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# BINDING OF NAD+ AND NADH TO RABBIT-MUSCLE GLYCERALDEHYDE-PHOSPHATE DEHYDROGENASE

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SUMMARY

1. The fourth NAD+ molecule bound to glyceraldehydephosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD+ oxidoreductase (phosphorylating), EC 1.2.1.12) contributes to the absorbance at 360 nm and the molecular ellipticity at 350 nm, but the absorbance coefficient of the NAD+–enzyme complex is 30% and the molecular ellipticity 40% of that found for the first three NAD+ molecules bound.

2. NAD+, present in excess of that sufficient to saturate the four specific binding sites, reacts with the enzyme in such a way as to give what is very probably a charge-transfer complex.

3. Ultracentrifugation and ultrafiltration showed that 4 moles of NADH may be bound to the enzyme. The first two molecules are bound stoicheiometrically within the experimental error ($K_D < 0.5$ $\mu$M), while the third and the fourth molecules are bound with dissociation constants of 2.5 and 50 $\mu$M, respectively.

4. The first three molecules of NADH bound to the enzyme cause the formation of a circular-dichroism band at 335 nm of equal intensity for each molecule. The fourth molecule does not contribute to the molecular ellipticity at 335 nm.

5. NADH inhibits the oxidation of glyceraldehyde by NAD+, in the presence of arsenate, competitively with respect to NAD+. The $K_i$, 90 $\mu$M, is similar to that of the dissociation constant of the fourth molecule of NADH.

## INTRODUCTION

Rabbit-muscle glyceraldehydephosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD+ oxidoreductase (phosphorylating), EC 1.2.1.12) is a tetramer composed of four identical sub-units[1] each binding one molecule of NAD+ (refs. 2,3). The binding shows negative cooperativity in the sense that the third molecule is bound less firmly than the first two (that are bound too firmly to allow a determination of the dissociation constant) and the fourth molecule is bound less firmly than the third[2,3].

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Binding of $NAD^+$ to this enzyme results in the formation of a broad absorption band with a peak at 360 nm (ref. 4), thought to be a charge-transfer band[5]. The first three $NAD^+$ molecules bound to the enzyme give rise to a band of equal intensity[6,3], while from our previous work it appeared that the fourth molecule bound to the enzyme gave rise to little, if any, increase of absorption at this wavelength[3,6-9]. The enzyme–$NAD^+$ complex is optically active[10], the first three molecules of $NAD^+$ bound to the enzyme causing an equal increase in the molecular ellipticity at 350 nm, the peak of the circular-dichroism band, whereas the fourth molecule, according to DE VIJLDER AND HARMSEN[10], gives no circular-dichroism signal.

VELICK[11] reported also that only three $NAD^+$-binding sites contribute to the quenching of the protein fluorescence. PRICE AND RADDA[12] have, however, recently shown that the fourth binding site was not occupied in VELICK's experiments, and that all four bound $NAD^+$ molecules quench the fluorescence equally. In view of this finding kindly communicated to us personally, we have determined quantitatively the contribution of the fourth binding site to the optical and circular-dichroism spectrum.

We have also measured the binding constants of NADH to the enzyme, parameters that are equally important for an understanding of its mechanism of action. The only publication on the binding constants of NADH with the enzyme is that of VELICK[11] based on the quenching of the fluorescence of NADH bound to the enzyme. Since this method contains the implicit assumption that the degree of quenching is the same for each molecule bound, it is desirable to have an independent measurement of the binding constants. Determinations made by ultracentrifugation and ultrafiltration are described in this paper.

## RESULTS

### *The contribution to the absorption at 360 nm of the fourth molecule of $NAD^+$ specifically bound to glyceraldehydephosphate dehydrogenase and of $NAD^+$ non-specifically bound or unbound*

Fig. 1A shows the effect on the absorbance at 360 nm of increasing amounts of $NAD^+$, up to 50 moles per mole enzyme, added to glyceraldehydephosphate dehydrogenase. The first two molecules of $NAD^+$ added result in a linear increase in absorbance. The curve bends between 2 and 5 molecules, and a new straight line, of much smaller slope than the initial, is found between 5 and 50 moles. In Fig. 1B the results are plotted on the basis of bound $NAD^+$, calculated from the dissociation constants previously reported (see Table I). Three phases may be distinguished in Fig. 1B: (1) a linear increase with absorption coefficient of 0.97 $mM^{-1}\ NAD^+ \cdot cm^{-1}$ (*cf.* 0.96 reported by DE VIJLDER AND SLATER[3]) up to 3 moles bound $NAD^+$ per mole enzyme; (2) a linear increase with absorbance coefficient 0.30 $mM^{-1}\ NAD^+ \cdot cm^{-1}$ between 3 and 4 moles $NAD^+$ bound per mole enzyme; (3) an increase in $A_{360\ nm}$ independent of the amount of $NAD^+$ bound. The effect of $NAD^+$ in excess of the four molecules of $NAD^+$ specifically bound is shown on an extended scale of absorbance in Fig. 1C. The absorbance coefficient is 0.011 $mM^{-1}\ NAD^+ \cdot cm^{-1}$.

