general very small. Excitation spectra were, however, corrected for the variation in the lamp intensity with wavelength using Rhodamine B (3 g/l in ethylene glycol) as a quantum counter [21] with the fluorimeter adapted for straight-through fluorescence measurements. In addition, where the incident light was absorbed by a non-fluorescent component, corrections were made for the attenuation of incident light by control measurements on *N*-acetyl tryptophan. Polarization measurements were made using polarisers in the excitation and emission beams. The polarization, P, was calculated from the expression

$$P = \frac{F_0 - F_{90}}{F_0 + F_{90}}$$

where the subscripts indicate the plane of the polarised fluorescence with respect to that of the exciting light, and F is the intensity of the fluorescence.

NAD^+ (grade I) and triethanolamine hydrochloride were obtained from Boehringer. NADH (grade III) and dithiothreitol were obtained from Sigma. Other reagents were the highest grade commercially available. All solutions were made up with double glass-distilled water, and except for enzyme solutions, were filtered through sintered glass prior to use. All the experiments were carried out at 25°C in 0.1 M triethanolamine buffer, pH 7.6, containing 1 mM EDTA, in which coenzyme binding studies were made previously [10].

Results

Effect of NAD^+ on the fluorescence of enzyme-bound NADH

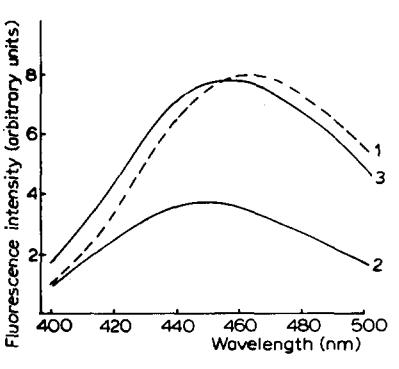
The fluorescence emission spectra of NADH (1.8 μM) in buffer and in the presence of an excess of enzyme (11.3 μM), excited at 340 nm, are shown in Fig. 1. The emission maximum for free NADH is at 460 nm. In the presence of enzyme, there is a marked quenching of the fluorescence and the emission maximum is shifted to 445 nm. The NADH will be totally bound by the excess of enzyme, because the dissociation constant for the binding of the first NADH molecule to the tetramer is $8 \cdot 10^{-9}$ M [10]. Also shown in Fig. 1 is the emission spectrum of enzyme-bound NADH when 2.3 mol NAD^+ per mol of enzyme is also added. Under these conditions 2 mol of NAD^+ will be bound per

mol of enzyme [10]. The emission maximum of the small amount of NADH is at 455 nm, and the intensity at this wavelength is the same as that of free NADH. However, it is evident from the emission spectrum, especially in the region 400—450 nm, that these changes are not due to displacement of NADH from the enzyme of NAD^+, and this is confirmed in the experiments described later.

The ratio Q, of the specific fluorescence of enzyme-bound NADH to that of free NADH, was determined by titrating a fixed concentration of the coenzyme (1.5 μM) with enzyme (Fig. 2). The fluorescence emission at 450 nm, corrected for the fluorescence of the enzyme, is progressively quenched by the enzyme until a plateau is reached from which a value of 0.52 is obtained for Q. This is in agreement with the results of Fig. 1.

A small amount of NADH was used as a fluorescent probe of enzyme conformation during titration of the enzyme with NAD^+, in the manner described previously [15], and the results are shown in Fig. 3. The concentration of NADH is less than 1% of the concentration of enzyme subunits, and it will be almost totally bound by the enzyme, whilst some 3% of the enzyme tetramers at any instant will be labelled with a single NADH molecule. To establish that all of the NADH is bound under the conditions shown in Fig. 3, Q was also determined at each NAD^+ concentration used by varying the protein concentration, keeping the relative concentrations of protein and NAD^+ constant in a manner similar to that described in Fig. 2. There is little change of Q on addition of NAD^+ until a stoichiometry of 1.5 mol NAD^+ per mol of enzyme is reached. Q then increases sharply from 0.54 to 1.05 after 2.5 mol NAD^+ per mol enzyme have been added. There is little further change on further addition of NAD^+.

The results of a similar titration in which the polarisation of the fluorescence of the small amount of NADH was measured instead of Q, are also shown



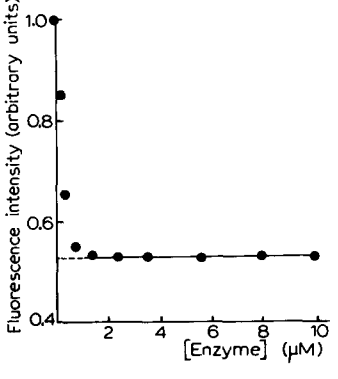


Fig. 1. Fluorescence emission spectra of 1.8 μM NADH in buffer (curve 1), in the presence of 11.3 μM glyceraldehyde-3-phosphate dehydrogenase (curve 2), and in the presence of 11.3 μM enzyme and 26 μM NAD^+ (curve 3). The buffer was 0.1 M triethanolamine, pH 7.6, containing 1 mM EDTA. The excitation wavelength was 340 nm.

