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# PREPARATION AND PROPERTIES OF RABBIT-MUSCLE GLYCERALDEHYDE-PHOSPHATE DEHYDROGENASE WITH EQUAL BINDING PARAMETERS FOR THE THIRD AND FOURTH $NAD^+$ MOLECULES

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## Summary

1. A method of preparing rabbit-muscle glyceraldehyde-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:$NAD^+$ oxidoreductase (phosphorylating), EC 1.2.1.12) is described which yields a preparation differing in important respects from those previously described and resembling the enzyme isolated from sturgeon muscle.

2. Direct binding measurements at 25°C by equilibrium gel filtration fit dissociation constants for the first two molecules that are too low to be measured by this technique and 0.9 $\mu$M for the third and fourth molecules. The dissociation constant of the fourth molecule is much lower than that previously reported for the rabbit-muscle enzyme.

3. In contrast to previous results with the rabbit-muscle enzyme, the increase in absorbance at 360 nm between three and four molecules of $NAD^+$ bound to the enzyme was, within experimental error, the same as that with each of the first three molecules.

4. Data on the quenching of the protein fluorescence by $NAD^+$ at 15°C at different enzyme concentrations closely fit dissociation constants of 0.028 $\mu$M for the first two molecules and 0.27 $\mu$M for the third and fourth molecules.

## Introduction

Glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate: $NAD^+$ oxidoreductase (phosphorylating), EC 1.2.1.12) contains four NAD-binding sites, one on each of the identical subunits in the tetramer. The weakening in the rate [1] and strength [2,3] of the binding of successive $NAD^+$

Abbreviation: MOPS, 3-(*N*-Morpholino)propanesulphonate.

molecules was first reported for the enzyme isolated from rabbit muscle by De Vijlder and Slater [1,2] and by Conway and Koshland [3], and has since been confirmed by many authors using enzyme isolated from this or other muscles [4—12]. Two models have been proposed to explain this phenomenon. In one, the asymmetry is assumed to be present in the apoenzyme [13]; in the other, the negative-interaction model, the asymmetry is thought to be induced by ligand binding [2,3,14,15]. Boers and Verhoeven [16], Bell and Dalziel [17] and Long and Dahlquist [12] have provided evidence in favour of an induced conformation change, the transition, revealed by the absorption band of the charge-transfer complex with *N*-methylnicotinamidium [16], the fluorescence of enzyme-bound NADH [17], or the NMR spectrum of trifluoroacetonylated enzyme [12], occurring near half saturation with $NAD^+$. Furthermore, although asymmetry has been reported to be present in the subunits of the muscle holoenzyme, which is best described by an $(\alpha\alpha')_2$ structure [18,19], none was detected in the enzyme from *Bacillus stearothermophilus* [20].

While all enzyme preparations isolated from muscle clearly show a weaker binding of the third and fourth $NAD^+$ molecules bound compared with the first two, important differences have been reported. Published data for the enzyme isolated from rabbit skeletal muscle and lobster-tail muscle suggest a sequential decrease in binding constants. Although the first two $NAD^+$ molecules are so firmly bound that direct measurement of the binding constant by equilibrium dialysis [3] or ultracentrifugation [2] is not possible, Bell and Dalziel [11] have calculated dissociation constants of $10^{-8}$ M and $9 \cdot 10^{-8}$ M for the first and second molecules, respectively, on the basis of the effect of $NAD^+$ on the protein fluorescence. Both this method and direct binding studies yield dissociation constants of 0.3 [3] —3 [5] $\mu$M at 5°C and 0.4 [5], 1.7 [6], 1.9 [21] or 4 [2] $\mu$M at 20—25°C for the third molecule, and 19 [5] —26 [3] $\mu$M at 5°C and 28 [21], 35 [2,6] or 55 [5] $\mu$M at 20—25°C for the fourth molecule. Kinetic measurements by the temperature-jump method gave values consistent with those values [7]. Similar constants have been reported for binding of the $NAD^+$ analogue, 1,$N^6$-etheno-adenine dinucleotide [14]. For the lobster-tail enzyme, values of 0.6 and 13 $\mu$M, respectively, have been reported [4]. On the other hand, Seydoux and co-workers [9,10] have shown that only two classes of binding sites are present in sturgeon-muscle enzyme, two molecules being bound strongly and two weakly, with dissociation constants, determined fluorimetrically, of 0.09 [10] —0.18 [12] and 1.1 [10]— 1.4 [12] $\mu$M, respectively, at 25°C. The enzyme isolated from *B. stearothermophilus* has somewhat similar properties [21]. Although different temperatures, pH values and ionic strengths were used in the different studies, there is sufficient overlap in the conditions to make it unlikely that this is the reason for the large discrepancy between the dissociation constants of the different preparations.