Fig. 2 shows that the first two phases observed in the titration of $A_{360\ nm}$ against $NAD^+$ are also obtained in a titration of $[\theta]_{350\ nm}$. For the two phases, $[\theta]_{350\ nm}$

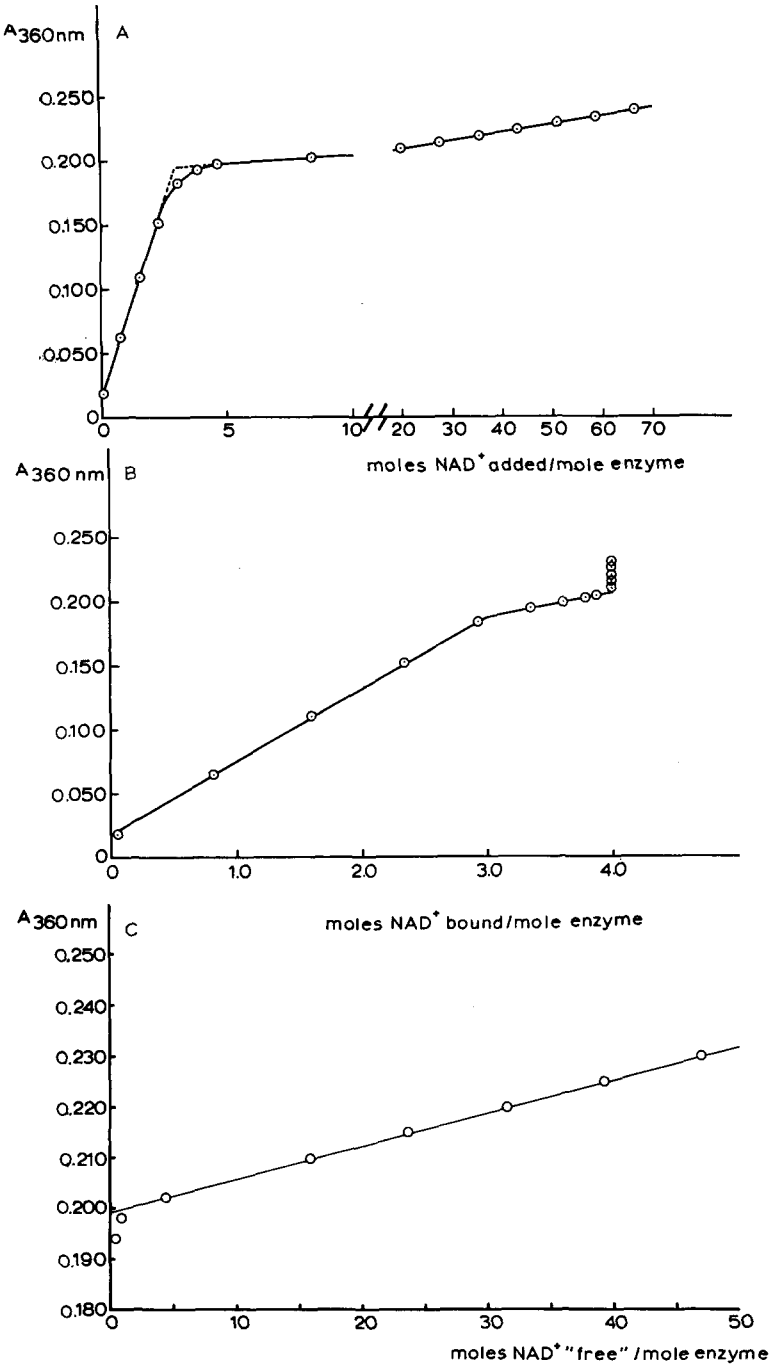


Fig. 1. Titration of charcoal-treated glyceraldehydephosphate dehydrogenase (60.7 $\mu$M) with $NAD^+$ at 360 nm. The enzyme was dissolved in 100 mM Tris–HCl buffer (pH 8.2), containing 5 mM EDTA; temp., 23°. A. Plotted against $NAD^+$ added. B. Plotted against $NAD^+$ bound, calculated from the dissociation constants 0.3 $\mu$M (ref. 2) for the third molecule and 35 $\mu$M (ref. 3) for the fourth one, as shown in Table I. C. Plotted against $NAD^+$ free, calculated from the same dissociation constants as in B.