Fig. 2. Estimation of Q, the ratio of the specific fluorescence of enzyme-bound NADH to that of free NADH, by fluorescence titration of 1.5 μM NADH with enzyme. The fluorescence emission was measured at 450 nm. Other conditions were as in Fig. 1.

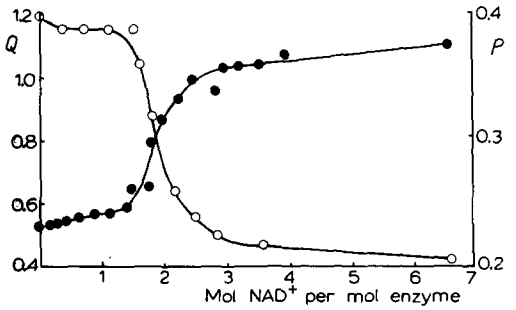


Fig. 3. The effects of NAD^+ on the relative fluorescence yield, Q (●———●), and the polarisation of fluorescence, P (○———○), of NADH bound to excess glyceraldehyde-3-phosphate dehydrogenase. The NADH concentration was 1.1 μM and the enzyme concentration 35 μM. The fluorescence was excited at 340 nm and measured at 450 nm, in 0.1 M triethanolamine buffer, pH 7.6, containing 1 mM EDTA.

in Fig. 3. Like the quantum yield, this property of the probe is not proportional to the fractional saturation of the oligomer with NAD^+, but changes sharply in the region of half-saturation, from 0.44 to 0.22. It should be noted that the latter value is significantly greater than that for free NADH (0.12).

Adenosine diphosphoribose also binds quite tightly to two of the four subunits of the enzyme molecule with dissociation constants $K_1 = 3.5 \cdot 10^{-8}$ M and $K_2 = 2.6 \cdot 10^{-7}$ M [10]. In a similar titration with adenosine diphosphoribose, however, there was no change of the fluorescence of the NADH tracer probe (Fig. 4), even though the nucleotide binds essentially stoichiometrically to two of the four subunits under the conditions of the experiment.

Effects of NAD^+ on the fluorescence spectrum of the enzyme

The fluorescence emission spectra of the apoenzyme excited at 280 and 295 nm are shown in Fig. 5. With excitation at 280 nm, (and also at 260 nm) the emission maximum is at 340 nm, whilst with excitation at 295 nm it is at 343 nm. This suggests that the tryptophan residues in the enzyme are in environments of different polarity [22], and that the "blue" tryptophans in the more hydrophobic environment are preferentially excited by irradiation at shorter wavelengths, possibly by energy transfer from tyrosine or phenylalanine.

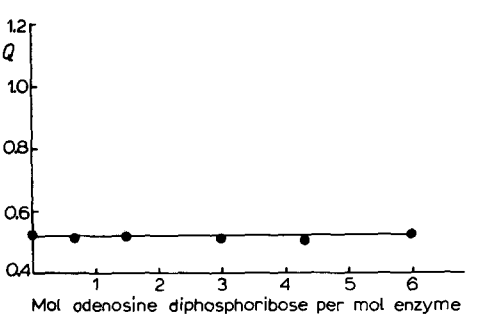
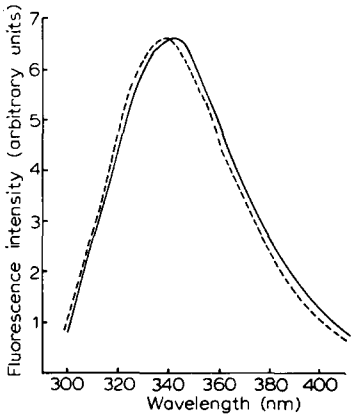


Fig. 4. The effects of adenosine diphosphoribose on the relative fluorescence yield, Q, of NADH bound to excess enzyme. The conditions were as in Fig. 3.



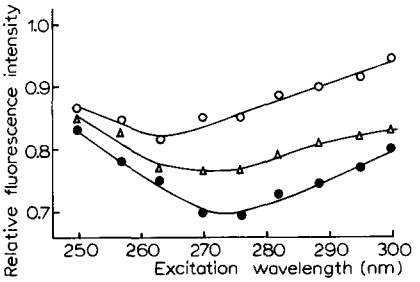


Fig. 5. Fluorescence emission spectra of glyceraldehyde-3-phosphate dehydrogenase excited at 280 nm (- - - - -) and 296 nm (———). The apoenzyme concentration was 2.9 μM. Other conditions were as in Fig. 1.

