Biochem. J. (1975) **151**, 747–749
Printed in Great Britain

Short Communications

The Binding of Nicotinamide–Adenine Dinucleotide to Glyceraldehyde 3-Phosphate Dehydrogenase from *Bacillus stearothermophilus*

By GEOFFREY ALLEN* and J. IEUAN HARRIS
Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K.

(*Received* 3 *September* 1975)

The binding of NAD^+ to glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) from *Bacillus stearothermophilus* has been studied by measurement of protein fluorescence quenching. Slight negative co-operativity was observed in the binding of the third and fourth coenzyme molecules to the tetrameric enzyme. The first two coenzyme molecules were tightly bound. In this respect the enzyme resembles that from sturgeon muscle rather than that from yeast.

The binding of NAD^+ to the glyceraldehyde 3-phosphate dehydrogenases from yeast and from rabbit, lobster and sturgeon muscle has been extensively studied, by many techniques. Binding to the rabbit muscle enzyme, measured by spectrophotometry (Boers *et al.*, 1971), equilibrium dialysis (Conway & Koshland, 1968), ultracentrifugation (De Vijlder & Slater, 1968), fluorescence quenching (Price & Radda, 1971; Velick *et al.*, 1971) and calorimetry (Velick *et al.*, 1971), displays negative co-operativity, whereas binding to the yeast enzyme shows positive co-operativity (Kirschner *et al.*, 1971; Kirschner, 1971; von Ellenrieder *et al.*, 1972), mixed positive and negative co-operativity (Cook & Koshland, 1970) or no co-operativity (Velick *et al.*, 1971), depending on the conditions of measurement. Lobster and sturgeon muscle enzymes display negative co-operativity (De Vijlder *et al.*, 1969; Seydoux *et al.*, 1973).

Studies on the enzyme from *Bacillus stearothermophilus* have revealed similarities in properties and structure to both yeast and muscle enzymes (Suzuki & Harris, 1971, and unpublished work; Singleton *et al.*, 1969), and the binding of the coenzyme was of interest as another comparison of the properties of these enzymes, and particularly in relation to X-ray-crystallographic studies in progress (A. J. Wonacott & R. M. Sweet, unpublished work, reported in Suzuki & Harris, 1971), which have shown that the form of the crystal depends on the amount of coenzyme bound.

Materials and methods

Glyceraldehyde 3-phosphate dehydrogenase was isolated from extracts of *B. stearothermophilus* (from the Microbiological Research Establishment, Porton, Wilts., U.K.) by the method of Suzuki & Harris (1971). Rabbit muscle glyceraldehyde 3-phosphate dehydrogenase was from Boehringer (Mannheim, West Germany). NAD^+ (Boehringer; grade II) was purified chromatographically by the method of Dalziel (1963). Apoenzymes, with absorbance ratios (E_{280}/E_{260}) 1.9–2.0, were prepared from the holoenzymes by charcoal treatment (Suzuki & Harris, 1971). Concentrations of enzyme and NAD^+ were determined spectrophotometrically. The solutions were passed through Millipore filters before fluorescence measurements were made. Fluorescence studies were performed on a Hitachi–Perkin–Elmer recording spectrofluorimeter with a thermostatically controlled cell compartment. The method followed was that of Price & Radda (1971), with N^{α}-acetyl-L-tryptophan solution of the same absorbance at 300nm as the apoenzyme in the control cuvette. The excitation wavelength was 300nm and emission was at 340nm; the bandwidths were 5nm and 16nm respectively. The fluorescence of the N^{α}-acetyl-L-tryptophan solution was scarcely decreased at concentrations of NAD^+ less than 0.1mM. The titrations were performed in a 0.1M-Tris–1mM-EDTA buffer, adjusted to pH8.2 with HCl, at 24.2°C. The protein concentrations were 0.22mg/ml for the rabbit muscle enzyme, and 0.22 and 0.192mg/ml for the *B. stearothermophilus* enzyme, in different experiments. The molecular weight of subunits of both proteins was taken to be 36000 (Suzuki & Harris, 1971),

Results and discussion

The fluorescence excitation and emission spectra of the *B. stearothermophilus* apoenzyme were similar to those of the rabbit muscle apoenzyme, with the emission maximum at 329nm. However, the fluorescence intensity was 63% greater, and less light-

* Present address: National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K.