TABLE I

DISSOCIATION CONSTANTS OF NAD BOUND TO RABBIT-MUSCLE GLYCERALDEHYDEPHOSPHATE DEHYDROGENASE

Dissociation constants given in $\mu$M.

| *Dissociation constant* | *NADH* | | *NAD+* | | |
|---|---|---|---|---|---|
| | *Ultracentrifugation*[a] | *Ultrafiltration*[b] | *Ultracentrifugation*[c] | *Equilibrium dialysis*[d] | *Quenching of protein fluorescence*[e] |
| $K_1$ | <1 | <0.5 | <0.05 | — | — |
| $K_2$ | <1 | <0.5 | <0.05 | <0.01 | — |
| $K_3$ | 5 | 2.5 | 4 | 0.3 | 1.7 |
| $K_4$ | 50 | 50 | 35 | 26 | 34 |

[a] At 5°, pH 8.2.
[b] At 5° and 20°, pH 8.2.
[c] At 20°, pH 8.2; from ref. 3.
[d] At 3°, pH 8.5; from ref. 2.
[e] At 25°, pH 8.2; from ref. 12.

was found to be 5000 and 2000° · $M^{-1}$ $NAD^+$ · $cm^{-1}$, respectively. The first value agrees closely with that of DE VIJLDER AND HARMSEN[10]. In contrast to the data plotted by these authors, however, $[\theta]_{350\ nm}$ like $A_{360\ nm}$ continues to increase after three $NAD^+$

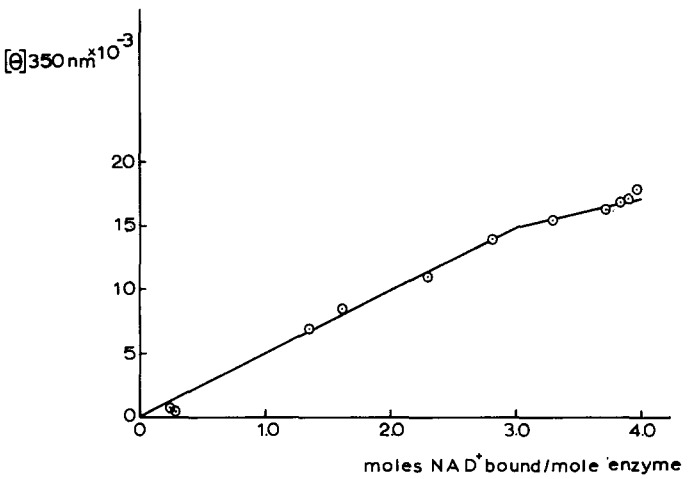


Fig. 2. Circular-dichroism titration of charcoal-treated glyceraldehydephosphate dehydrogenase (70 $\mu$M) at 350 nm. The enzyme was dissolved in 100 mM Tris–HCl buffer (pH 8.2), containing 5 mM EDTA; temp., 20°. $NAD^+$ bound was calculated from the dissociation constants as used in Fig. 1B.

molecules have been bound. The difference in the results resides in the fact that DE VIJLDER AND HARMSEN[10] applied a correction for the circular dichroism of $NAD^+$ itself, whereas in our hands $NAD^+$ shows no optical activity beyond 300 nm.

*Dissociation constants of NADH bound to glyceraldehydephosphate dehydrogenase*

In previous work both equilibrium dialysis[2,7] and ultracentrifugation[3] were used to determine the dissociation constants of $NAD^+$ bound to the enzyme. The former method is more accurate, but since a prolonged dialysis is required to reach equilibrium, it can be carried out only at low temperature, whereas most of the kinetic parameters of the enzyme have been measured at 20–25°. Ultracentrifugation can be carried out at the latter temperatures with apo-enzyme in the presence of $NAD^+$. Since, however, the apo-enzyme is unstable in the presence of NADH[13] and, moreover, it catalyses the slow conversion of NADH into its hydrate[14], ultracentrifugation could be carried out in the presence of NADH only at 5°, and equilibrium dialysis could not be used, even at this temperature. For this reason, the more rapid ultrafiltration was used, both at 20° and 5°.

