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THE EFFECT OF SUBSTRATES, EFFECTORS, ACETYL PHOSPHATE AND IODOACETATE ON THE BINDING OF NAD^+ AND NADH TO RABBIT-MUSCLE GLYCERALDEHYDEPHOSPHATE DEHYDROGENASE

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SUMMARY

1. None of the substrates (glyceraldehyde, acetaldehyde, phosphate or arsenate) or effectors (ADP and ATP) tested have an appreciable effect on the binding of NAD^+ to rabbit-muscle glyceraldehydephosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12). Glyceraldehyde, glyceraldehyde 3-phosphate, arsenate, phosphate, ADP and ATP have also no effect on the binding of NADH.

2. The product, acetyl phosphate, which acetylates the active-centre thiol group, and the inhibitor iodoacetate, which carboxymethylates it, both decrease the affinity of the NAD^+ molecule for the enzyme. Iodoacetate also decreases the affinity of at least the third and fourth NADH molecule to the enzyme (acetyl phosphate could not be tested with NADH). However, even after acetylation or carboxymethylation, the binding of both NAD^+ and NADH remains negatively co-operative.

3. The binding constants for NAD^+ and NADH do not differ appreciably under any of the conditions tested.

4. Acetyl phosphate decreases the absorbance coefficient of the 360-nm band for the third NAD^+ molecule bound to the enzyme. Between pH 7.0 and 7.5 the coefficient for this molecule becomes equal to that of the fourth NAD^+ molecule bound to the enzyme. Acetyl phosphate has no effect on the absorbance coefficient of the first, second or fourth molecule of bound NAD^+. Iodoacetate abolishes the 360-nm band given by all four NAD^+ molecules.

5. None of the substrates or effectors tested has any effect on the circular-dichroism band at 335 nm, caused by the binding of NADH to the enzyme. However, this band is completely abolished by iodoacetate.

6. None of the substrates, effectors or product tested has any effect on the degree of quenching of the protein fluorescence, caused by the binding of either NAD^+ or NADH to the enzyme. Nor does iodoacetate have any effect. The dissociation constants derived in this way are in good agreement with those calculated from direct separation techniques.

INTRODUCTION

Direct measurements of the binding of NAD^+ to rabbit-muscle glyceraldehyde-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12), whereby free NAD^+ is separated from the enzyme-bound nucleotide by ultracentrifugation[1] or equilibrium dialysis[2], have shown that NAD^+ is bound to the tetrameric enzyme molecule negatively co-operatively. This was the first example of negatively co-operative binding of a substrate to an enzyme, a behaviour that cannot be accommodated by the allosteric model of Monod *et al.*[3]. Paradoxically, Kirschner *et al.*[4,5] found that the kinetics of the binding of NAD^+ to the yeast enzyme at pH 8.5 and 40 °C, studied by the temperature-jump method of Eigen and De Maeyer[6], fit the model of Monod *et al.*[3] very well.

Two lines of evidence show that the different behaviour of the muscle and yeast enzyme is real, and is not due to the different experimental techniques used. First, Hammes *et al.*[7], using the temperature-jump method, have confirmed that the dissociation constant of the complex between NAD^+ and the rabbit-muscle enzyme increases as successive subunits are saturated. Secondly, we have confirmed by equilibrium dialysis, ultrafiltration and fluorescence quenching that the dissociation constant of the complex between NAD^+ and the yeast enzyme decreases as successive subunits are saturated (Boers, W. and Boersma, W. J. M., unpublished). Ellenrieder *et al.* have reported similar results.

The muscle and yeast enzymes also appear to differ with respect to the binding of NADH. Thus, Boers *et al.*[9] have shown that NADH is bound in a negatively co-operative fashion to the muscle enzyme, the binding constants of the four molecules being similar to those with NAD^+. According to Ellenrieder *et al.*[8], the binding of NADH to the yeast enzyme, on the other hand, is described by a simple hyperbolic relation and the kinetics of binding are also described by a single reaction.

Glyceraldehydephosphate dehydrogenase plays a key role in both glycolysis and gluconeogenesis. It catalyses the reduction of NAD^+ to NADH which in muscle is reoxidized by pyruvate bound to lactate dehydrogenase and in yeast by acetaldehyde bound to alcohol dehydrogenase. There is no evidence that enzyme-bound pyruvate or acetaldehyde can directly react with NADH bound to glyceraldehyde-phosphate dehydrogenase and steric considerations make it unlikely. Thus, efficient glycolysis requires a sufficiently rapid dissociation of NADH from glyceraldehyde-phosphate dehydrogenase. Similarly, efficient gluconeogenesis requires a sufficiently rapid dissociation of NAD^+ from this enzyme. In view of the very high binding constants for the first two, and to a less extent for the third, molecules of NAD^+ and NADH bound to the muscle enzyme, we[10] have considered it unlikely that the first two or three molecules play an important role in the catalytic reaction. A weakness of this argument is that the previous measurements of the binding of NAD^+ and NADH to the enzyme were made in the absence of the other substrates or products of the enzyme (aldehyde, acyl phosphate and phosphate) or of effectors such as ADP or ATP[11]. This paper describes the effect of these compounds and of the classical inhibitor, iodoacetate[12], and uncoupler of the phosphorylation, arsenate[13], on the binding constants and on certain optical properties of the NAD^+ and NADH complexes.