In this connection, a recent study by Gennis [22] of the yeast enzyme is of interest. This enzyme binds $NAD^+$ much less firmly than the muscle enzymes, and conflicting reports of positive [23] and mixed postive and negative interactions [24,25] have appeared. Gennis [22] has shown that the negative interaction is only apparent, being due to the presence in the enzyme preparations of molecules with a lower affinity for $NAD^+$. A homogeneous preparation shows only positive interactions. This report has made it desirable to examine to what

extent the sequential decline in binding constants of $NAD^+$ to preparations of rabbit-muscle enzyme is due to what Gennis has called affinity heterogeneity (see also ref. 26) in the enzyme preparations used. Indeed, Kelemen et al. [10] have suggested that the differences in properties between their preparation of sturgeon-muscle enzyme and those of other investigators are due to micro-heterogeneity of other muscle-enzyme preparations.

## Materials and Methods

*Isolation of glyceraldehyde-phosphate dehydrogenase from rabbit skeletal muscle*

Since the properties of the enzyme preparation used in this paper differ in important respects from those described previously, its isolation will be described in detail. A rabbit was killed by a blow on the head, and the skeletal muscles rapidly removed and minced into a solution containing 30 mM KOH, 5 mM EDTA and 1 mM dithiothreitol at 5°C. All subsequent steps were carried out between 5 and 10°C. After stirring for 15—20 min, the extract was separated by centrifugation at 30 000 × *g* for 20—30 min and the mince re-extracted with fresh medium. The combined extracts, the pH of which was about 7, were fractionated with ammonium sulphate (Merck, Suprapur) as described by Bloch et al. [26]. However, after the last addition of $(NH_4)_2SO_4$ the pH was brought to 7.8—8.0 with aq. $NH_3$. After standing overnight the suspension was centrifuged (60 min at 30 000 × *g*) and the pellet dissolved in 50 ml of a buffer (pH 6.4) containing 5 mM MOPS, 1 mM EDTA and 1 mM dithiothreitol. The deep-red clear solution was desalted by passing through a column (length, 40 cm; diameter 4 cm) of Sephadex G-50 (fine grade), equilibrated with the same buffer. The filtrate was brought on to a column of CM-Sephadex (C-25) of the same dimensions (cf. ref. 26). After washing with the same buffer until the first protein appeared in the eluate, the enzyme was eluted with a buffer (pH 7.5), containing 5 mM MOPS, 1 mM EDTA, 1 mM dithiothreitol and 0.1 M $(NH_4)_2SO_4$. The eluate containing 7—10 mg/ml enzyme at pH 6.5 was brought to pH 7.0 with aq. $NH_3$. After addition of 1 mM $NAD^+$, the solution was brought to 75% satn. with solid $(NH_4)_2SO_4$ and kept at 5°C. The enzyme crystallised within a few days. From 100 g of muscle, about 300 mg of enzyme was obtained.

The holoenzyme was collected by centrifugation of the suspension, dissolved in a small volume of the buffer to be used and passed through a column of Sephadex G-50 equilibrated with the same buffer. When the concentration of enzyme in the filtrate exceeded 10 mg protein/ml, it contained 3.8—4.0 mol $NAD^+$ per mol tetramer.

To prepare $NAD^+$-free enzyme, the suspension in 75% $(NH_4)_2SO_4$ was centrifuged, the pellet dissolved in the MOPS/EDTA/dithiothreitol buffer (pH 6.4) and the solution passed through a small Sephadex G-50 column, equilibrated with this buffer. The filtrate was applied to a small CM-Sephadex C-25 column, equilibrated with the same buffer, and the $NAD^+$ eluted by the same buffer. When the elution was complete, as judged by the absorbance at 260 nm, the apoenzyme was eluted with the MOPS/EDTA/dithiothreitol buffer (pH 7.5) containing 0.1 M $(NH_4)_2SO_4$. It is important to note that no steps are intro-

duced to prepare $NAD^+$-free enzyme that were not used in the isolation of the $NAD^+$-containing enzyme.

The $NAD^+$-free enzyme contained 3.8 rapidly titratable -SH groups per tetramer, measured by the method of Seydoux et al. [9].