Fig. 6. Quenching of the protein fluorescence of glyceraldehyde-3-phosphate dehydrogenase, excited at several wavelengths, by NAD^+. The relative fluorescence intensity is the ratio of the measured value in the presence of NAD^+ to that for the apoenzyme, measured at 342.5 nm. The enzyme concentration was 2.9 μM, and the NAD^+ concentrations were 2.9 μM (○———○), 5.8 μM (△———△) and 11.5 μM (●———●). Other conditions were as in Fig. 1.

Quenching of the protein fluorescence emitted at 342.5 nm caused by the binding of NAD^+ was studied with excitation at several wavelengths (Fig. 6). With 1 mol NAD^+ per mol enzyme, the quenching is greater with excitation at 250—265 nm than with excitation at 285—295 nm, suggesting that the "blue" tryptophans are quenched preferentially. The binding of subsequent molecules of NAD^+ causes about the same degree of quenching as the first when the fluorescence is excited at 285—295 nm, as was also shown previously [10,12], but a much smaller decrease when the fluorescence is excited at 260 nm (Fig. 6).

Discussion

It has been shown that discontinuous changes of the fluorescence quantum yield and polarisation of a small amount of NADH, totally bound by an excess of glyceraldehyde-3-phosphate dehydrogenase, accompany titration of the bulk enzyme with NAD^+. These results and their interpretation are similar to those presented earlier for glutamate dehydrogenase [15], and suggest that a conformational transition of the oligomer occurs near half-saturation with NAD^+. The changes in the properties of the NADH "tracer probe" must reflect a change of tertiary structure of a subunit to which NADH can bind, induced by the binding of NAD^+ to other subunits in the oligomer. Moreover, since the measured properties of the NADH are those of its nicotinamide moiety, it is evident that the structural change affects the active centre region, which in the crystallographic structure of the lobster muscle enzyme is deep inside the subunit [23]. These results are therefore not compatible with the conclusion of Schlessinger and Levitzki [12] that conformational changes at the nicotina-

mide subsites accompanying the binding of NAD^+ occur according to a simple sequential model at each liganded subunit independently, that is, are purely of the induced-fit type and do not affect the active-centre regions of unliganded subunits.

By contrast, it has been shown that the binding of adenosine diphosphoribose to two subunits of the oligomer does not cause a change of tertiary structure of other subunits detectable by the NADH probe. It therefore appears that binding of the nicotinamide moiety of NAD^+ is essential for the conformational transition at half-saturation and the concomitant change of tertiary structure of other subunits that affects their nicotinamide binding regions. This conclusion is not incompatible with the fact that the intensity of the Racker band and other properties of the charge-transfer complex formed with NAD^+ are linearly related to the fractional saturation of the oligomer with NAD^+ [10,12]. These latter findings merely indicate the close similarity of the structures of the nicotinamide binding sites in all four subunits after their combination with NAD^+. The earlier conclusions drawn from these facts, that structural changes resulting from the binding at the nicotinamide subsites are not transmitted to other subunits, and therefore cannot be involved in the negative cooperativity [12], do not appear to be justified, therefore.

Four dissociation constants are needed to describe the binding of NAD^+ to rabbit muscle glyceraldehyde dehydrogenase, and their values are $K_1 = 10^{-8}$ M, $K_2 = 9 \cdot 10^{-8}$ M, $K_3 = 4 \cdot 10^{-6}$ M and $K_4 = 3.6 \cdot 10^{-5}$ M at pH 7.6 and 25°C [10]. Thus the largest difference of affinity is between the binding of the second and third molecules of NAD^+, and is equivalent to a negative interaction energy of 2.2 kcal/mol. It seems reasonable to relate this to the conformational transition at half-saturation with NAD^+ detected by the NADH probe, as in the case of glutamate dehydrogenase [15]. For the latter enzyme, this correlation between the conformational transition at half-saturation with NAD^+ or $NADP^+$, detected both by the NADH probe and by circular dichroism measurements, and the negative cooperativity observed in coenzyme binding and in kinetic studies, was greatly strengthened by the finding that the allosteric effector ADP eliminated all these phenomena [15]. Unfortunately, similar additional evidence for the correlation is not yet available for glyceraldehyde-3-phosphate dehydrogenase.