RESULTS

Binding of NAD+ and NADH

Neither glyceraldehyde nor acetaldehyde has an appreciable effect on the dissociation constants of the four NAD+ molecules (Table I). Separate control experi-

TABLE I

DISSOCIATION CONSTANTS OF NAD+ BOUND TO GLYCERALDEHYDEPHOSPHATE DEHYDROGENASE IN THE PRESENCE OF SUBSTRATES, EFFECTORS, ACETYL PHOSPHATE OR IODOACETATE

The dissociation constants were measured by equilibrium dialysis at 20 °C, pH 8.2. The values are the means of usually 3 (sometimes 2) independent determinations. The enzyme concentration was 20 μM, while the substrates, effectors and the inhibitor were present in a concentration of 1 mM.

Addition	*Dissociation constant (μM)*			
	K_1	K_2	K_3	K_4
None*	$\leqslant$0.05	$\leqslant$0.05	4	35
Acetaldehyde	0.01	0.1	2.7	37
Glyceraldehyde	0.1	0.1	2.8	42
Acetyl phosphate	0.5	0.5	15	75
Phosphate	$\leqslant$0.05	$\leqslant$0.05	1.3	28
Arsenate	$\leqslant$0.05	$\leqslant$0.05	1.8	32
ADP	$\leqslant$0.05	0.1	2.2	43
ATP	$\leqslant$0.05	0.1	2.3	43
Iodoacetate	0.5	0.5	25	200

* ref. 1.

ments showed that NAD+ was not appreciably reduced by these substrates during the measurement of the binding. However, the effect of glyceraldehyde 3-phosphate on the binding of NAD+ could not be tested since, because of the presence of inorganic phosphate as an impurity, NADH was formed rapidly. Phosphate, arsenate, ADP and ATP also have no effect on the binding. The product acetyl phosphate which, according to Malhotra and Bernhard[14], acetylates two of the four cysteine –SH groups in the "active centres" of the enzyme, appreciably increases all four dissociation constants, but the negative co-operativity remains. Iodoacetate, which carboxymethylates all four cysteine –SH groups in the "active centre"[15–17], has an even greater effect on the dissociation constants of the third and fourth molecules.

None of the substrates tested, including glyceraldehyde 3-phosphate, has any effect on the binding of NADH (Table II). Acetyl phosphate could not be tested, since it rapidly oxidizes NADH, in the presence of the enzyme. Iodoacetate has the same effect on the binding of NADH as on that of NAD+. Indeed, under all conditions tested, the binding constants of NADH do not appreciably differ from those of NAD+.

360-nm absorption band of enzyme-NAD+ complex

Binding of NAD+ to glyceraldehydephosphate dehydrogenase results in the appearance of a broad absorption band with a maximum at 360 nm (ref. 18). The first three molecules of NAD+ bound give rise to a band of equal intensity for each

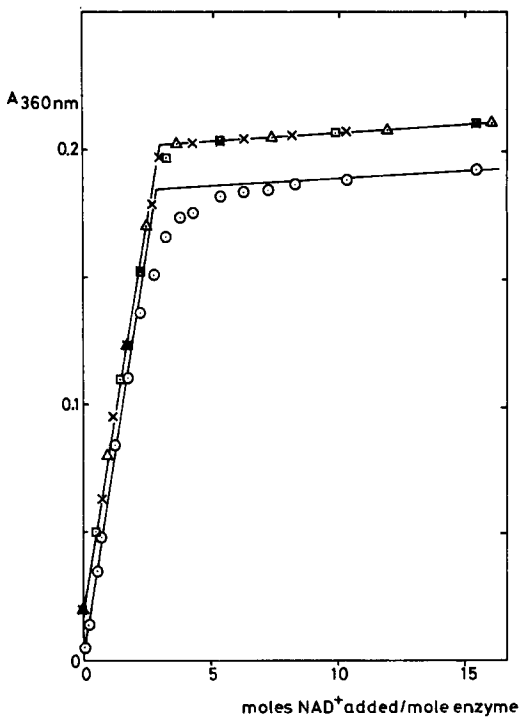

Fig. 1. Titration of charcoal-treated glyceraldehydephosphate dehydrogenase (61 μM) with NAD^+ at 360 nm in the presence of ATP (△), phosphate (×), glyceraldehyde (□) or acetyl phosphate (○). The enzyme was dissolved in 100 mM Tris–HCl buffer (pH 8.2), containing 5 mM EDTA; 23 °C.

molecule[1], whereas the fourth molecule has an effect on the absorbance at 360 nm equal to only about 30% of that of the first three molecules[9].