The protein concentration was determined by the absorbance at 280 nm, using the extinction coefficients reported by Fox and Dandliker [27]. Molar concentrations of tetramer were calculated, assuming a molecular weight of 145 000. The $NAD^+$ content was determined enzymically with ethanol and alcohol dehydrogenase (EC 1.1.1.1) on the neutralized supernatant, after precipitating with 4% trichloroacetic acid.

The specific activity, determined by the method of Ferdinand [28], but not correcting for incomplete saturation with $P_i$, was 140—160 μmol/min per mg protein. [$^3$H]β-$NAD^+$ was obtained from New England Nuclear. [$^3$H]$NAD^+$-labelled enzyme was prepared either by addition of a tracer amount of carrier-free [$^3$H]$NAD^+$ to the holoenzyme, or by addition of [$^3$H]$NAD^+$ together with carrier to the apo-enzyme. Both methods gave identical results.

*Equilibrium gel filtration.* For the determination of the dissociation constants of the weak binding sites, the enzyme was equilibrated with different concentrations of $NAD^+$ in the MOPS/EDTA/dithiothreitol buffer (pH 7.0), with or without 0.1 M $(NH_4)_2SO_4$. Gel filtration was used, since equilibration is much faster than by dialysis. A column (length, 20—50 cm; bed volume, 16—40 ml) of Sephadex G-50 (superfine) was equilibrated with the $NAD^+$-containing buffer, 0.5 ml of 30 μM holoenzyme was applied and the column eluted with the same buffer. The protein peak (about 1.5 ml) was pooled and the enzyme concentration (about 10 μM) and the total $NAD^+$ content determined as described above. The free $NAD^+$ concentration was assumed to be the same as that in the solution with which the column was equilibrated. Trial experiments showed that, under our conditions, with about 10 μM enzyme in the eluate and a bed volume of 40 ml, the ratio of the concentrations of "weak" binding sites and free ligand should not exceed 25 for reliable measurements. Otherwise, there is a danger that equilibration is not complete, which is revealed by an overlap between the protein and ligand curves. The dissociation constant found for the weak binding sites (approx. 0.9 μM) is close to the lower limit that can be measured by the technique described.

This method is similar in principle to the method of Hummel and Dreyer [29] used by Seydoux et al. [30] for measuring the binding of $NAD^+$ to the acylated sturgeon enzyme. The main difference is that both protein and $NAD^+$ are determined in the protein fraction, making it irrelevant whether the enzyme was in the holo- or apo-form before equilibration.

*Optical measurements.* The increase in absorbance at 360 nm when $NAD^+$ was added to the enzyme solution was measured with an Aminco DW-2 spectrophotometer, in the split-beam mode, with 1-cm light path. The reference cuvette contained buffer, and successive amounts of $NAD^+$ were added to each cuvette. Protein fluorescence was measured with a Perkin-Elmer spectrofluorimeter MPF 2a, using 290 nm exciting light (slit width, 1—3 nm) and observing at 340 nm (slit width, 40 nm).

## Results

*Binding of $NAD^+$*

The binding of $NAD^+$ to the enzyme was studied by separating enzyme-bound $NAD^+$ from free $NAD^+$ by gel filtration through Sephadex G-50. Fig. 1A shows that when [$^3$H]$NAD^+$-containing holoenzyme is passed through the column the elution pattern of radioactive $NAD^+$ is the same as that of protein, measured by absorbance at 280 nm. When, however, unlabelled $NAD^+$ is present in the buffer solution with which the Sephadex column is equilibrated, the radioactivity is eluted long after the protein, and the peak in the radioactivity is close to a minimum in the absorbance at 280 nm (Fig. 1B). It may be concluded that the equilibrium between bound and free $NAD^+$ is established within the separation time of the gel filtration, under the conditions of this experiment. Thus, although this procedure cannot be used to measure the dissociation rate constants of even the tightly bound $NAD^+$ molecules, it can be used, in principle, to determine the binding constants of $NAD^+$. In the method described in detail in Materials and Methods, the enzyme is allowed to equilibrate with $NAD^+$ present in the fluid with which the column is bathed, and the amount of $NAD^+$ and protein in the protein peak in the eluate measured. It is assumed that the concentration of free $NAD^+$ is equal to that in the eluting fluid. In practice this method can only be used to measure the binding constants of the weaker sites. Indeed, the data from measurements carried out with this technique at 25°C, plotted in the form of a Scatchard plot in Fig. 2, show that between 2.95 and 4 mol $NAD^+$ were bound per tetramer. The curved Scatchard plot, convex to the abscissa, confirms the weakening in binding of successive molecules of $NAD^+$ which is always found with the muscle enzyme. The plot can be resolved into two straight lines, presenting stoicheiometric bind-

