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BINDING AND PROPERTIES OF NAD+ IN GLYCERALDEHYDEPHOSPHATE DEHYDROGENASE FROM LOBSTER-TAIL MUSCLE

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SUMMARY

1. The extinction coefficient at 280 nm of charcoal-treated lobster-muscle glyceraldehydephosphate dehydrogenase is 0.80 $cm^2 \cdot mg^{-1}$.

2. The $A_{280\ nm}:A_{260\ nm}$ of NAD+-free enzyme was found to be 1.99.

3. Equilibrium dialysis 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 ($K_D < 5 \cdot 10^{-9}$ M), whereas the third and fourth molecules are bound with dissociation constants of respectively $6 \cdot 10^{-7}$ M and $13 \cdot 10^{-6}$ M.

4. The first three NAD+ molecules contribute equally to the absorption at 360 nm, whereas the fourth makes little if any contribution.

5. On mixing NAD+ with the enzyme, the maximum increase of absorption at 360 nm is reached within 3–5 msec, independently of the amount of NAD+ added. This corresponds to a second-order rate constant of more than $1 \cdot 10^{10}$ $M^{-1} \cdot sec^{-1}$.

6. The reduction of acetyl phosphate by NADH, and its arsenolysis, catalysed by the enzyme, require NAD+. NAD+ has no effect on the esterase activity.

INTRODUCTION

The enzyme glyceraldehydephosphate dehydrogenase (D-glyceraldehyde 3-phosphate:NAD+ oxidoreductase (phosphorylating), EC 1.2.1.12) isolated from lobster-tail muscle is a tetramer, composed of four identical protomers, each with a molecular weight of 36 000 (ref. 1). The amino acid sequence has been elucidated by DAVIDSON *et al.*[2], and X-ray studies[3] show that it possesses at least one 2-fold axis of symmetry.

The enzyme isolated from rabbit muscle is also a tetramer composed of four identical protomers[4]. Though there are differences in amino acid composition[5], it has been shown that the amino acid sequence in the active centre of the two enzymes is

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identical[1,2]. Both enzymes bind NAD^+ firmly, with formation of a broad absorption band with a maximum at 360 nm[6,7].

Since research on the structure of this enzyme has been concentrated on the lobster enzyme[1-3], it became desirable to extend our previous studies[8,9] on the binding of NAD^+ to the rabbit-muscle enzyme to the lobster-muscle enzyme.

RESULTS

Protein determination and measurement of NAD^+ content

In agreement with ALLISON[7] it was found that the extinction coefficient at 280 nm for the NAD^+-containing enzyme (3.9 moles NAD^+ per mole enzyme) was 0.96 $cm^2 \cdot mg^{-1}$. For the charcoal-treated enzyme we found at 280 nm an extinction coefficient of 0.80 $cm^2 \cdot mg^{-1}$. Determinations of protein by the Kjeldahl and biuret methods agreed within 0.5%.

The isolated enzyme contained 3.9 moles NAD^+ per mole, as was also found by TRENTHAM[10]. When all (< 0.02 mole remaining) of NAD^+ was removed with charcoal, the $A_{280\ nm}:A_{260\ nm}$ ratio was 1.99, which is close to the value (2.00–2.03) previously found by DE VIJLDER AND SLATER[8] for the rabbit-muscle enzyme. This ratio is much lower than the value of 2.13 found by MURDOCH AND KOEPPE[11] for rabbit-muscle enzyme and by KIRSCHNER *et al.*[12] for yeast enzyme.

Stability

The lobster enzyme is much less stable in dilute solution than that isolated from rabbit muscle. At a concentration of 10 μg/ml in 5 mM EDTA (pH 7.4) the enzyme is totally inactivated in about 1 h at 0°, even in the presence of 1 mg/ml serum albumin. Rabbit-muscle enzyme lost about 15% of its activity under the same conditions. Charcoal-treated lobster enzyme in a concentration of 3 mg/ml in 5 mM EDTA (pH 7.4) lost about 1.5% of its activity in 1 h at 0°; with NAD^+-containing enzyme there was practically no inactivation under these conditions, even after 10 h.

Binding of NAD^+ to charcoal-treated enzyme as measured by equilibrium dialysis and ultracentrifugation

By equilibrium dialysis and ultracentrifugation it was found that the lobster-muscle enzyme can bind 4 molecules NAD^+ per molecule enzyme. Even on addition of excess NAD^+ (11.3 moles/mole of enzyme) 3.9 moles NAD^+ per mole of enzyme were bound.