Fig. 1 shows a titration at 360 nm of the enzyme with added NAD^+, in the presence of ATP, P_i, glyceraldehyde or acetyl phosphate at pH 8.2. In Fig. 2, the results have been replotted, with on the abscissa the amount of bound NAD^+, calculated from that added and the appropriate binding constant given in Table I. In all four cases, the first two molecules of NAD^+ bound give rise to an increased absorption with an absorbance coefficient of 0.98 $(mM\ NAD^+)^{-1}\cdot cm^{-1}$, exactly the same as obtained in the absence of substrate or effector[9].

In the presence of ATP, P_i or glyceraldehyde (Fig. 2A), the absorbance coefficient of the third molecule is the same, whereas that of the fourth molecule is less (0.30 $(mM\ NAD^+)^{-1}\cdot cm^{-1}$), but also the same as in the absence of any addition[9]. In the presence of acetyl phosphate (Fig. 2B), however, the absorbance increase corresponding to the third molecule bound (0.74 $(mM\ NAD^+)^{-1}\cdot cm^{-1}$) is substantially less than in the absence of any addition, whereas the fourth molecule gives rise to a normally low coefficient (0.29 $(mM\ NAD^+)^{-1}\cdot cm^{-1}$). The absorbance coefficient of the third molecule bound is very pH dependent, becoming equal to that of the fourth molecule at pH 7.5. No further effect was seen on lowering the pH to 7.0. In the absence of acetyl phosphate, varying the pH between 8.2 and 7.0 has no effect on the absorbance coefficients. The dissociation constants, measured by equilibrium

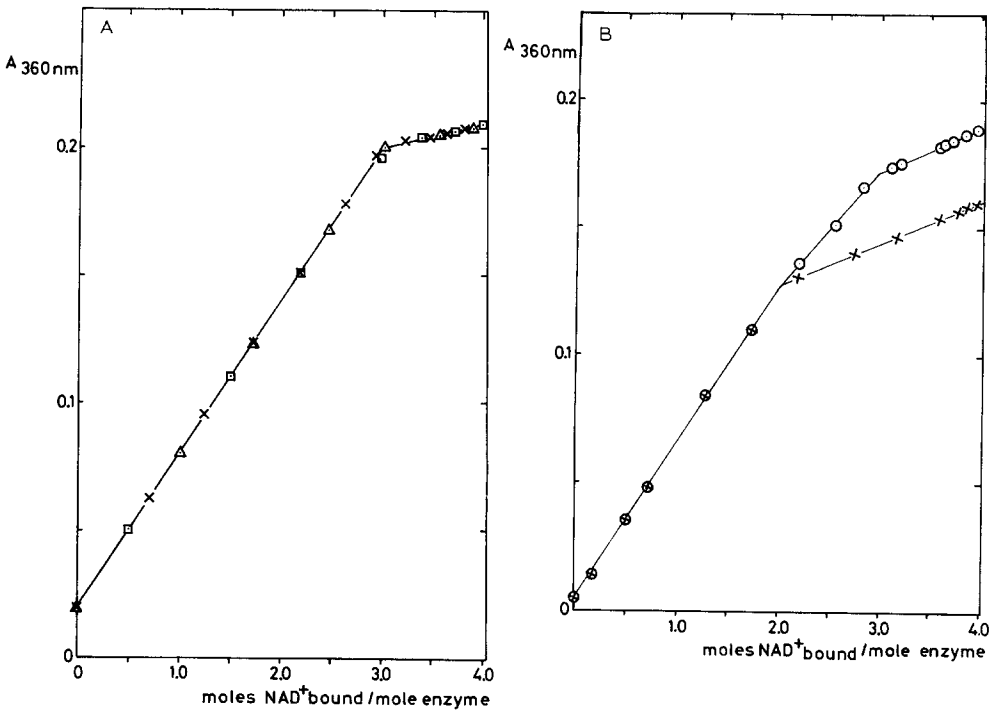

Fig. 2. (A) Data of Fig. 1 (in the presence of ATP, phosphate and glyceraldehyde) calculated for moles NAD^+ bound per mole enzyme. The absorbance at 360 nm is plotted against NAD^+ bound, calculated from the amount added and the dissociation constants given in Table I. (B) Data of Fig. 1 (acetyl phosphate) calculated for moles NAD^+ bound per mole enzyme, calculated from the amount added and the dissociation constants given in Table I, are given by points ○. The points × refer to a similar experiment carried out at pH 7.5.

TABLE II

DISSOCIATION CONSTANTS OF NADH BOUND TO GLYCERALDEHYDEPHOSPHATE DEHYDROGENASE IN THE PRESENCE OF SUBSTRATES, EFFECTORS AND IODOACETATE

The dissociation constants were measured by ultrafiltration at 20 °C, pH 8.2. The values are the means of usually 3 (sometimes 2) independent determinations. The enzyme concentration was 20 μM, while the substrates, effectors and the inhibitor were present in a concentration of 1 mM.