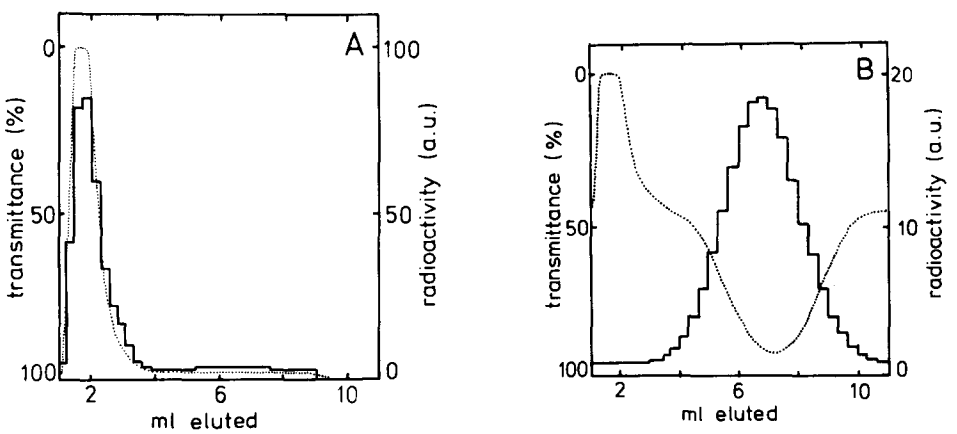


Fig. 1. Exchange of enzyme-bound $NAD^+$ with added $NAD^+$. A column (7 ml) of Sephadex G-50 (medium) was equilibrated with buffer (pH 7.0), containing 5 mM MOPS, 1 mM EDTA and 1 mM dithiothreitol and (in B only) 50 $\mu$M $NAD^+$. 0.5 ml of 14 $\mu$M enzyme (containing 3.5 mol [$^3$H]$NAD^+$/mol) in the MOPS/EDTA/dithiothreitol buffer was applied to the column, which was eluted at 2 ml/min with the same buffer. The transmittance of the eluate at 280 nm was recorded continuously (dotted line) and samples were taken for measurement of radioactivity (expressed in arbitrary units (a.u.); 100 = 12 500 dpm, solid line). The experiment was carried out at 5°C.

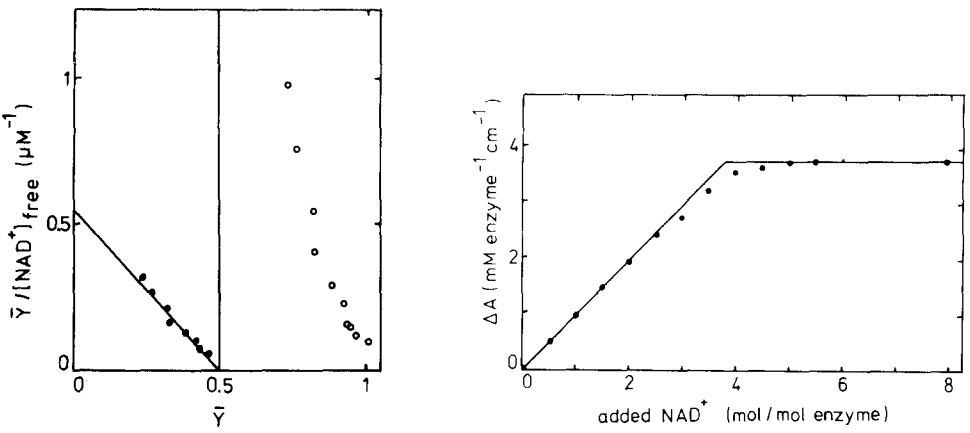


Fig. 2. Determination of dissociation constant of third and fourth $NAD^+$ molecules bound to glyceraldehyde-phosphate dehydrogenase. Equilibrium gel filtration was carried out at 25°C as described in Materials and Methods. Sephadex G-50 column, 20—50 cm long with 16—40 ml bed volume, equilibrated with buffer (pH 7.0) containing 5 mM MOPS, 1 mM EDTA, 1 mM dithiothreitol, 0.1 M $(NH_4)_2SO_4$ and different concentrations (0.75—10.1 $\mu$M) $NAD^+$. Each point represents a different filtration experiment. The concentration of the enzyme in the eluate varied between 8 and 12 $\mu$M. The flow rate was 15 ml/h. The ordinate is bound $NAD^+$ (expressed as saturation function ($\bar{Y}$) of the binding sites), measured in protein eluate, divided by free $NAD^+$ concentration (equal to that added to equilibration buffer). The abscissa is $\bar{Y}$. The open circles show the unresolved plot, the closed circles represent binding to the weak sites, assuming stoicheiometric binding to the first two sites.

