BBA 65787

THE REACTION BETWEEN NAD+ AND RABBIT-MUSCLE GLYCERALDEHYDEPHOSPHATE DEHYDROGENASE

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(Received April 5th, 1968)

SUMMARY

1. The $A_{280\ m\mu} : A_{260\ m\mu}$ ratio of NAD+-free rabbit-muscle glyceraldehydephosphate dehydrogenase was found to be 2.00–2.03.

2. Ultracentrifugation showed that 4 moles of NAD+ may be bound to the enzyme per mole (mol. wt. 145 000). The first two molecules are bound stoicheiometrically within the experimental error ($K_D < 0.05\ \mu M$), while the third and fourth molecules are bound with dissociation constants of 4 and 35 μM, respectively.

3. The first three molecules of NAD+ bound to the enzyme cause the formation of a band at 360 mμ of about equal intensity for each molecule. The fourth molecule causes little further increase of absorption at 360 mμ.

4. A plot of the rate of reduction of NAD+ by glyceraldehyde in the presence of arsenate against the NAD+ concentration shows a sharp break in the curve at 4 moles NAD+ per mole enzyme.

5. Stopped-flow experiments showed that when up to 1 mole of NAD+ is added to the enzyme, maximum absorbance increase at 360 mμ is reached within 3 msec. This corresponds to a second-order reaction constant of more than $10^8\ M^{-1} \cdot sec^{-1}$. With 2 moles of NAD+ per mole of enzyme, 81% of the reaction is over in 3 msec, and with 3 or more moles NAD+ about 75%. The reaction requires about 1 sec for completion.

6. Prior treatment with NAD+ speeds the change of absorbance obtained with subsequent additions of NAD+. The final value obtained, however, is unaltered.

7. For the muscle enzyme under our experimental conditions, a model in which the binding of 1 NAD+ molecule to one protomer affects the conformation of a second protomer, either before or after binding with NAD+, appears more appropriate than the allosteric model, which requires that the binding of any one ligand molecule is intrinsically independent of the binding of any other.

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Abbreviation: PCMB, *p*-chloromercuribenzoate.

INTRODUCTION

The enzyme glyceraldehydephosphate dehydrogenase (D-glyceraldehyde 3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12) isolated from rabbit skeletal muscle is a tetramer, the protomer having a molecular weight of 36 300 (refs. 1, 2). It binds NAD^+ so firmly that, even after repeated recrystallization, the isolated enzyme contains large amounts of NAD^+ from which it may be freed by treatment with charcoal[3]. RACKER AND KRIMSKY[4,5] and VELICK[6] showed that the combination of the enzyme with NAD^+ is accompanied by the appearance of a broad absorption band at 360 mμ, which disappears on treatment of the enzyme with -SH-binding agents (such as iodoacetate or *p*-chloromercuribenzoate (PCMB)) or with the phosphorylated product of the enzyme reaction, 1,3-diphosphoglycerate. Preliminary X-ray crystallographic data on the enzyme isolated from lobster muscle have been published[7].

In view of the tetrameric structure of glyceraldehydephosphate dehydrogenase, one would expect it to bind 4 NAD^+ molecules. Indeed, titrations with NAD^+ of the yeast enzyme, using the absorption at 360 mμ as a measure of the binding, have been reported in support of this view[8,9]. Similar titrations of the rabbit-muscle enzyme, however, interpreted in the same way, have given lower results, ranging from 2.7 (ref. 10) to 3.2 (refs. 6, 11) moles NAD^+ per 145 000 g enzyme.

This paper reports an investigation of the binding of NAD^+ with muscle glyceraldehydephosphate dehydrogenase studied in four different ways: (1) by ultracentrifugation of enzyme solutions to which varying amounts of NAD^+ were added; (2) by titration of the effect of various amounts of NAD^+ on the absorption at 360 mμ; (3) by titration of the effect of various amounts of NAD^+ on the rate of its reduction by glyceraldehyde; (4) by determining in a stopped-flow apparatus the rate of reaction of NAD^+ with the enzyme.

RESULTS

Measurement of NAD^+ content of glyceraldehydephosphate dehydrogenase

The NAD^+ content of the enzyme was determined in protein-free extracts obtained by addition of perchloric or trichloroacetic acid, either enzymically with ethanol and alcohol dehydrogenase or by measurement of the absorption in the ultraviolet ($A_{260\ m\mu}$ *minus* $A_{280\ m\mu}$). The absorption spectrum of the acid extract, before and after reduction with ethanol and alcohol dehydrogenase, was identical with that of NAD. The two methods gave results in good agreement (Table I).