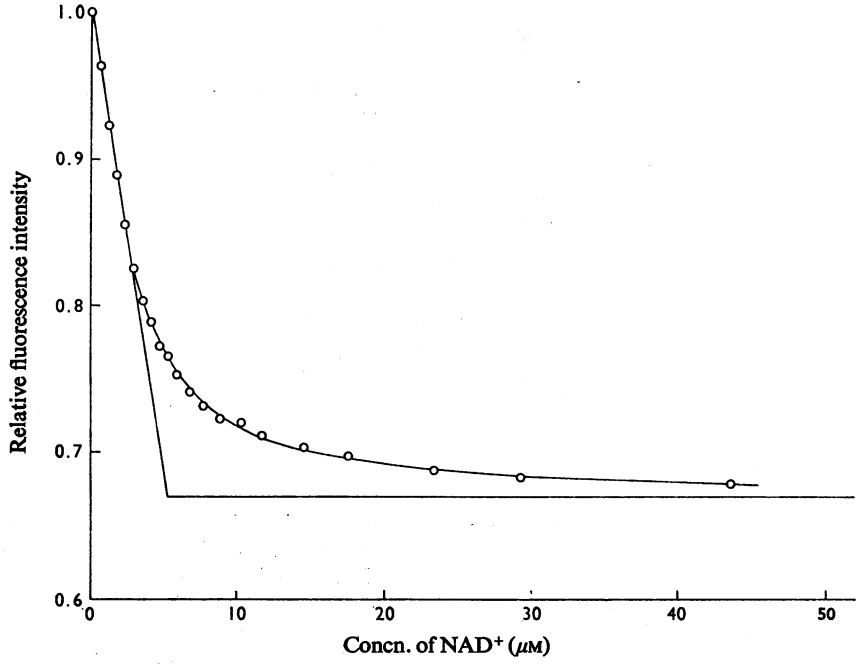

Fig. 1. *Relative fluorescence intensity of B. stearothermophilus glyceraldehyde 3-phosphate dehydrogenase as a function of the total NAD+ concentration*

The initial concentration of apoenzyme was 0.192mg/ml. For other conditions see the text. The intersecting straight lines represent the theoretical dependence of the fluorescence intensity for binding at four sites on the enzyme with infinite association constants and with the limiting fluorescence quenching observed at high NAD^+ concentrations, assuming equal contributions to quenching at each site. The curve through the experimental points is theoretical for dissociation constants K_1 and $K_2 < 10^{-7}$M, K_3 1.3μM and K_4 3.5μM.

scattering was observed than with rabbit muscle enzyme of the same concentration (0.22mg/ml in this experiment). The limiting fluorescence quenching observed at high NAD^+ concentrations was 38% for the rabbit enzyme, and 33% for the bacterial enzyme. It is assumed that the fluorescence quenching caused by each bound NAD^+ molecule is identical. This is likely to be so, since when 2.0 NAD^+ molecules are added per tetramer, and are tightly bound, the fluorescence quenching is exactly half the maximal quenching (Fig. 1).

The results for the rabbit muscle enzyme are similar to those published (Price & Radda, 1971); the first two NAD^+ molecules are tightly bound ($K_{dis.} < 10^{-7}$M), and K_3 and K_4 were 1.9 and 28μM respectively, as determined from the Scatchard plot (Fig. 2*a*). K_3 refers to $[E(NAD^+)_2][NAD^+]/[E(NAD^+)_3]$, and K_4 to $[E(NAD^+)_3][NAD^+]/[E(NAD^+)_4]$. The dependence of the corrected relative fluorescence intensity of the bacterial enzyme on total NAD^+ concentration is shown in Fig. 1. The first two molecules of NAD^+ are bound stoicheiometrically ($K_{dis.} < 10^{-7}$M), whereas there is only slight negative co-operativity in the binding of the third and fourth coenzyme molecules, as shown by the slight upward curvature of the Scatchard plot (Fig. 2*b*). Asymptotic tangents could not be readily drawn to fit this curve, so values of K_3 and K_4 were adjusted to give the best fit to the experimental results using the equation:

$$\bar{\nu} = \frac{[NAD^+]}{K_3 + [NAD^+]} + \frac{[NAD^+]}{K_4 + [NAD^+]}$$

where $\bar{\nu}$ is defined in the legend to Fig. 2. This treatment assumes that each subunit binds 1.0 molecule of NAD^+.

