EFFECT OF THE CONCENTRATION OF D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE ON THE REDUCIBILITY OF ITS FIRMLY BOUND NAD

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D-glyceraldehyde-3-phosphate dehydrogenase (GAPD) is composed of 4 identical subunits [1,2] and it binds and also crystallizes with 3.5 to 4 moles NAD per mole of enzyme [3–6]. Several authors have found that only two moles of firmly bound NAD per mole of GAPD were reducible [7–10] or even only one mole [11].

Our present results show that the amount of reducible firmly-bound NAD depends on the concentration of GAPD, when measured with D-glyceraldehyde-3-phosphate in the presence of arsenate ions.

Four times recrystallized GAPD isolated from swine muscle according to Elödi and Szörényi [13] was dissolved in 0.1 M glycine buffer pH 8.5. The concentration of the protein was determined by measuring the optical density at 280 nm. The amount of firmly bound NAD was 3.6 mole per mole of GAPD as determined from the change in absorbancy of the GAPD-NAD complex at 360 nm (Racker-band) after adding NAD to the GAPD solution ($\sim 10^{-5}$ M). The molecular weight was taken as 140,000 [14]. The molar extinction coefficient of NADH at 340 nm is 6.22×10^3 [15]. D-glyceraldehyde-3-phosphate was prepared from fructose-1,6-diphosphate [16] and the contaminating inorganic phosphate was removed as described earlier [17]. In the assay medium D-glyceraldehyde-3-phosphate was used in $\sim 10^{-3}$ M concentration in the presence of 10^{-3} M arsenate ion. The measured values were corrected for the decrease in absorbancy due to the disappearance of the Racker-band that, in turn, was caused by the transformation of NAD in the GAPD-NAD charge-transfer complex into NADH *. In the fluorimetric determination of NADH another correction was made for the quenching effect of GAPD. The latter was determined independently with NADH-solution, the concentration of which was measured enzymically.

When the concentration of GAPD is relatively high ($\sim 10^{-4}$ M) only about two moles of firmly bound NAD can be reduced, while at about 10^{-7} M GAPD the amount of reducible NAD equals the total amount of NAD firmly bound to the enzyme (fig. 1). In the absence of arsenate and phosphate, there is no difference in the amount of reducible bound NAD; using the above GAPD concentration range, i.e. from 10^{-4}M to 10^{-7} M, about two moles of NAD per mole of enzyme only are reduced (fig. 1). The reduction of bound NAD molecules, both in the presence and absence of arsenate, is complete in the first second.

Keleti et al. [20] and Chance and Park [21] suggested that there are two different types of firmly bound NAD in GAPD. Listovsky et al. [22] concluded from optical rotatory dispersion studies that the NAD binding sites are not equivalent. Vijlder and Slater [19] and Koshland and Neet [23] found that two out of the four firmly bound NAD molecules were bound strongly, one moderately and one relatively weakly. A similar difference between the bind-

* The molar extinction coefficient of the Kacker band at 360 nm is $\epsilon = 9.6 \times 10^2$ [18,19]. The correction was calculated as follows. The number of reduced NAD molecules

$$n = \frac{\Delta E(340\text{ nm, measured}) + \Delta E(340\text{ nm, correction})}{\frac{6.22 \times 10^3}{[\text{GAPD}]}}$$

where $\Delta E(340\text{ nm, correction}) = n[\text{GAPD}]\,9.6 \times 10^2$ (without taking into account the very little difference between the ϵ(340 nm) and ϵ(360 nm) of the Racker band). From this

$$n = \frac{\Delta E(340\text{ nm, measured})}{[\text{GAPD}]\,5.26 \times 10^3}.$$

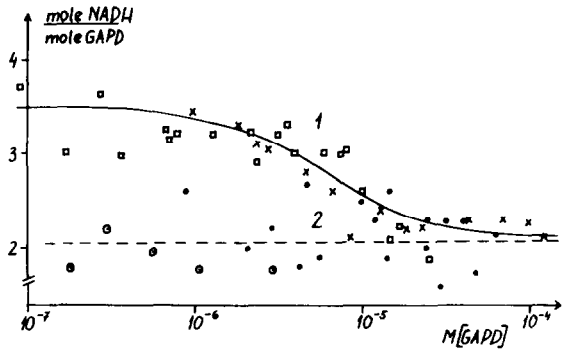

Fig. 1. *Effect of GAPD concentration on the reducibility of firmly bound NAD.*

1: determination of the amount of reducible firmly bound NAD in the presence of arsenate. □ spectrofluorimetrically (excitation at 335 nm, measurement at 470 nm); X spectroscopically at 340 nm.

2: the same as in 1 but determined without arsenate. ● spectrophotometrically at 340 nm; ⊙ spectrofluorimetrically (excitation at 335 nm, measurement at 470 nm).

An Opton PMQ II spectrophotofluorimeter and automatic recording was used.

ing sites of NAD was observed by means of gel filtration of the partially carbethoxylated enzyme. In this case two out of the four moles of firmly bound NAD can be removed from the protein [12]. There is some parallelism between these data and our present findings which show that two moles of firmly bound NAD are reducible in the presence of arsenate in relatively high concentration of GAPD, i.e. ~ 10^{-5} M (the same protein concentration was used by the authors who found also only two moles of firmly bound NAD to be reducible in GAPD [7–9]) or in the absence of arsenate or phosphate independent of GAPD concentration. However, in the presence of arsenate the total amount of bound NAD becomes reducible at low concentrations of GAPD.

Since the four subunits of GAPD are identical [24] the differences in the reducibility of the four firmly bound molecules of NAD may indicate an asymmetrical association of the subunits (perhaps type $\alpha_2\alpha'_2$ as supposed by Malhotra and Bernhard [25]) and/or a possible dissociation of GAPD into protomers during dilution as determined by Constantinides and Deal [26,27].

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