That treatment with perchloric acid gives quantative extraction of the NAD^+ was shown in two ways. First, additional treatments of the precipitated protein with $HClO_4$ yielded negligible extra NAD^+ (2% in the first treatment, and this can be accounted for by liquid entrapped in the precipitate; less than 0.3% in subsequent treatments). Secondly, the addition of PCMB prior to the $HClO_4$, in order to detach the NAD^+ from the enzyme[6], yielded exactly the same amount of NAD^+ as without treatment with mercurial.

The removal of NAD^+ from the enzyme by charcoal is often monitored by measurement of the ratio $A_{280\ m\mu} : A_{260\ m\mu}$ which increases with decreasing NAD^+ content. MURDOCH AND KOEPPE[12] have reported a value of 2.13 for this ratio with

TABLE I

DETERMINATION OF NAD^+ CONTENT OF DIFFERENT PREPARATIONS OF GLYCERALDEHYDEPHOSPHATE DEHYDROGENASE

The NAD^+ content was measured in protein-free supernatants both from measurements of $A_{280\ m\mu}$ and $A_{260\ m\mu}$, and with alcohol dehydrogenase.

$\frac{A_{280\ m\mu}}{A_{260\ m\mu}}$	*NAD+ content (moles/mole enzyme)*	
	Enzymically	*From $A_{260\ m\mu}$ minus $A_{280\ m\mu}$*
1.99	0.01	0.01
1.85	0.16	0.18
1.16	2.79*	2.84*
	2.85*	2.80*

* Duplicates.

completely NAD-free muscle enzyme and KIRSCHNER *et al.*[8] have reported a similar value for the yeast enzyme. In our hands, this ratio for muscle enzyme is 2.00–2.03 (Fig. 1), and we are unable to explain the difference with MURDOCH AND KOEPPE[12]. It is unlikely to be due to a spectrophotometric error in our measurements since both the $A_{280\ m\mu}$ and the $A_{260\ m\mu}$, with 1 cm light path, were proportional to the protein concentration up to 1.8 mg/ml. The ratio was practically independent of pH between 6 and 9. Below pH 6 and above pH 9, the ratio declined, the effect being greater with charcoal-treated than with untreated enzyme.

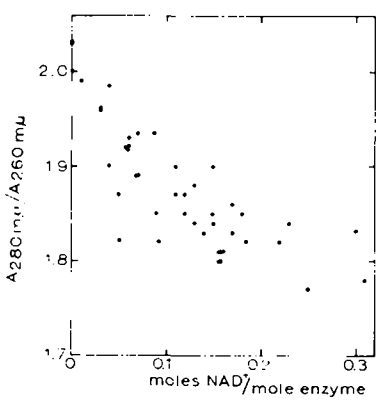


Fig. 1. The $A_{280\ m\mu}:A_{260\ m\mu}$ ratio of charcoal-treated enzyme as a function of NAD^+ content. The enzyme was dissolved in 1 mM EDTA (pH 8.0) or in 0.1 M Tris–HCl buffer (pH 8.2 or 8.8), containing 5 mM EDTA.

Binding of NAD^+ to charcoal-treated glyceraldehydephosphate dehydrogenase as measured in the ultracentrifuge

The binding of NAD^+ to charcoal-treated enzyme at 20–25°, as measured in the ultracentrifuge, is shown in Fig. 2. With up to 2 moles added NAD^+ per mole enzyme, no NAD^+ was detectable in the protein-free supernatant after centrifugation.

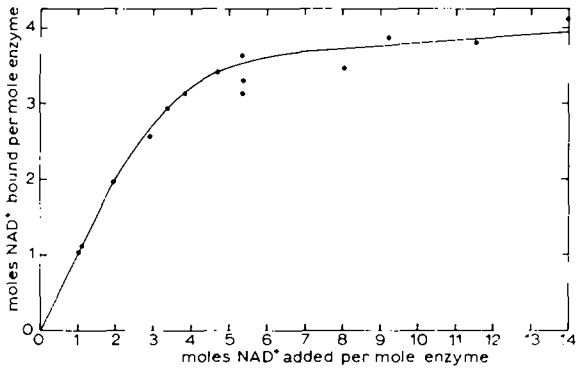


Fig. 2. The binding of NAD^+ to glyceraldehydephosphate dehydrogenase dissolved in 0.1 M Tris HCl buffer, containing 5 mM EDTA (pH 8.2) at 20–25°. The enzyme concentration was between 20.4 and 26.3 μM. The amount of NAD^+ found in the supernatant after centrifugation of mixtures of various amounts of enzyme and NAD^+ was determined as described in EXPERIMENTAL.