Fig. 3. Titration of Racker band at 360 nm on addition of $NAD^+$ to glyceraldehyde-phosphate dehydrogenase. 9.13 $\mu$M enzyme in 5 mM MOPS/1 mM EDTA/1 mM dithiothreitol/0.1 M $(NH_4)_2SO_4$ (pH 7.0, 15°C).

ing to the first two sites ($K_1 = K_2 = 0$) and equal binding to the weaker sites ($K_3 = K_4 = 0.9$ $\mu$M).

*Titration of Racker band*

Fig. 3 shows the titration of the Racker band at 15°C. The extinction coefficient with the strong binding sites occupied ($A_{360nm} = 1.00$ $mM^{-1}$ $NAD^+ \cdot cm^{-1}$) lies between those found by Boers et al. [31] (0.97) and Bell and Dalziel [11] (1.05). At the highest concentration of added $NAD^+$ (73 $\mu$M) the contribution to the absorbance of the non-specifically bound $NAD^+$ [31,16] is negligible ($\Delta A = 0.0004$, compared with $\Delta A_{obs} = 0.0342$). Calculated on the basis of a dissociation constant at 15°C (see below) of 0.27 $\mu$M for $NAD^+$ bound to the third and fourth specific binding sites, these sites are 99.3% occupied with this concentration of $NAD^+$. The $\Delta A_{360nm}$ is 94.1% of the value expected if the extinction coefficient of the weak binding sites were the same as that of the strong. If it is only the fourth bound $NAD^+$ that has a weaker Racker band, as is the case with previous preparations of the rabbit-muscle enzyme, then the extinction coefficient of this band is 100 (3.986 · 0.941—2.993)/0.993 = 76.2% that of the other three. This is much higher than previously reported and, in fact, in view of the accumulation of errors (including that in determining the protein) in this type of calculatuon, is equal to 100% within the experimental error. Thus, it may be concluded that, with the preparation of rabbit-muscle glyceraldehyde-phosphate dehydrogenase used in this work, like those from

sturgeon muscle reported by Seydoux and coworkers [9,10] the intensity of the Racker band is proportional to the concentration of bound $NAD^+$.

*Quenching of protein fluorescence*

It has been shown previously [32] that even when the intensity of the Racker band given by the fourth molecule was much less than that with the other molecules, all four molecules equally quench the protein fluorescence (see also refs. 10—12 and 14). It may be assumed, then, that this is also the case with the enzyme preparation used in this work. As pointed out by Bell and Dalziel [11], the fluorescence quenching can be measured at such low protein concentrations that the dissociation constants of the tight binding sites can also be determined by this method.