Addition	*Dissociation constant (μM)*			
	K_1	K_2	K_3	K_4
None*	$\leqslant$0.5	$\leqslant$0.5	2.5	50
Glyceraldehyde	$\leqslant$0.5	$\leqslant$0.5	3.3	52
Glyceraldehyde 3-phosphate	$\leqslant$0.5	$\leqslant$0.5	2.8	52
Phosphate	$\leqslant$0.5	$\leqslant$0.5	2.2	48
Arsenate	$\leqslant$0.5	$\leqslant$0.5	2.5	52
ADP	$\leqslant$0.5	$\leqslant$0.5	4.7	62
ATP	$\leqslant$0.5	$\leqslant$0.5	4.2	60
Iodoacetate	$\leqslant$0.5	$\leqslant$0.5	25	200

* ref. 9.

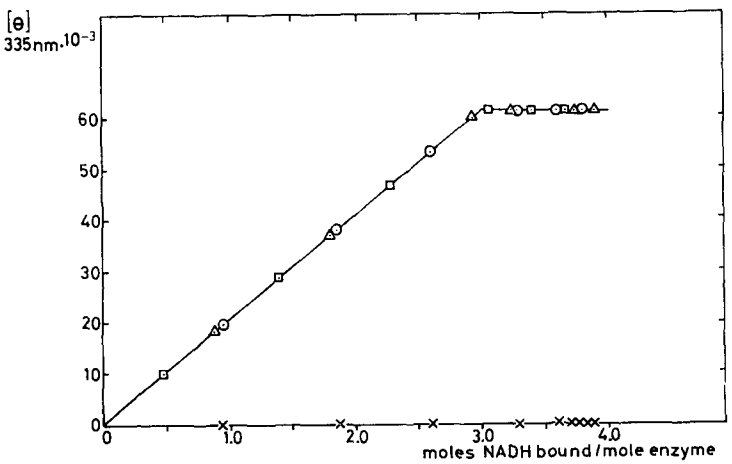

Fig. 3. Changes in the molecular ellipticity $[\theta]$ at 335 nm of charcoal-treated glyceraldehyde-phosphate dehydrogenase (83 μM) on adding NADH, in the presence of glyceraldehyde 3-phosphate (□), phosphate (△), ATP (○) and iodoacetate (×). $[\theta]_{335\ nm}$ is plotted against NADH bound, calculated from the dissociation constants given in Table II and the amount NADH present, calculated from NADH added, assuming that NADH is converted into its hydrate with a rate constant of 0.01 min^{-1} and 0.014 min^{-1} in the presence and absence of phosphate, respectively, as measured in a separate control experiment. The molecular ellipticity at 335 nm is corrected for the signal of free NADH (*cf.* ref. 9). The enzyme was dissolved in 100 mM Tris–HCl buffer (pH 8.2), containing 5 mM EDTA; temp. 20 °C.

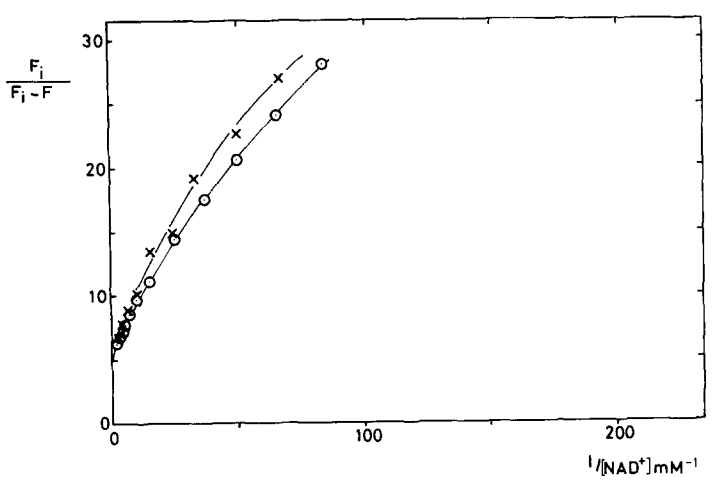

Fig. 4. Double-reciprocal plot of the fluorescence of charcoal-treated glyceraldehydephosphate dehydrogenase (4.95 μM) on addition of NAD^+ (relative to E–$(NAD^+)_2$ as 1.0), in the presence of 1 mM acetyl phosphate (○) or 1 mM iodoacetate (×). F_i is the initial fluorescence and F the fluorescence at any point in the titration. The enzyme was dissolved in 100 mM Tris–HCl buffer (pH 8.2) containing 5 mM EDTA; temp. 20 °C.

dialysis in the presence of acetyl phosphate, are not pH dependent within the range 7.0 to 8.2.

In agreement with earlier work[18], iodoacetate abolishes the 360-nm band.