Maximum binding—about 4 moles NAD^+ per mole enzyme—required the addition of about 10 moles NAD^+ per mole enzyme. The dissociation constants of the third and fourth molecules bound to the protein, as calculated from Fig. 2, are about 4 and 35 μM, respectively*. The first two molecules are much more firmly bound, with an estimated dissociation constant of less than 0.05 μM.

Our ultracentrifugal results do not agree with the conclusion of VELICK, HAYES AND HARTING[13], who used a similar procedure at 0°, that the K_D decreases with increasing added NAD^+. However, on recalculating their results on the basis of a molecular weight of 145 000, the agreement with ours is very close, except for one point, with 1.7 mole NAD^+ per mole enzyme, where VELICK, HAYES AND HARTING[13] reported an appreciable amount of NAD^+ in the supernatant. We do not find any with this amount of NAD^+. Using a fluorimetric method, VELICK[14] found that all (three) NAD^+ molecules are bound with the same dissociation constant, *viz.* 0.06 μM.

The 360 mμ band of NAD^+-containing glyceraldehydephosphate dehydrogenase

Recrystallized glyceraldehydephosphate dehydrogenase contains up to 3.7 moles NAD^+ per mole enzyme (Table II). Repeated recrystallization is necessary to remove contaminating protein as indicated in Table II by the decreasing $A_{280\ m\mu}$: $A_{260\ m\mu}$ ratio, increasing NAD^+ content and increasing specific activity. The specific activity doubled or more than doubled between the first and the sixth recrystallization. The catalytic-centre activity (*i.e.* enzyme activity per mole bound NAD^+)

* These dissociation constants refer to the equations

$$E\text{-}(NAD)_2 + NAD^+ \rightleftharpoons E\text{-}(NAD)_3;\ K = \frac{[E\ (NAD)_2]\ [NAD^+]}{[E\ (NAD)_3]}$$

and

$$E\text{-}(NAD)_3 + NAD^+ \rightleftharpoons E\text{-}(NAD)_4;\ K = \frac{[E\text{-}(NAD)_3]\ [NAD^+]}{[E\text{-}(NAD)_4]}$$

respectively. In the calculation of the dissociation constant of the third molecule it was assumed that the first two sites are completely occupied when between 2 and 3 molecules are bound and the fourth site is not occupied. In the calculation for the fourth molecule, it was assumed that the first three sites are completely occupied when 3 molecules are bound. The estimated standard errors of these constants are 0.5 and 0.9 μM, respectively.

TABLE II

PROPERTIES OF GLYCERALDEHYDEPHOSPHATE DEHYDROGENASE AFTER REPEATED RECRYSTALLIZATION

The $A_{280\ m\mu}:A_{260\ m\mu}$ ratio was measured as in Fig. 1. The $\Delta A_{360\ m\mu}$ is the decline of absorbance after adding a 80-fold excess sodium iodoacetate to a solution of the enzyme in 0.1 M Tris·HCl buffer (pH 8.2), containing 5 mM EDTA.

Expt. No.	*Number of recrystallizations*	$\frac{A_{280\ m\mu}}{A_{260\ m\mu}}$	$\frac{\Delta A_{360\ m\mu}}{A_{280\ m\mu}}$	*NAD+ (moles/ 145 000 g protein)*	*Specific activity***	*Catalytic-centre activity****
1	1	1.14	0.015	2.4	57	3.4
	2	1.13	0.020	2.2	80	5.4
	3		0.022		—	
	4	1.09	0.022	3.1	100	4.7
	5	1.07	0.021	3.6	113	4.5
	6	1.06	0.021	3.7	115	4.5
2	1	1.02	0.011	1.9	46	3.5
	2	1.20	0.016	2.4	71	4.3
	3	1.23	0.020	2.8	80	4.2
	4	1.21	0.017	2.8	100	5.2
	5*	1.12	0.019	3.3	115	5.1
3	1	1.17	0.012	2.2	45	2.9
	2	1.17	0.017	2.6	59	3.3
	3	1.09	0.020	3.1	82	3.8
	4	1.07	0.020	3.5	92	3.8
	5	1.07	0.022	3.6	90	3.7

* NAD+ added to aid crystallization.
** Moles added NAD+ reduced per min per mg protein.
*** Moles added NAD+ reduced per min per mole enzyme NAD+.

remained fairly constant after the first few recrystallizations. It was always low after only one recrystallization, indicating the presence of contaminating NAD+ at this stage of the purification. In all other experiments described in this paper, glyceraldehydephosphate dehydrogenase recrystallized at least 5–6 times was used.

The purification may also be followed by the ratio between $\Delta A_{360\ m\mu}$, the decrease of absorbance at 360 mμ brought about by the addition of iodoacetate, and $A_{280\ m\mu}$, which is a measure of the intensity of the NAD+–enzyme band.