Fig. 4 shows a titration carried out by this method, at 15°C, at three widely

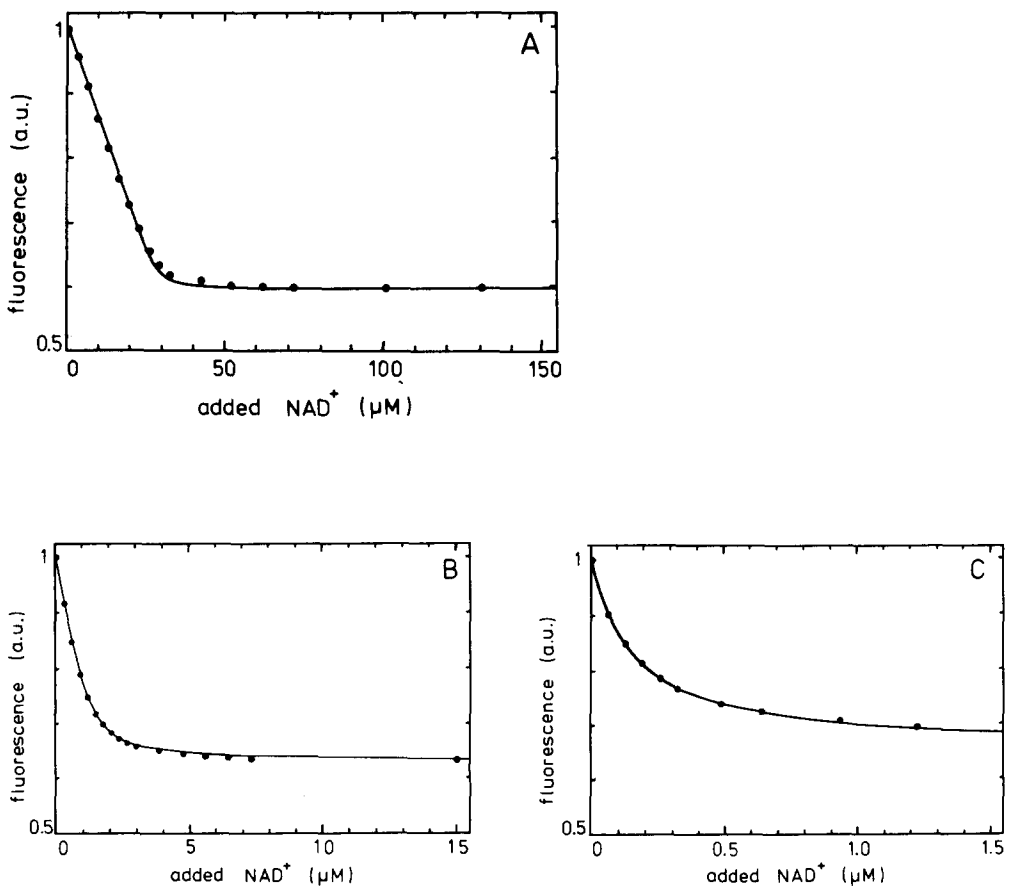


**Fig. 4. Quenching of protein fluorescence by added $NAD^+$. 7.80, 0.38 and 0.039 $\mu$M tetramer in A, B and C, respectively, in 5 mM MOPS/1 mM EDTA/1 mM dithiothreitol/0.1 M $(NH_4)_2SO_4$ (pH 7.0, 15°C). A small decline in fluorescence was observed after saturating amounts of $NAD^+$ were added due to inner-filter quenching of the fluorescence by $NAD^+$. The quenching coefficient was calculated from the slope of the line obtained with saturating amounts of $NAD^+$ and the points shown were corrected for this quenching. The continuous lines are drawn for dissociation constants of 0.028 $\mu$M for the first two $NAD^+$ molecules bound to the tetramer, and 0.27 $\mu$M for the third and fourth molecules.**

differing protein concentrations. At the highest concentration (Fig. 4A), the binding is stoicheiometric for a large part of the titration. The stoicheiometry of the $NAD^+$ binding (3.71 mol/mol tetramer) was used in simulations of the data obtained at the lower concentrations (Figs. 4B and 4C). (The departure from the theoretical value of 4 was assumed to be due either to an error in measuring the protein or to the presence of impurities in the enzyme preparation.) A good fit was obtained with microscopic dissociation constants of 0.028 $\mu$M for the first two "tight" sites and 0.27 $\mu$M for the third and fourth "weak" sites, assuming no intersite interaction. Alternatively, if negative interaction across one of the areas of contact between the subunits is assumed, the data are fitted by a single microscopic dissociation constant of 0.051 $\mu$M for all binding sites with an interaction energy of 2.6 kJ (0.63 kcal)/mol site. According to the latter model two weak binding sites, with a dissociation constant of 0.15 $\mu$M, are induced by the binding of the first two molecules of $NAD^+$.

The calculated value of 0.27 $\mu$M at 15°C for the "weak" binding sites is directly comparable with the value of 0.9 $\mu$M at 25°C obtained by direct binding measurements. This difference is probably at least partly explained by the difference in temperature. The factor $K_{25°C}/K_{15°C} = 3.3$ may be compared with $K_{25°C}/K_{16°C} = 2.2$ reported by Keleman et al. [10] for the sturgeon-muscle enzyme. Velick et al. [5] found $K_{25°C}/K_{5°C} = 2.9$ for the fourth site, but less than 1 for the third site on the rabbit-muscle enzyme.

The value of 0.028 $\mu$M for the strong ($K_1 = K_2$) binding sites lies between those reported by Bell and Dalziel [11] for the first two sites, namely 0.01 and 0.09 $\mu$M at 25°C. The value of 0.27 $\mu$M for the weak sites ($K_3 = K_4$) are, however, much lower than reported by Bell and Dalziel [11], 4 $\mu$M and 35 $\mu$M, respectively. Our data were consistent with identical values for $K_1$ and $K_2$, respectively, but insufficient data were collected at low saturation to exclude the difference of about 10-fold reported by Bell and Dalziel [11].