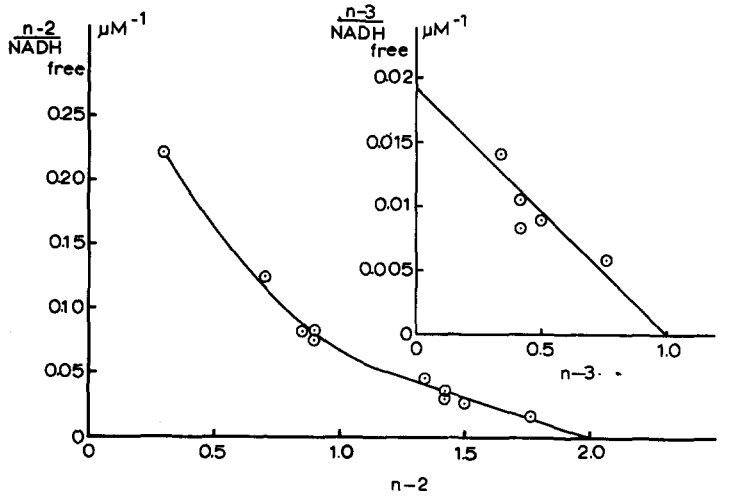


Fig. 3. Scatchard plot of binding of third and fourth molecules of NADH to charcoal-treated glyceraldehydephosphate dehydrogenase, measured by ultrafiltration at 20°, and calculated on the assumption that the first two sites are completely occupied before NADH is bound to the other sites. The enzyme was dissolved in 100 mM Tris–HCl buffer (pH 8.2), containing 5 mM EDTA. Inset: Scatchard plot of the fourth molecule, calculated on the assumption that the first three sites are completely occupied before NADH is bound to the fourth site. $n$ = number of molecules NADH bound per molecule enzyme.

Fig. 3 shows the results of such studies, carried out at 20°, plotted in the form of a modified Scatchard plot[2] for the third and fourth molecule NADH. With up to 2 moles of NADH added per mole of charcoal-treated enzyme, no NADH could be detected in the filtrate. Based on a sensitivity of 2 $\mu$M NADH, it is calculated that the dissociation constant of these two molecules is $<0.5\ \mu$M. Four molecules are bound per mole enzyme. The curve, convex to the abscissa, shows a negative cooperativity between the third and fourth molecule. The inset shows the data for the fourth

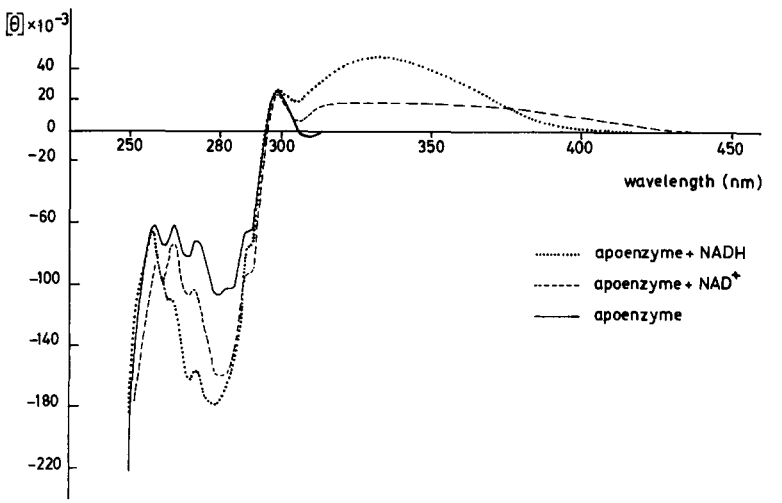


Fig. 4. Circular dichroism of charcoal-treated glyceraldehydephosphate dehydrogenase (75–80 $\mu$M). ———, spectrum of the apoenzyme; — — —, spectrum of the apoenzyme + 7.5 moles $NAD^+$ added per mole enzyme; · · · · · ·, spectrum of the apoenzyme + 7.5 moles NADH added per mole enzyme. 80 $\mu$M enzyme in 100 mM Tris–HCl buffer (pH 8.2), containing 5 mM EDTA; temp., 20°.

molecule. The dissociation constants calculated from this experiment, from an ultrafiltration at 5° and from ultracentrifugation at 5° are assembled in Table I.

The dissociation constants for NADH are similar to those of $NAD^+$, which is not surprising if it is the adenine moiety that is primarily involved in the binding, as suggested by YANG AND DEAL[15].