Fig. 3 shows the effect of increasing amounts of NAD+ on the absorption at 360 mμ of the charcoal-treated enzyme. There is a linear increase of absorbance up to 2 moles of NAD+ per mole enzyme. The $\Delta\varepsilon_{mM}$ (360 mμ) for this portion of the titration is 0.96*. The absorbance increase per mole NAD+ declines with further addition of NAD+, and the absorbance reaches a maximum with about 4 moles of NAD+. The intersection point of this titration is 3.0 moles (this varied between 2.7 and 3.0 in 11 different experiments).

If it could be assumed that each molecule NAD+ bound to glyceraldehydephosphate dehydrogenase causes the same increase in $A_{360\ m\mu}$, it would be concluded that the enzyme binds only 3 moles of NAD+ per mole. However, the ultracentrifugal experiments showed clearly that 4 moles of NAD+ can be bound to the enzyme, two strongly, one moderately and one weakly. When the $\Delta A_{360\ m\mu}$ is plotted against the

* The mean value for 11 titrations was 0.98.

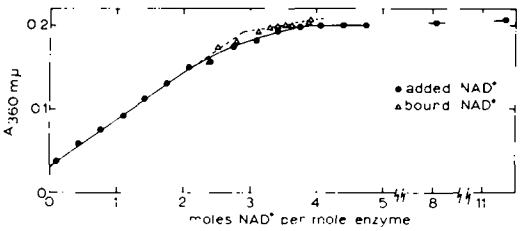


Fig. 3. Titration of glyceraldehydephosphate dehydrogenase (57.2 μM) with NAD^+ at 360 mμ. The enzyme was dissolved in 100 mM Tris–HCl buffer (pH 8.2) (containing 5 mM EDTA); temp. 25°. ●, added NAD^+; △, bound NAD^+ calculated from the dissociation constants calculated from Fig. 2.

bound NAD^+ (calculated from the added NAD^+ by use of the dissociation constants calculated from Fig. 2), it can be clearly seen that the third (moderately bound) NAD^+ molecule has almost the same effect on $A_{360\ m\mu}$ as the first two, whereas the fourth molecule causes only a small increase in absorbance.

Rate of reduction of added NAD^+ by glyceraldehyde in presence of glyceraldehydephosphate dehydrogenase

Fig. 4 shows the effect of increasing amounts of NAD^+ added to the enzyme on the rate of reduction of the NAD^+ (measured by $A_{340\ m\mu}$) on the addition of glyceraldehyde in the presence of arsenate. In this case the sharp break in the curve occurs at 3.9–4.0 moles NAD^+ per mole enzyme, in agreement with MURDOCH AND KOEPPE[12], who carried out titrations under the same conditions. FAHIEN[15] obtained a similar result, but FURFINE AND VELICK[16] found a break at 3.1 moles NAD^+ per mole enzyme (recalculated to a molecular weight of 145 000). The reason for this difference is not apparent.

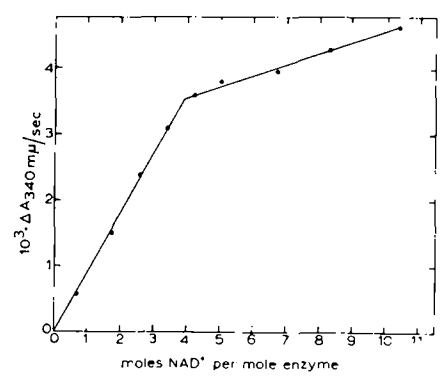


Fig. 4. The effect of NAD^+ concentration on the velocity of NAD^+ reduction with glyceraldehyde at 25°, measured at 340 mμ. The medium contained 100 mM Tris–HCl buffer, 43 mM Na_2HAsO_4, 1 mM EDTA, 0.1% serum albumin, 10.1 μM glyceraldehydephosphate dehydrogenase and 8.6 mM DL glyceraldehyde. Final pH, 8.8.