The binding showed the same characteristics as with the rabbit-muscle enzyme[8,13]. With up to two moles added NAD^+ per mole of enzyme no NAD^+ was detectable in the dialysate and the estimated dissociation constant was less than $5 \cdot 10^{-9}$ M. The third and the fourth molecule were more loosely bound as can be seen in Table I (column 1). In Table I the dissociation constants measured are compared with those previously reported for the rabbit-muscle enzyme[8,13,14].

Titration with NAD^+

The change in absorption at 360 nm as a function of the added and bound NAD^+, as shown in Fig. 1, is similar to that obtained for the rabbit-muscle enzyme (*cf.* refs. 8, 15). Up to 2 moles NAD^+ per mole enzyme cause a linear increase in absorption with

TABLE I

DISSOCIATION CONSTANTS OF NAD^+ BOUND TO GLYCERALDEHYDEPHOSPHATE DEHYDROGENASE

*Dissociation constant**	*Lobster (equilibrium dialysis)*	*Rabbit*	
		Ultracentrifugation[8]	*Equilibrium dialysis*[13,14]
K_1 (M)	$< 5 \cdot 10^{-9}$	$< 5 \cdot 10^{-8}$	$< 1 \cdot 10^{-11}$
K_2 (M)	$< 5 \cdot 10^{-9}$	$< 5 \cdot 10^{-8}$	$< 1 \cdot 10^{-9}$
K_3 (M)	$6 \cdot 10^{-7}$	$4 \cdot 10^{-6}$	$3 \cdot 10^{-7}$
K_4 (M)	$13 \cdot 10^{-6}$	$35 \cdot 10^{-6}$	$26 \cdot 10^{-6}$

* As defined in ref. 8.

an ε_{mM} (at 360 nm) = 1.02 per mole NAD^+ for this portion of the titration. On further addition of NAD^+ the absorbance increase per mole added NAD^+ declined. The intersection point of the titration with lobster-muscle enzyme was found at 3.0 moles NAD^+ per mole enzyme. From the plot of $A_{360\ nm}$ against bound NAD^+ (as calculated from added NAD^+ by the use of the dissociation constants given in Table I) it can be seen that the absorbance band at 360 nm is almost entirely caused by three NAD^+ molecules, the fourth molecule making only a slight contribution, if any. This is also the case with the rabbit-muscle enzyme. The effect of NAD^+ concentration on the rate of oxidation of glyceraldehyde at 25° was also similar to the result obtained with rabbit-muscle enzyme[8,11,16,17].

Kinetics of NAD^+ binding to charcoal-treated enzyme

Because of the lower stability of the lobster enzyme the incorporation studies were done at 10°. For comparison the rate of incorporation of NAD^+ into rabbit-muscle enzyme at this temperature was also measured.

The course of the reaction between NAD^+ and the enzyme was followed at 360 nm in a stopped-flow apparatus. As can be seen in Fig. 2 the reaction with lobster-muscle enzyme was virtually complete in about 3–5 msec, the mixing time of the instrument, independent of the amount of NAD^+ added. The calculated second-order rate constant for this reaction must be greater than $1 \cdot 10^{10}\ M^{-1} \cdot sec^{-1}$.

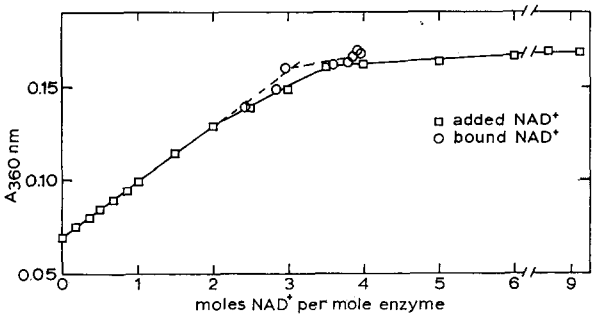
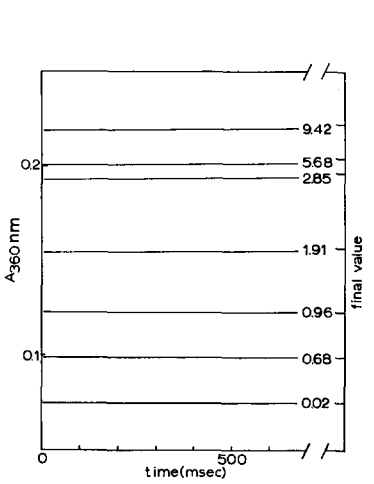


Fig. 1. Titration at 360 nm with NAD^+ of charcoal-treated glyceraldehydephosphate dehydrogenase (28.3 μM) isolated from lobster muscle. The enzyme was dissolved in 100 mM Tris–HCl buffer (pH 8.2) containing 5 mM EDTA. Temp., 23°. □, added NAD^+; ○, bound NAD^+ calculated from the dissociation constants in Table I.