## Discussion

In previous papers from this and other laboratories, it has been shown that not only is the fourth $NAD^+$ or NADH molecule much more weakly bound to the tetrameric glyceraldehyde-phosphate dehydrogenase than the other three, but that there are other differences, both qualitative and quantitative, in the effects of binding the fourth molecule. Although binding of all four molecules gives equal quenching of the protein fluorescence, the absorbance of the NAD-enzyme complex increases by only about 30% as much * and the molecular

* The low extinction coefficient of the Racker band, the relatively high dissociation constant of the fourth molecule with the earlier preparations, and the formation of a weak complex with higher $NAD^+$ concentration [31,16] make it difficult to detect the smaller coefficient for the fourth $NAD^+$ molecule unless the $\Delta A_{360nm}$ is plotted against the occupancy of the specific sites. Boers et al. [31] distinguished three extinction coefficients: 0.97 $mM^{-1} \cdot cm^{-1}$ for the first 3 sites, 0.30 $mM^{-1} \cdot cm^{-1}$ for the fourth, and 0.011 $mM^{-1} \cdot cm^{-1}$ for the weak complex. Peczon and Spivey [8] claimed that the extinction coefficient for the fourth molecule is the same as that for the first three, but Boers and Slater [32] showed that when Peczon and Spivey's data were calculated against occupancy of the binding sites, the extinction coefficient for the fourth molecule varied in different experiments quoted from 0 to 43% of that of the other three molecules. Bell and Dalziel [11] assumed for the calculation of the dissociation constants for the third and fourth molecule that the extinction coefficients for all four molecules are the same. However, it can be calculated that the highest $\Delta A$ reported in Fig. 1 of their paper, when corrected for

ellipticity by about 40% on binding the fourth molecule [31]. The thermodynamic parameters of binding the fourth molecule are also quite different [5]. Notably, $-\Delta G^{o}$ increases with increase in temperature for the third site and decreases for the fourth. Binding of the fourth molecule of NADH results in no further increase in the intensity of the circular-dichroism band at 335 nm [31].

The preparation of glyceraldehyde-phosphate dehydrogenase used in this paper differs in important respects from those described previously. Most important is the finding that the dissociation constant for the fourth molecule of $NAD^+$ bound is 0.9 $\mu$M at 25°C (0.27 $\mu$M at 15°C), compared with 35—55 $\mu$M at 20—25°C found previously (see Introduction). Although some of the previous measurements were made at pH 8.2, compared with pH 7.0 used in this paper, control experiments showed that the dissociation constant, measured by titration at 360 nm, is little affected by pH in this range (see, however, ref. 6). Since about 0.1 M $(NH_4)_2SO_4$, derived from the enzyme solution, was present in previous determinations, from this laboratory at least, this concentration of $(NH_4)_2SO_4$ was added to the buffer used in the present experiments. Replacement of the buffer used in this investigation by that used by previous workers also had little effect, as did the use of activated charcoal instead of column chromatography to prepare the apoenzyme. Moreover, the high dissociation constant for the fourth molecule bound to previous preparations has been found by five different methods: ultracentrifugation [3], equilibrium dialysis [2], quenching of protein fluorescence [5,6,11,32], measurement of $\Delta H$ [5] and the effect of $NAD^+$ on the fluorescence of 4-chloro-7-nitrobenzofurazan-modified enzyme [33]. Moreover, significant differences in the effect of $NAD^+$ on the $^{19}F$ NMR spectrum of trifluoroacetonylated enzyme have been reported for the rabbit-muscle [34,35] and sturgeon-muscle enzyme [12]. A similarly high dissociation constant for the fourth molecule of NADH has been found by ultrafiltration [31], quenching of protein fluorescence [11] and decline of absorbance of NADH at 340 nm [11]. There is little doubt, then, that the difference in NAD-binding properties is a property of the enzyme preparation, and not of the physical parameters measured or of the conditions.

The main difference in the isolation procedure used in this study, compared with previous ones, is its rapidity (2 instead of 7 days), the use of dithiothreitol throughout the procedure, the use of highly purified $(NH_4)_2SO_4$ and the use of adsorption on CM-Sephadex to purify the enzyme instead of repeated crystallizations from ammonium sulphate.