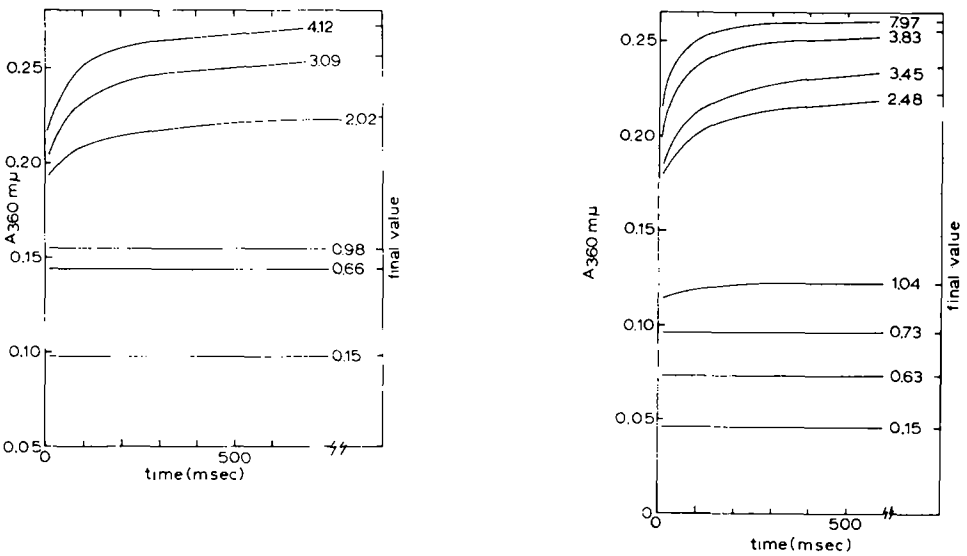


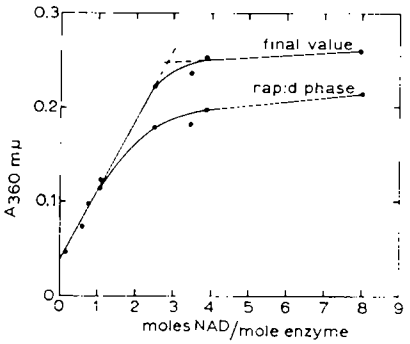
Fig. 5. Reaction of NAD+ with glyceraldehydephosphate dehydrogenase as measured in the Durrum stopped-flow apparatus. One of the two syringes contained 67 μM enzyme in 100 mM Tris-HCl buffer, containing 5 mM EDTA (final pH 8.2) and the other various amounts of NAD+ in the same Tris EDTA mixture containing 0.5% serum albumin. All solutions were at 25°. The curves are marked with the total amount of NAD+ (moles per mole enzyme) present. The final values reached are indicated on the right-hand side. The charcoal-treated enzyme contained 0.15 mole NAD+ per mole enzyme. The curves plotted are the averages of three runs. Light path, 2 cm.

Fig. 6. Reaction of NAD+ with glyceraldehydephosphate dehydrogenase as measured in the Durrum stopped-flow apparatus. A second experiment carried out in the same way as described in Fig. 5 with 68 μM enzyme and various NAD+ concentrations. The charcoal-treated enzyme contained 0.15 mole NAD+ per mole enzyme.

Rate of combination of NAD+ with charcoal-treated glyceraldehydephosphate dehydrogenase

Figs. 5 and 6 show the course of the reaction between NAD+ and the enzyme at 25°, as followed at 360 mμ in a stopped-flow apparatus. With less than 1 mole NAD+ per mole enzyme an almost maximal increase of $A_{360\ m\mu}$ is reached within about 3 msec, the mixing time of the instrument. The second-order rate constant for this reaction, on the basis that more than 90% of the reaction is complete in 3 msec, must be greater than $10^8\ M^{-1}\cdot sec^{-1}$. With 2 moles NAD+ per mole enzyme 81% of the reaction was complete within 3 msec (see Fig. 7), but the reaction required more than 1 sec to go to completion. With 3 or more moles NAD+ per mole enzyme about 75% of the reaction was complete in 3 msec.

In these two experiments, separate runs were made with different amounts of NAD+ added to NAD-free enzyme. Fig. 8 summarizes an experiment in which NAD+ was added to the enzyme already treated with NAD+. The lower curve, as in Fig. 7, records the $A_{360\ m\mu}$ 3 msec after addition of NAD+ to NAD-free enzyme. The middle curve was obtained with enzyme to which 1.05 moles NAD+ had already been added, and the upper curve with enzyme to which 1.8 moles NAD+ had previously been added. It is clear that prior treatment with NAD+ speeds the change of absorbance



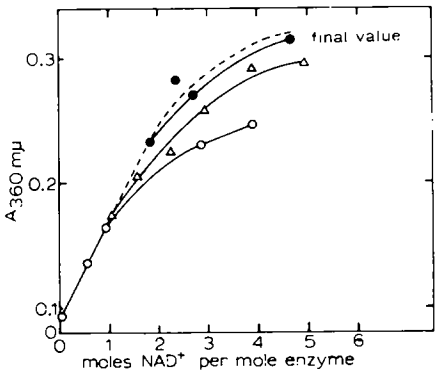


Fig. 7. The $A_{360\ m\mu}$ measured at the end of the rapid phase (3 msec) and the final value obtained after about 1 sec. The same experiment as in Fig. 6.