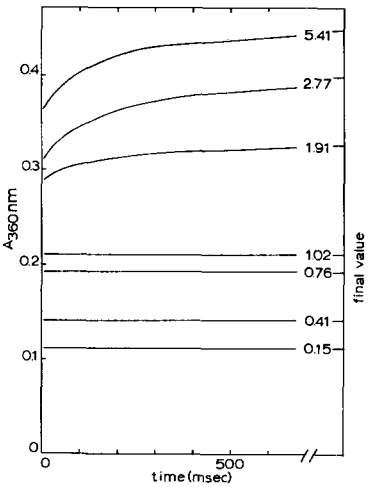


Fig. 2. Reaction of NAD^+ with lobster-muscle glyceraldehydephosphate dehydrogenase as measured in the Durrum stopped-flow apparatus. One of the two syringes contained 46 μM enzyme in 0.1 M Tris–HCl buffer–5 mM EDTA (pH 8.2), and the other various amounts of NAD^+ (indicated on the lines) in the same buffer with 0.5% serum albumin. Temp., 10°; light path, 2 cm.

Fig. 3. Reaction of NAD^+ with rabbit-muscle glyceraldehydephosphate dehydrogenase as measured in the Durrum stopped-flow apparatus. The experiment was carried out in the same way as described in Fig. 2 with 99 μM enzyme in 0.1 M Tris–HCl buffer–5 mM EDTA (pH 8.2) and various NAD^+ concentrations. Temp., 10°.

Fig. 3 shows the reaction course with rabbit-muscle enzyme at 10°. The results are similar to those obtained at 25° (ref. 8). With less than 1 mole NAD^+ per mole enzyme the end-value was practically reached within the mixing time of the instrument. With 2 moles NAD^+ added per mole of enzyme 80% of the final value was reached in 3–5 msec, and completion of the reaction required more than 1 sec.

Requirement for NAD^+ for catalytic activities

The reduction of acetyl phosphate by NADH in the presence of the lobster enzyme shows a distinct initial lag, as previously reported by HILVERS AND WEENEN[18] for the rabbit-muscle enzyme, and shown by them to be due to a requirement for NAD^+ for this reaction. It may be concluded that the lobster enzyme also has this requirement. Also in agreement with the experience with the rabbit enzyme[19], NAD^+ is required for the arsenolysis of acetyl phosphate catalysed by lobster enzyme. The esterase activity was not affected by NAD^+, in accordance with our experience with the rabbit-muscle enzyme (J. J. M. DE VIJLDER AND A. G. HILVERS, unpublished experiments) but in disagreement with PARK *et al.*[20] who found NAD^+ inhibitory.

DISCUSSION

As was to be expected, glyceraldehydephosphate dehydrogenase isolated from lobster muscle resembles more closely the enzyme of rabbit muscle than the yeast enzyme. It contains 3.9 moles NAD^+ per mole enzyme compared with 3.6–3.7 moles for the rabbit enzyme. The yeast enzyme as isolated contains only a trace of NAD^+

(refs. 21, 22). The most interesting difference between the enzyme from different sources relates to the kinetics of the reaction of NAD^+-free enzyme with NAD^+. All three molecules of NAD^+ react completely with the lobster enzyme within 3–5 msec at 10° (Fig. 2). No slow phase is present. In the case of the rabbit enzyme, two phases are detected with more than 1 mole NAD^+, both at 10° (Fig. 3) and 25° (ref. 8). The slow phase has only a relatively slight temperature dependency ($t_{\frac{1}{2}}$ = 250–300 msec at 5°, 70 msec at 35°; J. J. M. DE VIJLDER, unpublished observations). The slow phase is much slower with the yeast enzyme and it has a high temperature coefficient ($t_{\frac{1}{2}}$ = 100 sec at 0° and 1.2 sec at 40°, calculated from refs. 23 and 24, respectively; 11 sec at 8.5° and 2.4 sec at 25° (ref. 23).

