PRELIMINARY NOTES

BBA 61124

The reaction of glyceraldehydephosphate dehydrogenase with NAD^+

RACKER AND KRIMSKY[1] showed that crystalline glyceraldehydephosphate dehydrogenase, (D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase, EC 1.2.1.12), isolated from rabbit muscle, has a broad absorption band at 360 mμ that they attributed to enzyme-bound NAD. The band disappears on removal of the bound NAD by adsorption on active charcoal. Since it also disappears on the addition of iodoacetate, *p*-chloromercuribenzoate, acetyl phosphate or 1,3-diphosphoglycerate to the enzyme, RACKER AND KRIMSKY[1] suggested that the –SH group present in the active centre of the enzyme is the site of combination of NAD with the enzyme. KOSOWER[2] has suggested that the 360-mμ band is caused by a charge-transfer complex between the –SH group and the pyridine ring of NAD^+.

Rabbit-muscle glyceraldehydephosphate dehydrogenase contains four identical polypeptide chains of molecular weight of about 35 000 (refs. 3 and 4), each of which is capable of binding one molecule of NAD or of related compounds.

The rate of combination of NAD-free enzyme with added NAD^+, as determined by the stopped-flow procedure, is reported in this paper.

Glyceraldehydephosphate dehydrogenase was isolated from rabbit muscle according to the procedure of CORI, SLEIN AND CORI[5]. It was recrystallized 6 times from $(NH_4)_2SO_4$ solution containing 1 mM EDTA. NAD was removed by stirring a solution in 0.1 M Tris–5 mM EDTA (pH 8.2) with 0.2 g activated charcoal per ml of solution for 4 min at 2°. After centrifugation, the charcoal was washed once with the Tris–EDTA solution. The concentration of the enzyme was calculated from the absorbances at 280 mμ and 260 mμ, making use of the extinction coefficients reported by FOX AND DANDLIKER[6]. Control experiments with enzyme containing 0.1, 1.3 and 3.3 moles NAD/mole enzyme showed that this method agreed within 1% of the value obtained by the Kjeldahl method. A molecular weight of 145 000 was assumed (see ref. 4). Resi-

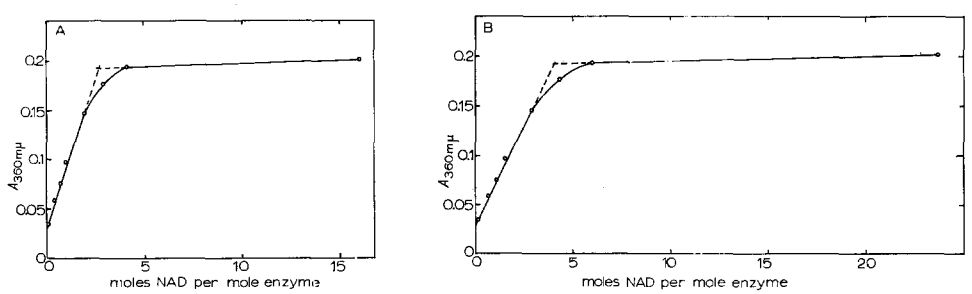

Fig. 1. Titration of glyceraldehydephosphate dehydrogenase with NAD^+. One of the two syringes of the stopped-flow apparatus contained 64.1 μM enzyme in 0.1 M Tris–HCl buffer (containing 5 mM EDTA; final pH 8.2), and the other various amounts of NAD^+ in the same Tris–EDTA mixture containing 0.5% serum albumin. All solutions were at 25°. A. The final changes in absorbance (calculated from the transmissions) reached after about 2 sec are plotted. Light path, 2 cm; wavelength, 360 mμ; band width, 12 mμ. B. Data recalculated on the assumption that native enzyme binds 4 molecules of NAD^+ per molecule of enzyme (see text).

dual NAD in the enzyme was determined by deproteinizing with $HClO_4$ and measuring the NAD^+ in the supernatant with alcohol dehydrogenase (EC 1.1.1.1). The charcoal-treated enzyme used in the experiments described in this paper contained 0.13 mole NAD^+ per mole enzyme. The $A_{280\ m\mu}:A_{260\ m\mu}$ was 1.90.

Rate measurements were made in a Durrum stopped-flow apparatus (Durrum Instrument Corporation). The mixing time is about 5 msec.

In Fig. 1A the final change of absorbance (after about 2 sec) at 360 mμ is plotted against the amount of NAD^+ added. The $\Delta A_{360\ m\mu}$ is linear with the amount of NAD^+ only up to about 2 moles NAD^+ per mole of enzyme. The intersection of the two straight lines, which gives the amount of NAD^+ bound by the charcoal-treated enzyme, is 2.7. This value was found to lie between 2.85 and 3.0 in 6 other experiments. The mean $\Delta\varepsilon_{mM}$ (360 mμ) per mole NAD^+/mole enzyme was found to be 0.97.