The conformational change induced by half-saturation with NAD^+ may also be related to the requirement for NAD^+ in the reductive dephosphorylation of diphosphoglyceric acid by NADH catalysed by the enzyme [24]. The facts that NAD^+ stabilises [1] and NADH destabilises [25] the apoenzyme already indicated that the oxidised and reduced coenzymes induce different conformations of the enzyme. The present experiments show that combination of NAD^+ with two subunits imposes a tertiary structure on the remaining subunits different from that induced by the normal binding of NADH. This tertiary structure may be essential for efficient reductive dephosphorylation. The fact that adenosine diphosphoribose, although firmly bound by the enzyme [10], cannot substitute for NAD^+ in activating the reductive dephosphorylation reaction [24] nor in bringing about the conformational transition detected by the NADH probe strengthens this tentative correlation.

A number of workers have reported that similar results were obtained in

various investigations with commercial enzyme preparations of relatively low specific activity and enzyme freshly prepared in the laboratory. It is perhaps worth mentioning, therefore, that this was not the case in the present experiments. We were unable to completely remove the bound nucleotide from commercial holoenzyme preparations, and in NADH probe experiments the value of Q increased from 0.7 to 2.0 after the addition of 2.5 mol NAD^+ per mol enzyme. The higher quantum yield of the bound NADH may be caused by modification of thiol groups in the commercial enzyme preparation, but has not been investigated further.

We have confirmed the findings of Schlessinger and Levitzki [12] that the quenching of protein fluorescence attributable to "red" tryptophans is proportional to the fractional saturation of the oligomer with NAD^+ (and with NADH or adenosine diphosphoribose [10]), whilst that attributable to "blue" tryptophans is quenched to a much greater extent on binding of the first NAD^+ molecule than on binding of subsequent molecules to the tetramer. These results might suggest that the major conformational change accompanies binding of the first NAD^+ molecule, rather than the second molecule as indicated by the NADH probe experiments. However, whilst the latter experiments can only reflect conformational changes at the active centre of one subunit caused by the binding of NAD^+ to other subunits in the tetramer, the quenching of protein fluorescence will include in addition the induced-fit changes of the subunits to which NAD^+ binds. Presumably the quenching of the fluorescence of the "red" tryptophans is mainly due to induced-fit changes of tertiary structure. The decreased quenching in the 250—265 nm region may indicate that the conformational changes induced by binding of the second NAD^+ molecule affect the absorption properties of the "blue" tryptophans specifically, possibly by affecting energy transfer to these residues from tyrosine or phenylalanine residues. It is not yet possible to identify the two types of tryptophan in the crystal structures of the enzymes from lobster muscle [24] or *Bacillus stearothermophilus* (Wonacott, Biesecker and Thierry, personal communication).

Negative cooperativity cannot be explained by the concerted symmetry model for allosteric proteins [26,16], and the conformation changes detected by the NADH probe are also not consistent with the simplest sequential induced-fit model [27]. A more complex sequential model is therefore indicated for glyceraldehyde-3-phosphate dehydrogenase, as well as for glutamate dehydrogenase [15]. Substantial evidence of the molecular basis of positive cooperativity has been obtained only for the case of oxygen binding to haemoglobin, from a knowledge of the crystal structures of oxyhaemoglobin and deoxyhaemoglobin. The model proposed by Perutz [28,29] for haemoglobin, in contrast to the concerted symmetry model, may be extended to explain negative as well as positive cooperativity. Oxygen-free haemoglobin is constrained in a tense structure by inter-subunit salt bridges. Combination of a subunit with oxygen causes a change of tertiary structure, triggered by a shift of the iron atom relative to the porphyrin, which results in fission of a salt linkage to a neighbouring subunit. When two subunits have been oxygenated, all the inter subunit salt linkages give way and the quaternary structure clicks over to the relaxed, oxyhaemoglobin structure. The energy required to break the intersubunit bonds is derived from combination of the first two oxygen molecules, and

combination of the remaining oxygen molecules with the relaxed quaternary structure therefore occurs with greater affinity. We suggest that in glyceraldehyde-3-phosphate dehydrogenase, the apoenzyme has a relaxed structure, and that the combination of two NAD^+ molecules, through an induced-fit change of tertiary structure of two subunits, allows the creation of new intersubunit bonds, the energy of which contributes to the high affinity of coenzyme binding, and causes a change of quaternary structure. Combination of the remaining NAD^+ molecules with the "tense" structure then occurs with lower affinity.

Experimental evidence of the molecular basis of negative cooperativity requires structural information about both apoenzyme and holoenzyme at atomic resolution, and this may soon be available for glyceraldehyde-3-phosphate dehydrogenase from *B. stearothermophilus* (Wonacott, Biesecker and Thierry, personal communication). Meanwhile, it is hoped that the energetics of coenzyme binding to this bacterial enzyme and the accompanying conformational changes can be studied in similar detail to the rabbit muscle enzyme.

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