A second difference in properties between the present and earlier prepara-

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weak complex, corresponds, for the fourth molecule, to 56% of the average measured with the first two molecules, whereas the fourth site, on the basis of their calculated dissociation constant (which is the same as that of de Vijlder and Slater [2]) would be 90% occupied. Thus, the extinction coefficient of the fourth $NAD^+$ molecule is 62% of that of the other molecules. Peczon and Spivey [8] have quoted a thesis of W.A. Bloch, which is not available to us, which is said to demonstrate equivalent absorptivity changes and optical rotary dispersion for saturation at all enzyme sites, and attributes the lower values previously found to partial alteration of enzyme-binding properties due to using older methods of enzyme purification.

tions is the intensity of the Racker band with the fourth $NAD^+$ molecule. Although, according to previously published data, the intensity of this band varies, in contrast to very close agreement in the extinction coefficient of the band formed with the first three molecules of $NAD^+$ bound, in all cases it is appreciably less intense than with the first 3 molecules. In our preparation, the difference has, within experimental error, disappeared. In this respect the present preparation of the rabbit-muscle enzyme resembles that isolated from sturgeon muscle [9,10].

These differences in $NAD^+$ binding are not reflected in a difference in enzyme activity. The activity of our preparation, 140—160 μmol/min per mg protein, measured under standard conditions, was about the same as that used by Boers and Slater [32] and Peczon and Spivey [8] (up to 160 μmol/min per mg protein), and is less active than that reported by Bell and Dalziel [11,17], namely 200 μmol/min per mg protein. Moreover, De Vijlder and Slater [2] showed that the specific activity does not decline during the repeated recrystallizations of the enzyme, and Boers and Slater [32] have reported that ageing of their preparations, resulting in a decline in activity from 165 to 120 μmol/min per mg protein, had no effect on either the binding constant or the absorbance coefficient of the fourth $NAD^+$ molecule.

It seems, then, that the classical method [36] of preparing rabbit-muscle glyceraldehyde-phosphate dehydrogenase leads to a change in the protein (possibly caused by binding of metal ions present in the $(NH_4)_2SO_4$) revealed by a change in some, but not all, of the characteristics of $NAD^+$ binding, but not in enzymic activity. Indeed, the possibility that this preparation contains denatured protein, incapable of binding $NAD^+$, was considered already in the first paper from this laboratory that drew attention to the fact that titrations of the Racker band did not yield 4 $NAD^+$-binding sites [1], but seemed to be excluded when 4 sites were found by direct binding (see, e.g. ref. 37). The available evidence does not allow us to decide whether all enzyme molecules present in these preparations are modified, or only some. The distinction is important, since if the former is the case the large amount of work carried out on the enzyme prepared in the classical way is still valid from the point of view of protein structure, and, in particular, the weakening in the binding of successive $NAD^+$ molecules retains its interest from this point of view even if its relevance to the enzymic mechanism in vivo becomes doubtful. If, however, the classical preparations show what Gennis [22] has termed affinity heterogeneity, the difference in binding between the third and fourth molecule of $NAD^+$ might only be apparent. For example, a mixture of 50% enzyme molecules with a dissociation constant equal to 1 μM for both the third and fourth $NAD^+$ molecules and 50% enzyme molecules with a dissociation constant equal to 35 μM for both $NAD^+$ molecules would yield a binding isotherm identical with that given by a homogeneous preparation with binding constants of 1 and 35 μM for the third and fourth molecule, respectively. Gennis [22] has suggested that this is the explanation for the apparent negative interaction reported for the yeast enzyme. This matter can possibly be resolved for the rabbit-muscle enzyme by fractionation of the classical preparation by affinity chromatography.

In any case, it is clear that the apparent difference between the rabbit-muscle

enzyme and that present in other muscles has disappeared. Only two types of binding site can be distinguished, tight and weak. No difference in binding constants of the two weak sites can be detected. Further work is necessary before one can be sure that there is no difference in binding constants between the two tight sites, and indeed, before affinity heterogeneity can be rigorously excluded as the explanation of the presence of tight and weak sites in our enzyme preparation.

Since the fluorescence quenching with 0.039 and 0.38 $\mu$M tetramer are fitted with the same dissociation constants, it is unlikely that any dissociation of the tetramer occurred under the conditions of these measurements (15°C). Slow [39] dissociation of the enzyme into dimers and monomers has been reported to occur at concentrations below 3 $\mu$M at lower temperatures (5°C) in the presence of 0.1 M phosphate [39—41].

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