Fig. 8. Reaction of NAD^+ with glyceraldehydephosphate dehydrogenase as measured in the Durrum stopped-flow apparatus. The medium was as in Fig. 5. One of the two syringes contained 75.3 μM enzyme, containing 0.03, (○), 1.05 (△) and 1.80 (●) moles NAD^+ per mole enzyme, respectively, and the other syringe contained various amounts of NAD^+ —, values after 3 msec; - - -, final values. The abscissa refers to the total amount of NAD^+.

obtained with subsequent additions of NAD^+, although the final value reached depends only upon the total amount of NAD^+ added.

DISCUSSION

Although glyceraldehydephosphate dehydrogenase is a tetramer it is apparent from the results reported in this paper that the four protomers do not react independently of one another with NAD^+. The differences between the reactions with successive molecules of NAD^+ are summarized in Table III.

The first molecule of NAD^+ reacts very rapidly and firmly with the enzyme and gives rise to the absorption band at 360 mμ that has been ascribed to a charge–transfer complex between the thiol group of the reactive cysteine and the pyridine ring of the NAD^+ (ref. 17). The spectral changes associated with binding of the second molecule are about 60% complete within 3 msec (the time required for maximal effect of the first molecule) and about 1 sec is required for the maximal effect. The intensity of the 360 mμ band with the second NAD^+ molecule is the same as with the first. The second molecule is also firmly bound to the enzyme. Although the kinetics of the spectral change brought about by combination of the third molecule of NAD^+ with the enzyme are very similar to those brought about by the second molecule, and the intensity of the 360 mμ band is about the same, this molecule is much less firmly bound to the enzyme. The fourth molecule is still less firmly bound and gives rise to only a small increase of $A_{360\ m\mu}$.

The following picture of the binding of NAD^+ with the muscle enzyme emerges. The first molecule of NAD^+ combines rapidly with the enzyme with a negative binding free energy ($-\Delta G_0'$) of more than 10 kcal (1.36 log K_D). A charge–transfer complex cannot alone be responsible for the binding of NAD^+ to the enzyme, because the $-\Delta G_0'$ value of charge–transfer forces does not exceed 2 kcal (ref. 18). The

formation of the charge–transfer complex may require a specific conformation of the polypeptide chain, induced by binding of the NAD^+. Measurements of optical rotatory dispersion indicate that NAD^+ induces changes in the tertiary structure of the enzyme[19–21] (see also ref. 13). LISTOWSKY *et al.*[19] found that the major change occurs on addition of 1 mole NAD^+.

If a conformation change is required in the first protomer that combines with NAD^+, it must be very rapid, since maximum development of the charge–transfer band is already reached within 3 msec. The second molecule of NAD^+ also reacts rapidly with and is firmly bound to the enzyme. However, although there is an immediate increase of $A_{360\ m\mu}$, the complete development of the charge -transfer band apparently requires a conformation change of the second protomer combining with NAD^+ which proceeds much more slowly than the initial binding or than any conformation change of the first protomer. The third molecule of NAD^+ behaves kinetically much the same as the second, but is less firmly bound to the protein. When three of the four protomers are combined with NAD^+, it becomes much more difficult to bind a fourth molecule, and this molecule is bound in such a way that little if any charge–transfer complex with the cysteine –SH group of this protomer is possible. It has earlier been suggested by HILVERS, VAN DAM AND SLATER[22] that one of the NAD^+ molecules behaves differently from the other three.

When the rate of reduction of NAD^+ by glyceraldehyde is studied, a sharp break in the activity–NAD^+ curve occurs when 4 moles NAD^+ are added per mole enzyme (Fig. 4), even though Fig. 2 shows that only about 3.2 moles of NAD^+ are bound when 4 moles are added. This result agrees with those of MURDOCH AND KOEPPE[12] and with FAHIEN[15], but not with that of FURFINE AND VELICK[16] who found a break at about 3 moles NAD^+ per enzyme. This discrepancy is puzzling, and until it is resolved discussion of this finding is premature. In any case, the fact that, under the conditions of Fig. 4, an interception point at 4 is obtained shows that the enzyme preparation used is pure and does not contain denatured material, which was one of the explanations considered in our preliminary note[10] for the unexpected result of the titration at 360 mμ.

The conclusions that we have drawn concerning the reaction of NAD^+ with muscle glyceraldehydephosphate dehydrogenase are not all applicable to the yeast enzyme. It has been known for some time that the yeast enzyme binds NAD^+ less firmly than the muscle enzyme. Other differences are the absence with the yeast enzyme of the sharp break in the titration curve illustrated in Fig. 4 for the muscle enzyme[9] and the evidence that 4 molecules of NAD^+ contribute to the 360 mμ band in the yeast enzyme[8,9]. The extinction coefficient calculated by KIRSCHNER *et al.*[8] for the yeast enzyme on the basis of 4 binding sites is very close to that found by us for the first 3 molecules of NAD^+ bound to the muscle enzyme.