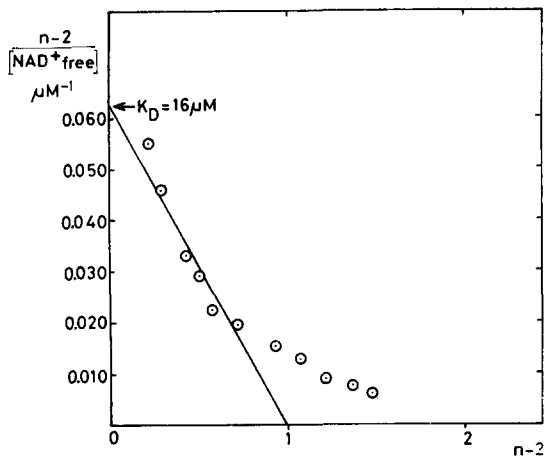

Fig. 5. Scatchard plot of the binding of the third and fourth molecule of NAD^+ to charcoal-treated glyceraldehydephosphate dehydrogenase in the presence of 1 mM acetyl phosphate, calculated from the data in Fig. 4, on the assumption that the first two sites are completely occupied before NAD^+ is bound to the other sites and assuming that the binding of the third and fourth NAD^+ molecule quenches equally the protein fluorescence. n = number of molecules NAD^+ bound per molecule enzyme.

335-nm circular-dichroism band of enzyme–NADH complex

Binding of NADH to glyceraldehydephosphate dehydrogenase causes a characteristic positive absorbance in the circular-dichroism spectrum from 300 to 400 nm, with a maximum at 335 nm (ref. 9). The first three NADH molecules bound to the enzyme contribute equally to this band, whereas there is no contribution from the fourth molecule[9]. Fig. 3 shows that glyceraldehyde 3-phosphate, phosphate and ATP have no effect on the formation of the band, which is, however, completely abolished by iodoacetate.

Quenching of fluorescence of glyceraldehydephosphate dehydrogenase on binding of NAD^+ or NADH

The fluorescence of glyceraldehydephosphate dehydrogenase is quenched on adding either NAD^+ or NADH. Although Velick's earlier data[19] seemed to show that the fourth molecule behaves differently from the other three, also in this respect, Price and Radda[20] have recently shown that, when allowance is made for the relatively low binding constant of the fourth molecule, this molecule also quenches the protein fluorescence.

Figs 4 and 5 show an experiment carried out to test whether, in the presence of acetyl phosphate, which as shown above lowers the affinity of the enzyme for NAD^+ without abolishing the co-operativity, the fluorescence of the third and fourth subunits are equally quenched when they bind NAD^+. Fig. 4 shows a double-reciprocal plot of the degree of quenching of the fluorescence of the enzyme–$(NAD^+)_2$ complex on the addition of various concentrations of NAD^+. The curve concave to the abscissa is to be expected since the fourth molecule is bound less firmly than the third. The fluorescence of the enzyme–$(NAD^+)_4$ complex was obtained by extrapolating this line to the ordinate.

Using this value, each point in Fig. 4 is recalculated for the Scatchard plot,

shown in Fig. 5, assuming that the third and fourth subunit are equally quenched. From this Scatchard plot and the formula derived by Price and Radda[20] it is possible to calculate dissociation constants of 16 and 90 μM, respectively, for the third and fourth molecules, values that are in good agreement with those obtained by equilibrium dialysis in the presence of acetyl phosphate (*cf.* Table I). Thus, the third and fourth molecules of NAD^+ quench the fluorescence of the enzyme equally.

Similar results were obtained in the presence of acetaldehyde, glyceraldehyde, phosphate, arsenate, ADP or ATP and with NADH instead of NAD^+. It may be concluded, then, that the differences between the third and fourth molecules of the nucleotide with respect to the 360-nm absorption band of the NAD^+ complex and to the 335-nm circular-dichroism band of the NADH complex are not reflected in quenching by the nucleotide of the protein fluorescence. Even iodoacetate, which completely abolishes these two bands, has little effect on the quenching of the protein fluorescence by NAD^+ added to $E-(NAD^+)_2$ (Fig. 4). It also has no effect on the quenching by the two first molecules that are firmly bound, even to carboxymethylated enzyme (Fig. 6). The smaller quenching by NAD^+ added in excess of 2 molecules per molecule enzyme reflects the weaker binding to carboxymethylated enzyme. From the Scatchard plot and the formula of Price and Radda, values of K_3 and K_4 of 17 and 225 μM, respectively, may be calculated, which are close to those found in the binding experiments (Table I).