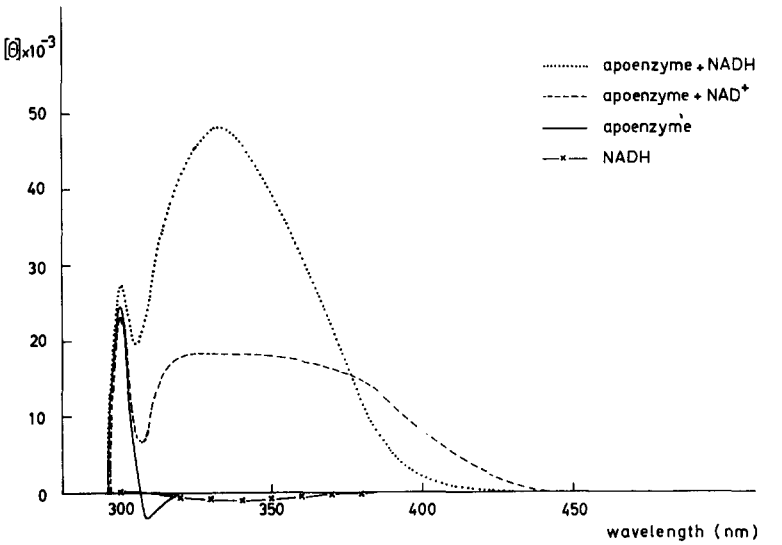


Fig. 5. Circular dichroism of charcoal-treated glyceraldehydephosphate dehydrogenase and of NADH. ×—×, spectrum of NADH. For the notation of the spectra of the enzyme and the conditions see Fig. 4.

*Effect of binding of NADH on optical properties*

NADH bound to glyceraldehydephosphate dehydrogenase absorbs at a somewhat longer wavelength and with a somewhat lower intensity than NADH free in solution (*cf.* VELICK AND FURFINE[16]; FISHER *et al.*[17]), but the effect is too small for accurate quantitative measurements, particularly since it is necessary to correct for the conversion of NADH into the hydrate catalysed by the enzyme[14].

Binding of NADH to the enzyme has a marked effect on its circular-dichroism spectrum. Fig. 4 shows the spectrum between 250 and 450 nm for apo-enzyme, apo-enzyme to which 7.5 moles NADH were added per mole enzyme (3.84 moles bound

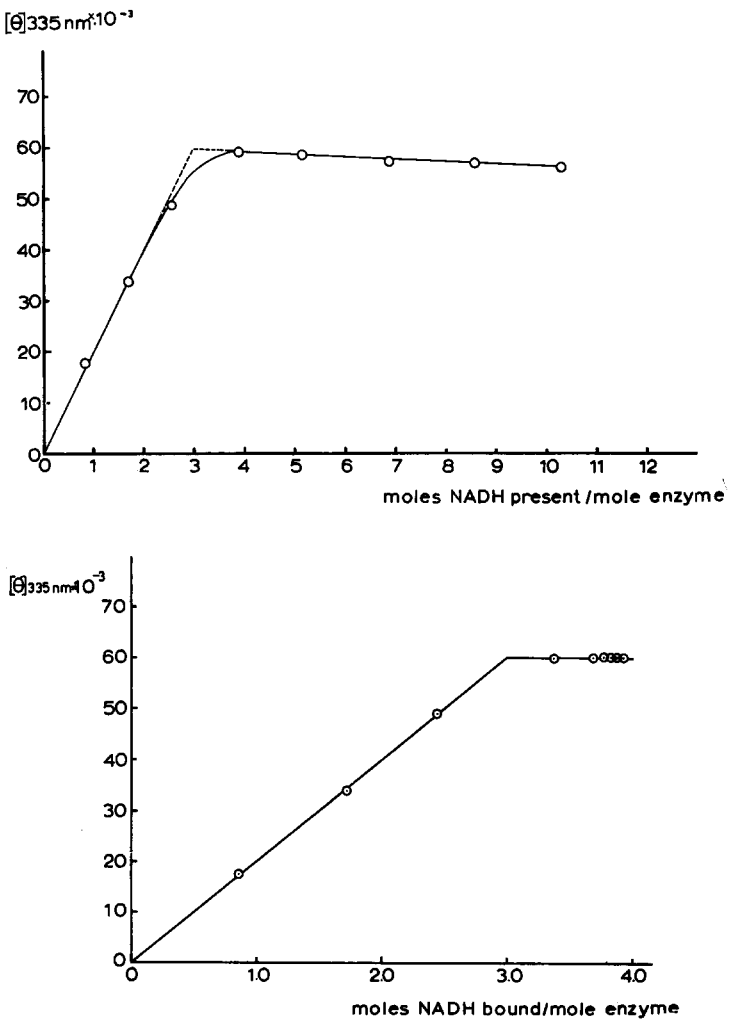


Fig. 6. Changes in the molecular ellipticity $[\theta]$ at 335 nm of charcoal-treated glyceraldehydephosphate dehydrogenase (82 $\mu$M) on adding NADH. For conditions see Fig. 4. A. $[\theta]_{335\ nm}$ plotted against NADH present, calculated from NADH added, assuming that NADH is converted into its hydrate with a rate constant of 0.01 $min^{-1}$ as shown in a separate control experiment. B. $[\theta]_{335\ nm}$ plotted against NADH bound, calculated from the dissociation constants as shown in Table I and corrected for the signal of free NADH.