CHANCE AND HARTING PARK[9] and KIRSCHNER *et al.*[8] found rapid and slow phases of the reaction between NAD^+ and the yeast enzyme, but both phases appear to be considerably slower than those observed by us for the muscle enzyme. At 40°, where the yeast enzyme shows a weakly sigmoidal response to increasing NAD^+ concentration, the reaction proceeds in three discrete steps[8]. KIRSCHNER *et al.*[8] have interpreted the kinetics in terms of the allosteric model of MONOD, WYMAN AND CHANGEUX[23]. The most rapid second-order reaction constant measured by KIRSCHNER

TABLE III

REACTION OF GLYCERALDEHYDEPHOSPHATE DEHYDROGENASE WITH SUCCESSIVE MOLECULES OF NAD^+

Molecule NAD^+	*K_D (μM)*	*$\Delta\varepsilon_{mM}$ (360 mμ)*	*Rate of reaction*
First	<0.05	0.98	Complete within 3 msec
Second	<0.05	0.98	Incomplete within 3 msec
Third	4	0.98	Incomplete within 3 msec
Fourth	35	0.18	Not studied

et al.[8] was $1.9 \cdot 10^7\ M^{-1} \cdot sec^{-1}$ at 40°, compared with more than $10^8\ M^{-1} \cdot sec^{-1}$ at 25° found by us for the muscle enzyme.

It is clear from our data that one of the basic assumptions of the allosteric model of MONOD, WYMAN AND CHANGEUX[23], namely that the binding of any one ligand molecule is intrinsically independent of the binding of any other, is not applicable to the muscle enzyme under the conditions of our experiments. A model in which the binding of one NAD^+ molecule to one protomer affects the conformation of a second protomer, either before or after binding with NAD^+, appears more appropriate than the allosteric model, which emphasizes the effect of ligand binding on the quaternary structure of the oligomer.

EXPERIMENTAL

Glyceraldehydephosphate dehydrogenase was isolated from rabbit muscle by the method of CORI, SLEIN AND CORI[24] slightly modified by HILVERS[25]. It was recrystallized 5–6 times from 70% satd. $(NH_4)_2SO_4$ (pH 8.0), containing 1 mM EDTA. NAD^+ was sometimes added before the fifth or sixth recrystallization in order to promote crystallization. NAD^+ was removed by stirring a solution containing 40 mg protein per ml with a 20% charcoal suspension for 4 min at 0–2° (*cf.* ref. 13). The method of MURDOCH AND KOEPPE[12] was also used to obtain some of the values given in Fig. 1. Charcoal treatment had no effect on the activity of the enzyme. The concentration of the enzyme was calculated from $A_{280\ m\mu}$ using the extinction coefficients for native and charcoal-treated enzyme reported by FOX AND DANDLIKER[11], *viz.* 1.000 and $0.829\ cm^2 \cdot mg^{-1}$ (*cf.* 0.86 for charcoal-treated yeast enzyme[8]), respectively. These extinction coefficients were checked by independent protein determination by the Kjeldahl method, using enzyme containing 0.1, 1.3 and 3.3 moles NAD^+ per mole enzyme. The two methods agreed within 1%. Concentrations are expressed as μM, based on a molecular weight of 145 000 (refs. 1, 2).

The activity of the enzyme was measured spectrophotometrically at 24° with a reaction mixture containing 1.0 mM DL-glyceraldehyde 3-phosphate (prepared from the barium salt of the diethylacetal, obtained from Sigma Chemical Co., as described by RACKER, KLYBAS AND SCHRAMM[26]), 1.0 mM NAD^+ (Sigma), 50 mM sodium phosphate, 2 mM EDTA, 1 mg/ml serum albumin and 0.1 M Tris(Sigma)–HCl buffer. The final pH was 8.8. The specific activity of purified enzyme in this assay was 90–115 μmoles NADH per min per mg protein.

NAD^+ determinations were made on supernatants obtained after precipitating

the enzyme with 6% $HClO_4$ or 4% trichloroacetic acid. The NAD^+ was determined either by measurement of the $A_{280\ m\mu}$ and $A_{260\ m\mu}$, by making use of the values of 4.0 and 17.8, respectively, for the molar extinction coefficients, or enzymically (after neutralization) with ethanol and alcohol dehydrogenase (EC 1.1.1.1).