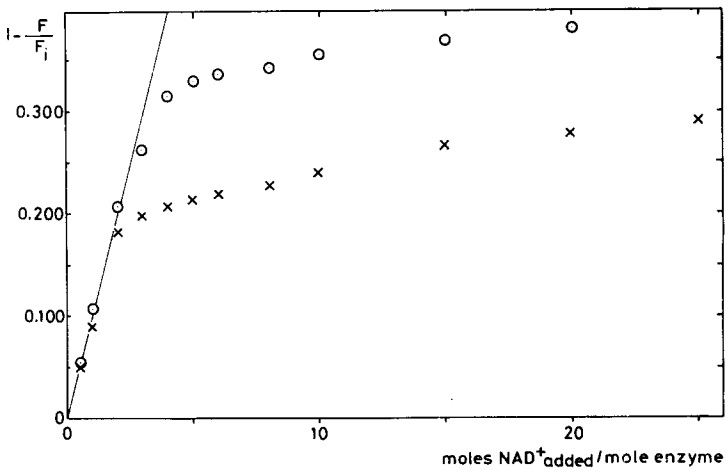

Fig. 6. Quenching of the fluorescence of charcoal-treated glyceraldehydephosphate dehydrogenase (4.95 μM) on addition of NAD^+, in the presence (×) or absence (○) of 1 mM iodoacetate. F_i is the initial fluorescence and F the fluorescence at any point in the titration. The enzyme was dissolved in 100 mM Tris–HCl buffer (pH 8.2), containing 5 mM EDTA; temp. 20 °C.

DISCUSSION

The most important conclusion to be drawn from the experiments described in this paper is that, under a variety of conditions, the binding constants of NADH to the four subunits of glyceraldehydephosphate dehydrogenase are about the same as for NAD^+. Most significantly, this is the case after carboxymethylation of the active-centre thiol groups by iodoacetate, which decreases the binding of both the

oxidized and reduced nucleotide to about the same extent. The effect of iodoacetate on the binding constants of NAD^+ shown in Table I is consistent with the finding of Friedrich[21] that iodoacetate-treated enzyme loses 35% of its NAD^+ content on passing over a column. Acetylation of the enzyme, in which half of these thiol groups seem to be involved[14], also decreases the affinity of NAD^+ for the enzyme, as predicted from kinetic studies by Bloch *et al.*[22] and Smith and Velick[23]. The effect of acetylation shown in Table I is rather less than might be expected from the report of Krimsky and Racker[24] who found that phosphoglyceryl enzyme lost 75% of its NAD^+ on passing through a Sephadex column. Although it was not possible directly to test the binding of NADH to acylated enzyme, it seems unlikely on the basis of the effects of carboxymethylation that the binding of NADH is less affected than that of NAD^+, as suggested by Bloch *et al.*[22] and Smith and Velick[23].

Our results, then, give no support to the suggestion of Bloch *et al.*[22] that a physiological role of the enzyme is to regulate the redox potential of NAD^+ by a differential effect of acylation on the binding of NAD^+ and NADH to the enzyme.

It is rather surprising that acetylation affects the intensity of the charge-transfer band of only one of the four subunits. Bernhard and co-workers[14,16,17,22] have shown that the enzyme reacts towards acylating agents as if it has a subunit structure $(\alpha\alpha')_2$ rather than α_4 and that only one subunit in each dimer is acylated. Our finding that acetylated enzyme containing 4 molecules of bound NAD^+ shows two bands of normal intensity and two bands of low intensity (equal to that given by the "fourth" NAD^+ molecule in non-acylated enzyme) is in agreement with this concept, and suggests the possibility that the "third" and "fourth" NAD^+ molecules combine with the subunits that are acylated.

The binding studies reported in this paper give us no reason to abandon our proposal[9,10] that the catalytically active subunit of the enzyme is the one that binds NAD^+ and NADH least firmly. This proposal is based partly on the finding that NADH is a competitive inhibitor for the enzyme-catalysed reduction of NAD^+ by glyceraldehyde, with a K_i (90 μM) close to the dissociation constant for the fourth molecule of NADH bound to the enzyme (50 μM for non-acylated enzyme, presumably 75 μM for acylated), and that NAD^+ is a competitive inhibitor for the enzyme-catalysed oxidation of NADH by acetyl phosphate with a K_i (45 μM) close to the dissociation constant for the fourth molecule of NAD^+ bound to the enzyme (35 μM for non-acylated enzyme, 75 μM for acylated). If the subunits that bind NAD^+ and NADH more firmly were predominantly involved in the enzymic reaction, product inhibition would be expected with lower K_i values. If both types of subunit were involved, linear Lineweaver–Burk and Dixon plots would not be expected. This conclusion has been criticized by Peczon and Spivey[25], who state that the degree of inhibition is governed by the ratio K_m/K_i, rather than by K_i alone. Although this statement is, of course, correct, it is irrelevant to our argument, since we determined K_i by conventional enzyme kinetics, and K_i is independent of K_m.

In our previous papers, we left open the possibility that, in addition to the fourth subunit, the third might play a limited role in the catalytic mechanism. The relatively high value of K_3 for the acetylated enzyme strengthens this possibility somewhat, at least for the catalysis of the oxidation of aldehyde by NAD^+. It is possible that the larger acyl group, phosphoglyceryl, would have a greater effect. The simultaneous transfer of hydride ion to NAD^+ and acylation of the enzyme

thiol by the dehydrogenated aldehyde might conceivably facilitate the dissociation of the NADH before phosphorylysis or arsenolysis of the thiol ester, after which NAD^+ will readily bind to the de-acylated enzyme. If this is the case, the competition between NAD^+ and NADH will be for the de-acylated enzyme. However, kinetic evidence suggests that dissociation of NADH is subsequent to decomposition of the thiol ester[26,27]. The dissociation constant of 75 μM (at 20 °C) for binding of NAD^+ to the fourth site of acetylated enzyme agrees reasonably well with 80–150 μM NAD^+ (at 37 °C) reported by Smith and Velick[23] for the concentration for half-maximal velocity for the reaction in the direction of oxidative phosphorylation.