NADH), and apo-enzyme to which 7.5 moles $NAD^+$ were added per mole enzyme (3.89 moles bound $NAD^+$). Fig. 5 shows the spectra between 295 and 450 nm, as well as that of free NADH in this region.

Whereas free NADH has a very weak negative circular-dichroism band at about 340 nm (molecular ellipticity: $-1000° \cdot M^{-1} \cdot cm^{-1}$), NADH bound to the enzyme has a large positive band with maximum at 335 nm (molecular ellipticity: $48\,000° M^{-1} \cdot cm^{-1}$), larger than the band for $NAD^+$ bound to the enzyme previously reported by DE VIJLDER AND HARMSEN[10]. A titration of the circular-dichroism band at 335 nm is shown in Fig. 6A. The abscissa shows the amount of NADH present, calculated from that added by allowing for the amount of NADH hydrate formed, as determined in a control experiment. The decline of $[\theta]$ after 4 moles NADH is due to

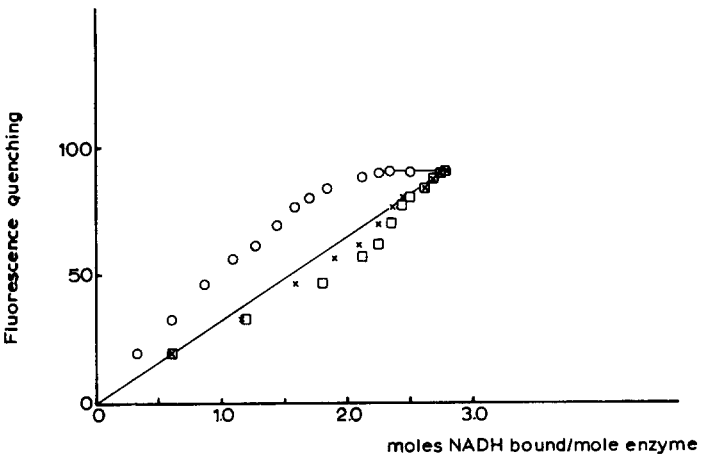


Fig. 7. Quenching of the fluorescence of NADH on adding to charcoal-treated glyceraldehydephosphate dehydrogenase, plotted against NADH bound. □, NADH bound calculated from the dissociation constants $K_{D1,2} < 0.01\ \mu M$, $K_{D3} = 2.5\ \mu M$; ○, NADH bound calculated from the dissociation constants $K_{D1,2,3} = 0.24\ \mu M$; ×, NADH bound calculated from the dissociation constants $K_{D1} < 0.01\ \mu M$, $K_{D2} = 0.24\ \mu M$ and $K_{D3} = 2.5\ \mu M$. The enzyme (1.12 $\mu$M) was dissolved in 100 mM Tris–HCl buffer (pH 8.2), containing 5 mM EDTA; temp., 23°.

the small negative ellipticity of free NADH. In Fig. 6B, the values of $[\theta]$ corrected for the contribution of free NADH are plotted against bound NADH, calculated from the binding constants given in Table I. It is clear that the first three molecules contribute equally to the circular-dichroism band, whereas, in contrast to the fourth $NAD^+$ molecule, there is no contribution from the fourth molecule whatsoever.

VELICK[11] has reported earlier, on the basis of the quenching of the fluorescence of NADH bound to the enzyme, that all three molecules are bound with a dissociation constant of 0.24 $\mu$M. Fig. 7 shows our data on the fluorescence quenching, plotted in three ways: (1) assuming stoicheiometric binding of the first two molecules (infinitesimally small dissociation constant) and a dissociation constant of 2.5 $\mu$M, as determined by ultrafiltration, for the third; (2) assuming, with VELICK[11], a dissociation constant of 0.24 $\mu$M for all three molecules; (3) assuming stoicheiometric binding of the first molecule, a dissociation constant of 0.24 $\mu$M for the second and 2.5 $\mu$M for the third. The results show that this third assumption fits the fluorescence data the most closely.