Since the slow phase is not found with the rabbit enzyme after pre-incubation with 1–2 moles NAD^+ per mole enzyme, we concluded that binding of 1 molecule of NAD^+ affects the conformation of the second protomer. The absence of a slow phase with the lobster enzyme, even without pre-incubation, suggests either that there is a difference in interaction between the two protomers in the two enzymes or that the change of conformation is very rapid with the lobster enzyme.

A second difference between the lobster and rabbit enzymes is the much greater instability of the former in dilute solution. This may be related to the difference in thiol groups. The lobster enzyme contains 5 cysteine residues per protomer[2], compared with 4 and 2 for the rabbit and yeast enzyme, respectively[25]. The yeast enzyme is the most stable of the three[26].

Apart from these differences, the lobster and rabbit enzymes are very similar. The intensities of the charge-transfer bands at 360 nm obtained on combination of the enzymes with the first three molecules of NAD^+ are identical, and in both cases further addition of NAD^+ causes only a slight increase in intensity of the band. CONWAY AND KOSHLAND[14] have shown that binding of the fourth molecule to the rabbit enzyme has no effect on the viscosity, indicating little if any effect on protein conformation. The dissociation constants of the four molecules of NAD^+ bound to lobster and rabbit enzyme are also quite similar (Table I).

It seems likely, then, that the 'sequential' model previously applied to the rabbit muscle[8,14] is also applicable to the lobster. On the other hand, the allosteric model of MONOD *et al.*[27] has been applied to the yeast enzyme[12].

EXPERIMENTAL

Glyceraldehydephosphate dehydrogenase was isolated from lobster-tail muscle *(Homarus europeënsis)* by essentially the method of ALLISON[7] with the difference that the filtration steps were replaced by centrifugation at 36 000 × *g*. The enzyme was recrystallized 1–2 times from 98–100% satd. $(NH_4)_2SO_4$ solution (pH 7.0) containing 1 mM β-mercaptoethanol and 1 mM EDTA*. NAD^+ was removed by stirring a solution containing 40 mg protein per ml in 5 mM EDTA with 20% (w/v) charcoal for 4 min at 0–2° (*cf.* ref. 28).

The activity was measured spectrophotometrically at 22° in the reaction mixture described by DE VIJLDER AND SLATER[8]. The specific activity obtained under these conditions was 130 ± 10 μmoles NADH per min per mg protein. In order to minimize

* Crystallization of the lobster enzyme was more difficult than of the rabbit enzyme. This procedure was followed in order to minimize inactivation on prolonged standing.

inactivation, the enzyme was diluted to about 15 μg/ml in 0.1 M Tris–HCl buffer (pH 8.0), containing 1 mg/ml serum albumin and 2 mM EDTA, 1 min before the reaction was initiated by adding this enzyme solution to 20 times its volume of reaction mixture.

NAD^+ determinations were made enzymically with ethanol and alcohol dehydrogenase (EC 1.1.1.1). If enzyme was present, protein was first denatured in 6% $HClO_4$. A molecular weight of 145 000 was assumed[1]. Acetyl phosphate was determined by the method of LIPMANN AND TUTTLE[29].

Equilibrium dialysis was carried out by placing a dialysis sac, containing 1.5 ml of the enzyme (80 μM) in 0.1 M Tris–HCl buffer (pH 8.2)–5 mM EDTA and 20–900 μM NAD^+, in a tube containing 6.5 ml of the Tris–EDTA solution. The contents of both the dialysis sac and the tube were continuously mixed. Control experiments with protein-free NAD^+ solutions showed that equilibrium was reached within 5–6 h.

In order to remove traces of heavy metals, the membranes were boiled three times in a 0.1 M EDTA solution before use.

The dissociation constants given in Table I were calculated from the smooth curve drawn by hand through points obtained with 2.9, 3.4, 3.8, 4.6, 6.5, 9.1 and 11.3 moles NAD^+ added per mole enzyme. The amount of bound NAD^+ was calculated from the amount added *minus* the amount of dialysable NAD^+ determined enzymically. Allowance was made for NAD^+ adsorbed to the dialysis membrane. For the calculation of K_3, the portion of the curve between 2.5 and 3.0 moles added NAD^+ per mole enzyme was used. It was assumed that the first two sites were fully occupied and the fourth site not occupied. For the calculation of K_4, the portion of the curve between 5 and 7 moles added NAD^+ per mole enzyme was used. It was assumed that the first three sites were fully occupied.

Ultracentrifugation was carried out in an MSE No. 65 centrifuge. The stopped-flow experiments were performed with an instrument of the Durrum Instrument Corporation following the method as described by DE VIJLDER AND SLATER[8].

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