Ultracentrifugation was carried out either in the MSE analytical ultracentrifuge at 262 000 × *g* (measured at the centre of the cell) for 120 min or in the MSE preparative ultracentrifuge No. 65, with an angle rotor, at 400 000 × *g* for 150 min. When the analytical ultracentrifuge was used photographs were taken with ultraviolet light (265 mμ) and the degree of blackening of the negative was measured with an Eppendorf photometer, and compared with standard NAD^+ solutions. When the preparative ultracentrifuge was used, 1-ml samples of the supernatant were siphoned off and the NAD^+ determined with ethanol and alcohol dehydrogenase. The absence of protein was checked by measuring the $A_{280\ m\mu} : A_{260\ m\mu}$ ratio (0.22 for NAD^+). The amount of bound NAD^+ was calculated as the difference between total NAD^+ added and that found in the supernatant. A correction was applied for the NAD^+ gradient that developed while centrifuging at this speed. This was determined in separate control experiments in which the same concentrations of NAD^+ were used as in the experiments in which enzyme was also present, and the NAD^+ content was determined at different distances along the tube.

Stopped-flow experiments were carried out in the instrument of the Durrum

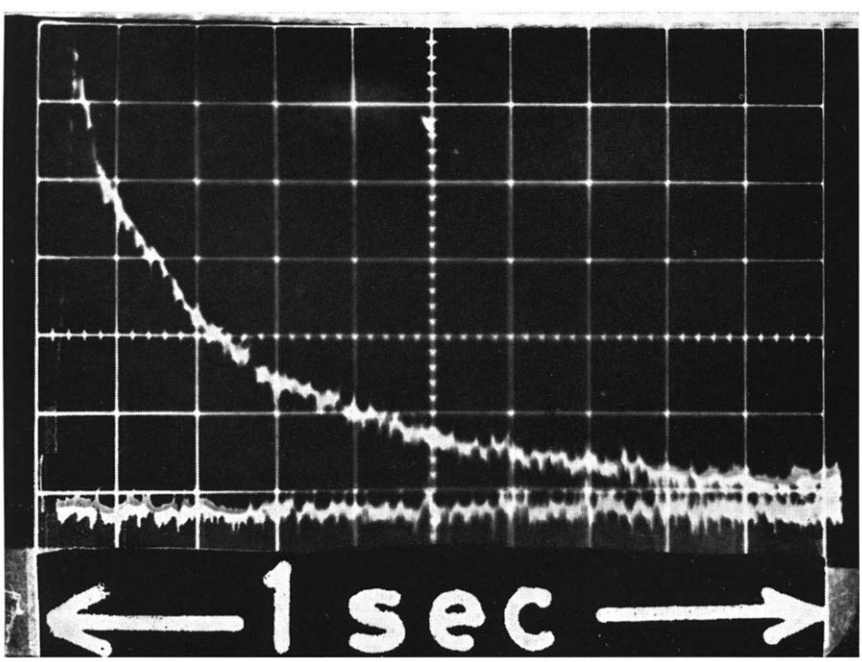


Fig. 9. Photograph of oscilloscope tracing of reaction between NAD^+ and glyceraldehydephosphate dehydrogenase. The syringes contained 76 μM enzyme and 192 μM NAD^+, respectively; other components as in Fig. 6. Immediately before the reaction the observation tube contained enzyme–NAD complex from the previous experiment. The lower trace records the transmission of this complex. The reaction between fresh enzyme and NAD^+ solution began at the left-hand margin of the figure. The upper trace shows that part of the reaction that is not completed within the mixing time of the instrument (3 msec).

Instrument Corp. The mixing time was 3–5 msec. The instrument was first calibrated with water to give 100% transmission at 100 mV per scale division, and the transmission of enzyme in the reaction mixture (but without added NAD^+) and of the enzyme–NAD complex at the end of the reaction was recorded at the same sensitivity. The instrument was set in balance at the transmission of the enzyme–NAD complex and the sensitivity increased to 5–10 mV per scale division by using the vertical amplifier, before starting the reaction with new solutions by operating the syringe plungers. A typical tracing is shown in Fig. 9. $A_{360\ m\mu}$ was calculated from the % transmission recorded on photographs of the storage oscilloscope.

In control experiments with an extended time scale (1 vertical division per 1 msec) in which water was mixed with dichlorophenol indophenol, the mixing time was found to be 3 msec. Since, in all cases, at least 75% of the reaction was complete within this mixing time, the maximum value of the transmission recorded in the trace has been given the time of 3 msec.

ACKNOWLEDGEMENTS

We wish to thank Dr. A. G. HILVERS for helpful discussion and Mr. S. VAN DALSEM for technical assistance. This work was supported in part by a grant from the Life Insurance Medical Research Fund.

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