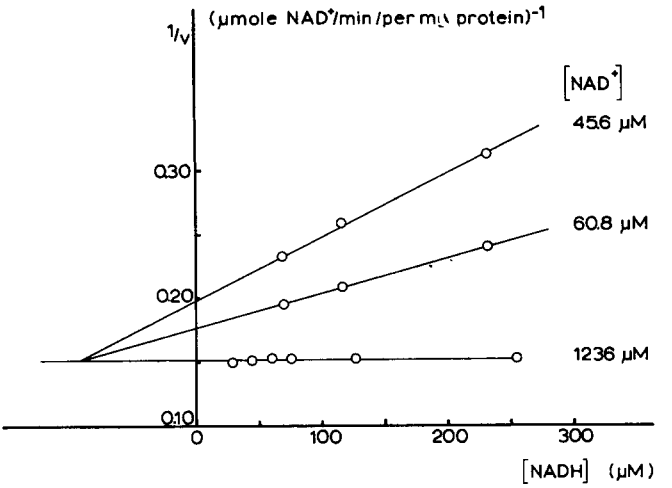


Fig. 8. Inhibition by NADH of the $NAD^+$ reduction by glyceraldehyde in the presence of arsenate[3]. Charcoal-treated glyceraldehydephosphate dehydrogenase (11 $\mu$M) was dissolved in 100 mM Tris–HCl buffer (pH 8.2); temp., 20°.

*Inhibition by NADH of the dehydrogenase activity*

DE VIJLDER *et al.*[18,19] found that $NAD^+$ inhibits the enzyme-catalysed oxidation of NADH by acetyl phosphate, competitively with respect to NADH. The $K_i$, 45 $\mu$M, was found to be about the same as the dissociation constant of the fourth molecule of $NAD^+$.

Fig. 8 shows that NADH inhibits the oxidation of glyceraldehyde by $NAD^+$, in the presence of arsenate, competitively with respect to $NAD^+$. The $K_i$, 90 $\mu$M, is similar to that of the dissociation constant of the fourth molecule of NADH.

## DISCUSSION

Although the third molecule of $NAD^+$ or NADH is bound to the enzyme less firmly than the first two, the effect of binding this third molecule is, except in one respect, quantitatively the same as the effect of each of the first two molecules. This is so with respect to the intensity of the charge-transfer band[3,7–9] between $NAD^+$ and the enzyme, its optical activity[10], the degree of quenching of the protein fluorescence by added $NAD^+$ (ref. 12), the degree of quenching of the fluorescence of NADH when bound to the enzyme (ref. 11 and Fig. 7 of this paper), the thermodynamic parameters of binding $NAD^+$ to the enzyme[20], and the stimulation of the dehydrogenase activity[18,19]. The exception relates to activation of the active-centre-SH groups, all four of which appear to be activated on binding one molecule of $NAD^+$ (ref. 2).

In most respects, however, binding of the fourth molecule of $NAD^+$ or NADH has, qualitatively or quantitatively, different effects than binding of the other 3. The absorbance of the $NAD^+$–enzyme complex increases by only about 30% as much (Fig. 1) and the molecular ellipticity by about 40% (Fig. 2). The thermodynamic parameters of binding of the fourth molecule of $NAD^+$ are also different[20]. Binding of the fourth molecule of NADH results in no further increase in the intensity of the circular-dichroism band at 335 nm. Because of the relatively low binding constant

for the fourth molecule it was not possible to test whether the fluorescence of this molecule is also quenched. The only physical parameter that remains the same for the fourth molecule as for the other three is the quenching of the fluorescence of the protein by $NAD^+$ (ref. 12). Thus, it appears that the conformation change detected by this technique is equally great for all four molecules.

In previous papers[3,6–9], the question was left open as to whether the small increase in the intensity of the 360-nm charge-transfer band seen on adding amounts of $NAD^+$ in excess of that necessary to saturate the first three sites is due to $NAD^+$ bound to the fourth site or to an effect of $NAD^+$ not bound specifically to the four sites. The titrations, carried out over a wider range of $NAD^+$ concentrations, shown in Fig. 1 show that both effects are present. The fourth molecule of $NAD^+$ bound to the enzyme gives rise to a charge-transfer band, of the same type as that obtained with the first three molecules, albeit with a smaller absorbance coefficient. In addition, $NAD^+$, present in excess of that sufficient to saturate the four specific binding sites, reacts with the enzyme in such a way as to form a charge-transfer band, with an absorption maximum at a slightly lower wavelength than that of the complex formed with the first four molecules. Two explanations of this band are possible: (1) it is due to a charge-transfer complex formed between $NAD^+$ and an electron-donating group in a binding site for $NAD^+$ with very low affinity. Since no deviation from a straight line can be detected between 0.3 and 3 mM bound $NAD^+$ in Fig. 1C, the dissociation constant of any loosely bound complex must exceed 5 mM, (2) it is due to 'contact' charge transfer of the type described by MULLIKEN[21] and ORGEL AND MULLIKEN[22]. These authors have shown that 'contact' charge-transfer absorption may occur during random encounters whenever a donor and an acceptor are sufficiently close to one another, without necessarily forming a complex. This is possible because the acception orbital in the negative ion of an acceptor may be considerably larger than the van der Waals size of the natural acceptor model. Since a charge-transfer spectrum becomes possible as soon as the donor orbital begins to overlap this orbital of the acceptor negative ion, this interaction can occur even at greater than van der Waals distances.