The continuous curve in Fig. 1 was drawn using the best-fit values $K_3 = 1.3\mu$M and $K_4 = 3.5\mu$M, with probable errors estimated from a series of less-well-fitting curves of $\pm 25\%$.

Thus the binding of NAD^+ to glyceraldehyde 3-phosphate dehydrogenase from *B. stearothermophilus* is qualitatively similar to that to the sturgeon muscle enzyme (Seydoux *et al.*, 1973), but different from that to the yeast enzyme. The negative co-operativity shown in the binding of the third and fourth coenzyme molecules is small, and the binding isotherm appears to result from the presence of two pairs of similar

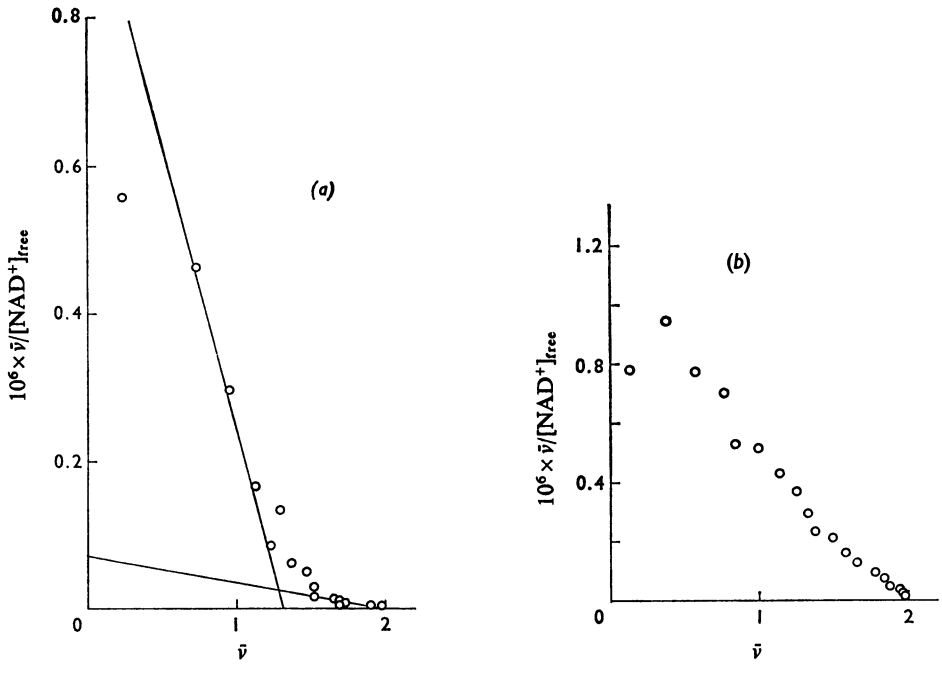

Fig. 2. *Scatchard plots (Scatchard et al., 1957) for the binding of NAD$^+$ to glyceraldehyde 3-phosphate dehydrogenase (a) from rabbit muscle and (b) from B. stearothermophilus*

$\bar{\nu}$ is the average number of mol of NAD$^+$ bound/mol of enzyme in excess of the first two, tightly bound, molecules. $[NAD^+]_{free}$ is the concentration of free NAD$^+$. The rabbit muscle enzyme concentration was 0.22mg/ml, and the *B. stearothermophilus* enzyme concentration was 0.192mg/ml. For other conditions see the text.

binding sites on the enzyme, one pair with a very low dissociation constant. The negative co-operativity displayed by the rabbit and lobster muscle enzymes extends more markedly to the binding of the fourth coenzyme molecule. For the enzyme from lobster muscle, K_1 and K_2 are less than 5×10^{-9}M, and K_3 and K_4 are 0.6 and 13μM respectively (De Vijlder *et al.*, 1969).

Thus negative co-operativity in the binding of NAD$^+$ was already developed in prokaryotes, and is not a control system developed only by eukaryotic organisms. The physiological role of this negative co-operativity is not well understood.

This work was performed during the tenure of a Salters' Company Fellowship by G. A.

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