The facilitation of the dissociation of NADH by acylation of the enzyme is no help in the catalysis of the oxidation of NADH by acyl phosphates. Indeed, the de-acylation of the enzyme consequent to the transfer of hydride from NADH will hinder the dissociation of NAD^+, and it remains difficult to understand how the enzyme catalyses this reaction if the concentrations of NAD^+ and NADH in the liver cytosol are 0.5 mM and 0.37 μM, respectively, as reported by Bücher[28]. If the liver enzyme has properties similar to those of the muscle enzyme, and Smith and Velick[23] indicate that this is the case, the oxidation of NADH would be very strongly inhibited. Smith and Velick[23] suggest as a solution of this dilemma that acylation preferentially facilitates the dissociation of NAD^+ compared with NADH but, as already indicated, our binding studies give no support to this suggestion, although they do not directly disprove it. Other possible solutions are: (i) the concentrations of NAD^+ and NADH, calculated by Bücher[28] are, for some reason, not the appropriate concentrations for glyceraldehydephosphate dehydrogenase; (ii) conditions in the liver, not realized *in vitro*, favour the dissociation of NAD^+ from the enzyme; (iii) a separate enzyme catalyses the oxidation of NADH by acyl phosphate in gluconeogenesis.

The charge-transfer band of the enzyme–NAD^+ complex at 360 nm and the circular-dichroism band of the enzyme–NADH complex appear to be sensitive probes of the conformation of the subunits. Carboxymethylation of the active-centre thiol group in each subunit modifies the conformation in such a way that NAD^+ and NADH are less firmly bound, and the charge-transfer and circular-dichroism bands of the native enzyme are completely absent. In the native enzyme, the binding of NADH to the last subunit resembles that in the carboxymethylated subunits *in that* the binding constant is lower and the circular-dichroism band is completely absent. NAD^+-bound to the last subunit still gives a charge-transfer band, but its intensity is less than that of NAD^+ bound to the other subunits[1,9].

In disagreement with our previous findings[1,9], Peczon and Spivey[25] claim that the fourth molecule of NAD^+ bound to the enzyme gives a charge-transfer band with an intensity equal to that of the first three, in analogy with the quenching of the protein fluorescence. They suggest that our results were due to our use of an enzyme preparation with a lower activity than that used by themselves or by Bloch *et al.*[22]. We wish to bring the following arguments against this conclusion of Peczon and Spivey. (1) The same enzyme preparation was used for our measurements of fluorescence quenching by NAD^+, in which there is no difference between the four molecules, and for the titration at 360 nm in which the fourth molecule behaves differently. (2) Price and Radda[20] found no difference in quenching behaviour between enzyme made by the method of Amelunxen and Carr[29] and a commercial preparation of relatively low activity. (3) Our preparations of enzyme (activity

145–165 units/mg) were about as active as those used by Peczon and Spivey (160 units/mg). Ageing of our enzyme preparation, which caused the specific activity to decline from 165 units to 120 units per mg, had no effect on the absorbance coefficient or on the binding constant of the fourth molecule of NAD^+. (4) Peczon and Spivey plot their absorbance data in terms of amount of added NAD^+ (*cf.* Fig. 1 in this paper). When their data are plotted against bound NAD^+, as in Fig. 2A of this paper, using our binding constants, the fourth molecule of NAD^+ bound to the most purified enzyme preparation used by Peczon and Spivey (160 units/mg) gives no absorbance increase at 340 nm in the experiment shown. In three experiments with the less purified preparation (145 units/mg), the absorbance coefficient of the fourth molecule is 43% of that of the other three. Although this large difference between the two preparations is difficult to explain, Peczon and Spivey's data agree with ours in showing that the absorbance increment on binding the fourth molecule is less than that of the other three. (5) Similarly, when their data for the quenching of the absorbance of NADH on adding to the enzyme are plotted in terms of bound NADH, using our binding constants, the fourth molecule of NADH is found to contribute little (0–20%) to the absorbance change, in agreement with our circular-dichroism measurements.

Acetylation of the active-centre thiol of the enzyme has a much smaller effect than carboxymethylation. It decreases the binding constant for NAD^+, but affects the charge-transfer band of only one subunit, the "third", reducing the intensity of the band at pH 7.5 to that of the "fourth" subunit.

Quenching of the protein fluorescence by NAD^+ or NADH is a much less sensitive indicator of subunit conformation. Thus, in both non-acetylated[20] and acetylated enzyme (this paper) all four bound NAD^+ or NADH molecules quench the fluorescence equally. Moreover, carboxymethylation of the active-centre thiol group has no effect on the degree of quenching. Apparently, the bound NAD^+ or NADH molecule is still sufficiently close to the fluorescing tryptophan residues, even in the modified subunits, to cause quenching of the fluorescence.