VELICK[11] has suggested that the quenching of the fluorescence of NADH by binding to the enzyme is due to the fact that bound NADH is in a more closed form than in solution. If this is so, this may be the reason for the development of asymmetry in the molecule revealed by the strong positive circular-dichroism band. It is also possible, of course, that this band is due to the asymmetric environment of the NADH when it is bound to the enzyme. In any case, the absence of any circular dichroism for the fourth molecule indicates that the conformation of the protein around the NADH-binding site on the fourth protomer is considerably different from that around the binding sites of the other protomers, even if this difference is not revealed by the effect of $NAD^+$ on the protein fluorescence[12].

As usually isolated, muscle glyceraldehydephosphate dehydrogenase contains about 3 moles of firmly bound $NAD^+$. This is not surprising in view of the low dissociation constants of the first three binding sites for $NAD^+$ and the high concentration of $NAD^+$ in the cell cytosol. The role of glyceraldehydephosphate dehydrogenase in glycolysis requires that the enzyme binds $NAD^+$ and that NADH dissociates from it. Its role in gluconeogenese requires that it binds NADH and ejects $NAD^+$. An efficient catalysis of both reactions requires that the binding constants for the oxidized and reduced nucleotide should be of the same order, as is, in fact, found experimen-

tally. An efficient catalysis requires too that the binding of the product should not be too strong. For this reason, we have suggested[7–9,18] that the catalytic reaction is largely carried out on the fourth binding site. According to this view, the binding of $NAD^+$ to three protomer sub-units in the tetramer induces a conformation in the fourth protomer with suitable binding constants. While this conclusion must remain tentative until experiments on the effect of the other substrates (aldehyde, acyl phosphate and phosphate) on the binding constants of $NAD^+$ and NADH have been completed (*cf.* ref. 23), it is given good support by the facts that the $K_i$ for the inhibition by $NAD^+$ of the oxidation of NADH by acetyl phosphate, catalysed by the enzyme, is equal to the dissociation constant of $NAD^+$ for the fourth binding site[18,19], and that the $K_i$ for inhibition by NADH of reduction of $NAD^+$ by glyceraldehyde is the same as the dissociation constant of NADH from the fourth site. If the other three binding sites played an important role in the catalytic mechanism, one would expect $NAD^+$ and NADH to be much more effective inhibitors of these two reactions.

## EXPERIMENTAL

Glyceraldehydephosphate dehydrogenase was isolated from rabbit muscle by the method of CORI *et al.*[24], slightly modified by HILVERS[25]. The specific activity, measured spectrophotometrically as previously described[3], was 100–120 $\mu$moles NADH per min per mg protein. $NAD^+$ was removed by stirring with charcoal (0.2 g per ml solution; *cf.* ref. 26). The enzyme concentration was calculated from absorbance measurements at 280 nm, using the extinction coefficients reported by FOX AND DANDLIKER[27]. $NAD^+$ and NADH were determined enzymically with alcohol 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 $mM^{-1} \cdot cm^{-1}$ (ref. 28). A molecular weight of 145 000 was assumed[1].

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. Fluorescence measurements were performed with a Hitachi Perkin Elmer spectrofluorimeter, Model MPF-2A, or with an Eppendorf spectrophotometer with a fluorescence attachment, excitation filter 313 and 366 nm, and emission filter 470–3000 nm, both equipped with front-face fluorescence.

Ultracentrifugation was carried out in the MSE analytical ultracentrifuge with an ultraviolet scanning system and multiple cell device. The concentrations of the free NADH were measured spectrophotometrically at 260 nm and 340 nm after 60 min centrifugation at 262 000 $\times$ *g*. The scanning system was calibrated with a standard NADH solution. A correction was applied for the NADH gradient that developed while centrifuging at this speed. Ultrafiltration was carried out in a UF cell from Amicon, equipped with a PM-30 filter. The NADH concentrations were measured enzymically, and compared with standard NADH solutions ultrafiltered in the same way.

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