Two alternative explanations may be considered for the fact that the enzyme used binds only 2.7–3.0 moles NAD^+ per mole of enzyme.

(1) Only three of the four chains in the molecule of glyceraldehydephosphate dehydrogenase can bind NAD^+. This appears unlikely, since up to 3.5 moles NAD^+ per mole enzyme have been found in enzyme crystallized 4 times from ammonium sulphate, and not treated with charcoal.

(2) Only $2.7/4 = 67.5\%$ of the enzyme preparation used for Fig. 1A is able to

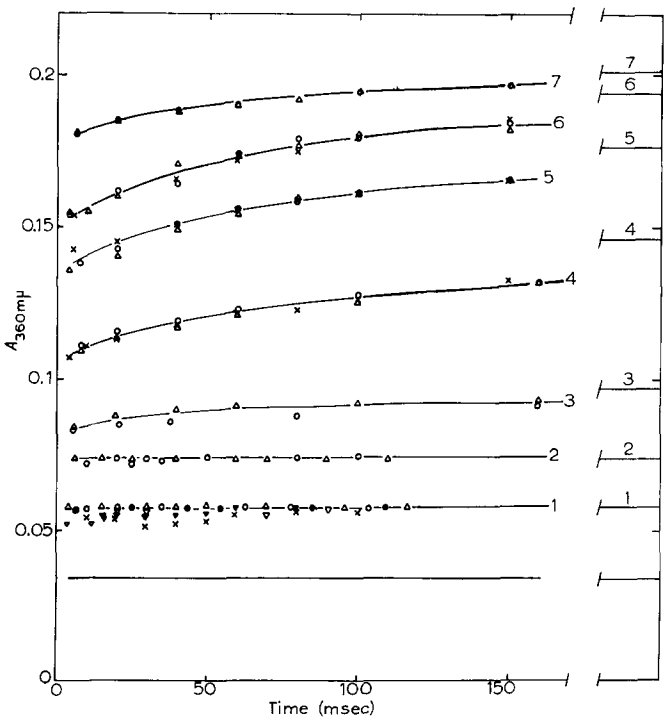

Fig. 2. Time course of the absorbance changes at 360 mμ in the experiment shown in Fig. 1. The lower horizontal line gives the absorbance of the enzyme without added NAD. Curves 1–7, with 0.42, 0.72, 1.02, 1.95, 2.95, 4.13, 16.0 moles NAD^+ per mole enzyme, respectively. The different points represent different runs. The short horizontal lines on the right-hand side represent the final values reached (after about 2 sec).

react with NAD^+, the rest being denatured. If this is so, Fig. 1B gives the titration curve of the active enzyme.

Fig. 2 shows the course of the reaction between different amounts of enzyme and NAD^+. With 0.42 and 0.72 mole NAD^+ per mole enzyme (0.62 and 1.1 according to the recalculation on the basis of the second explanation given above), the reaction was completed within the mixing time (5 msec) of the instrument. With the larger amounts of NAD^+, about 20–30% of the reaction was not completed within 6 msec.

Whatever the explanation of the lower than expected binding of NAD^+ to the enzyme, it would seem safe to conclude that the first molecule of NAD^+ reacts with the enzyme much more rapidly than the other molecules. This is of interest in connection with the mechanism of action of the enzyme proposed by HILVERS, VAN DAM AND SLATER[7], in which one of the four molecules of NAD combines with substrate without being reduced.

This study was supported in part by a grant from the Life Insurance Medical Research Fund. We wish to thank Professor C. VEEGER of the Laboratory of Biochemistry, Agricultural University of Wageningen, for making available the stopped-flow apparatus used, and Mr. S. VAN DALSEM for technical assistance.

Laboratory of Biochemistry.
*B.C.P. Jansen Institute**,
University of Amsterdam,
Amsterdam (The Netherlands)

J. J. M. DE VIJLDER
E. C. SLATER

1 E. RACKER AND I. KRIMSKY, *Nature*, 169 (1952) 1043.
2 E. M. KOSOWER, *J. Am. Chem. Soc.*, 78 (1956) 3497.
3 J. I. HARRIS AND R. N. PERHAM, *J. Mol. Biol.*, 13 (1965) 876.
4 W. F. HARRINGTON AND G. M. KARR, *J. Mol. Biol.*, 13 (1965) 885.
5 G. T. CORI, M. W. SLEIN AND C. F. CORI, *J. Biol. Chem.*, 173 (1948) 605.
6 J. B. FOX, JR. AND W. B. DANDLIKER, *J. Biol. Chem.*, 221 (1956) 1005.
7 A. G. HILVERS, K. VAN DAM AND E. C. SLATER, *Biochim. Biophys. Acta*, 85 (1964) 206.

Received October 24th, 1966

* Postal address: Plantage Muidergracht 12, Amsterdam-C.