The maximum effect of carboxymethylation on the binding constant of NAD^+ is about 10-fold, corresponding to a binding energy of 1.4 kcal/mole NAD^+. Since the charge-transfer band is abolished by carboxymethylation, this must represent the maximum binding energy to be attributed to a charge-transfer bond. This is indeed in the range of most charge-transfer bonds[30]. However, since NADH and NAD^+ are bound with about the same affinity, and the reduced pyridine ring in NADH cannot be the acceptor in a charge-transfer bond, it seems likely that the real contribution of the charge-transfer bond to the binding energy of NAD^+ to the enzyme is much less than 1.4 kcal/mole, compared with the total binding energy for the 4 molecules of NAD^+ of $\geqslant 10$, $\geqslant 10$, 7.34 and 6.05 kcal/mole, respectively. Indeed the isolation of active enzyme in which the NAD-binding sites are replaced by ADP–ribose[22] indicates that the pyridine ring is relatively unimportant for the binding to the enzyme.

The small effect of acetylation on the intensity of the charge-transfer band makes it unlikely that the active-centre thiol group is the donor to the NAD^+ in the charge-transfer complex. We shall return to this point in a later paper.

The energy for the binding of the fourth molecule of NAD^+ to the enzyme is at least 4 kcal/mole less than that of the first. This may be compared with haemoglobin in which the binding of the fourth molecule of oxygen is 4.6 kcal/mole more

than that of the first[31]. Perutz[32] has shown that the co-operative binding of oxygen to haemoglobin is due to the breaking of inter-subunit interactions, largely salt bridges, as a consequence of the binding of oxygen to the first subunits. By analogy, it might be expected that the negative cooperativity of NAD^+ and NADH binding to glyceraldehydephosphate dehydrogenase is caused by the creation, as a result of binding of nucleotide to the first subunits, of inter-subunit interactions that have to be broken before nucleotide molecules can bind to the other subunits. Simon[31] has shown by small angle X-ray scattering that after binding of each NAD^+ molecule the subunits are displaced from one another, the effect decreasing with each molecule bound.

EXPERIMENTAL

Glyceraldehydephosphate dehydrogenase was isolated from rabbit muscle by the method of Cori *et al.*[33], slightly modified by Hilvers[34]. The specific activity, measured spectrophotometrically using DL-glyceraldehyde 3-phosphate, NAD^+ and phosphate by the method of Ferdinand[35], was 145–165 μmoles NADH per min per mg protein. (The correction factor[35] of 1.07 for non-saturation with respect to phosphate was not used.) NAD^+ was removed by stirring with charcoal (0.2 g per ml solution; *cf.* ref. 36). The enzyme concentration was calculated from absorbance measurements at 280 nm, using the extinction coefficients reported by Fox and Dandliker[37]. A molecular weight of 145 000 was assumed[38]. NAD^+ and NADH were determined enzymically with ethanol and alcohol dehydrogenase (EC 1.1.1.1), and pyruvate and lactate dehydrogenase (EC 1.1.1.27), respectively, assuming that $A_{340\ nm}$ for NADH equals 6.22 $mM^{-1} \cdot cm^{-1}$.

[Carbonyl-^{14}C]NAD^+ was purchased from the Radiochemical Centre, Amersham, England. Iodoacetic acid (The British Drug Houses Ltd) was recrystallized from carbon tetrachloride and dried before use. All other materials were obtained from commercial sources and were used without purification.

Absorbance measurements were carried out with a Zeiss PMQ II spectrophotometer or with a Cary recording spectrophotometer, Model 17, and circular-dichroism measurements with a Cary spectropolarimeter, Model 60, with a 6002 CD attachment. In the titration experiments a correction for a "stirring effect"[25] was not necessary when the enzyme solution was allowed to settle for a moment. Fluorescence measurements were performed with a Hitachi Perkin Elmer spectrofluorimeter, Model MPF-2A. The fluorescence intensities were corrected for the attenuation of the incident light by added NAD^+ or NADH, using as blank a glycyl-L-tryptophan solution with the same absorbance at the excitation wavelength.

Ultrafiltration was carried out in a UF cell from Amicon, equipped with a PM-30 filter (see ref. 9). The NAD^+ or NADH concentrations were measured enzymically, and compared with standard NAD^+ or NADH solutions ultrafiltered in the same way. Equilibrium dialyses with enzyme and ^{14}C-labelled NAD^+ were carried out in 1-ml cells for 16 h at 20 °C (*cf.* refs 2, 8). Controls showed that equilibrium was reached in that time and assays of enzyme activity revealed that denaturation occurred only to a minor degree (5–15%).

The concentration of enzyme exceeded 5 μM in all cases. According to the data

of Lakatos *et al.*[39], the enzyme would be essentially in the tetrameric form at these concentrations.

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