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ROLE OF DEHYDROGENASE BINDING IN PROTECTION OF NICOTINAMIDE ADENINE DINUCLEOTIDE FROM ENZYMATIC HYDROLYSIS

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SUMMARY

Incubation of pig-brain NADase (NAD glycohydrolase, EC 3.2.2.5) with a mixture of free NAD and NAD bound to rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD oxidoreductase (phosphorylating), EC 1.2.1.12) results in destruction of only the free NAD. Enzyme-bound NAD is completely recovered by gel-permeation chromatography, thus confirming the protective effect of dehydrogenase binding. The binding of NAD to glyceraldehyde-3-phosphate dehydrogenase could be a mechanism for controlling the hydrolytic action of NADase *in-vivo*. However, quantitative considerations indicate that in muscle tissue about 80% of the intracellular NAD is unbound. Therefore, dehydrogenase binding of NAD would appear to only partially account for the regulation of intracellular NADase.

It has recently been proposed by KAPLAN[1] that the steady state concentrations of pyridine nucleotides in tissues are regulated in part by the hydrolysis of free NAD to nicotinamide and adenosine diphosphoribose by the enzyme NADase (NAD glycohydrolase, EC 3.2.2.5). The proposal predicts that NAD which is protein bound would be inaccessible to NADase and therefore would be protected from hydrolysis. The steady-state level of NAD would thus be related to the total number of intracellular binding sites with strong affinities for NAD. This theory is attractive because it partially accounts for the rapid metabolic turnover of NAD (ref. 2) and at the same time provides a function for the widely distributed NADase. The proposal would also explain why an enzyme like glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD oxidoreductase (phosphorylating), EC 1.2.1.12), which has strong binding sites for NAD (ref. 3), is present in tissues in quantities which seem inordinately high.

Studies on the hydrolysis of NAD bound to glyceraldehyde-3-phosphate dehydrogenase were first conducted by ASTRACHAN *et al.*[4] using the soluble NADase isolated from Neurospora. On the basis of initial-rate measurements of coenzyme destruction, ASTRACHAN *et al.*[4] concluded that in the pH range of 7.4–8.0, NAD which

is bound to glyceraldehyde-3-phosphate dehydrogenase is not hydrolyzed by Neurospora NADase.

It should be pointed out, however, that the mechanism of action of the Neurospora NADase is unlike that of mammalian NADase (ref. 5), and that the studies of ASTRACHAN *et al.*[4] did not involve the separation of the free and bound forms of NAD. Because of the new significance that the recent proposal of Kaplan[1] has placed on the protection of NAD by dehydrogenase binding, it was felt that a reinvestigation of the reaction was warranted.

NADase was an acetone powder of pig brain (Sigma Chemical Co., St. Louis, Mo.). It was homogenized in the cold with 20 vol. of 50 mM Tris–50 mM KH_2PO_4 (pH 7.5) and centrifuged at 27 000 × *g* for 20 min. The supernatant fraction was discarded, the process was repeated, and the final residue was resuspended in a minimal volume of buffer and stored at −20°. 1 mg of NADase protein hydrolyzed 14 nmoles of NAD per min at 30° in 1 ml of the above buffer containing 2 μmoles of NAD.

Glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle (Sigma) was a crystalline suspension which had a specific activity of 125 units/mg protein when assayed as described by FERDINAND[6]. By means of acid extraction, the enzyme was found to contain 1 μmole of NAD per 49 mg of protein. Protein was measured by the method of LOWRY *et al.*[7].

NAD was assayed by the cycling procedure of SLATER *et al.*[8]. Separate experiments showed that NAD values obtained by direct assay of dehydrogenase-bound NAD were only 72% of the values obtained after acid extraction. For acid extraction, 0.2 ml of 3 M $HClO_4$ was added to a 1.0-ml sample and the mixture was stirred for 2 min at 30°. The mixture was then neutralized with 0.2 ml of 1 M K_2HPO_4–KH_2PO_4 (pH 7.4) and 0.2 ml of 3 M KOH and centrifuged to obtain a clear extract.

Fig. 1 shows the time course of hydrolysis by pig-brain NADase of free NAD and NAD bound to glyceraldehyde-3-phosphate dehydrogenase. Whereas NADase caused a rapid destruction of free NAD, the same level of NADase had only a minimal

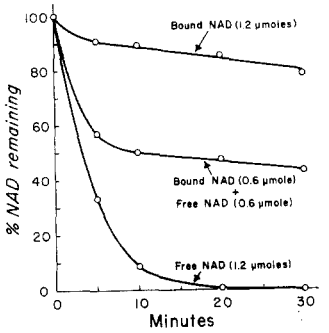

Fig. 1. Reactivity of pig-brain NADase with free and bound NAD. Each reaction mixture contained, in a final volume of 1.0 ml, 50 μmoles Tris–50 μmoles KH_2PO_4 (pH 7.5) and the quantities of NAD shown. The source of bound NAD was glyceraldehyde-3-phosphate dehydrogenase. Reactions were started by addition of 10 mg of NADase protein followed by incubation at 30° with constant stirring. At the times indicated, 0.1-ml samples were removed for acid extraction. The percentages shown are based on the NAD content of control reaction mixtures containing no NADase.

TABLE I

CHROMATOGRAPHIC ANALYSIS OF FREE AND BOUND NAD BEFORE AND AFTER NADase TREATMENT

Reactions were conducted in final volumes of 2.0 ml containing 45 mM Tris–45 mM KH_2PO_4 (pH 7.5), 0.3 mM EDTA, 54 mg of glyceraldehyde-3-phosphate dehydrogenase and, when present, 4 μmoles of NAD and 12 mg of pig-brain NADase protein, added to start the reaction. The mixtures were stirred for 20 min at 30° and were then chilled and centrifuged at 150 000 × *g* for 20 min. Each supernatant fraction (1.0 ml) was chromatographed on a 2.5 cm × 20 cm column of Sephadex G-50 (medium) at 5°. The column was eluted with 50 mM Tris–50 mM KH_2PO_4 (pH 7.5) at a rate of 2 ml/min, and fractions of 50 drops were collected. Bound NAD emerged from the column with the void volume, Fractions 10–14, and free NAD appeared in Fractions 21–37 (see Fig. 2).

Addition	*Recoveries of bound and free NAD (μmoles)*			
	Before NADase		*After NADase*	
	Bound	*Free*	*Bound*	*Free*
None	0.25	0.06	0.23	0.02
NAD (2 μmoles)	0.39	1.54	0.37	0.60

effect on the enzyme-bound NAD. The small rate of NAD destruction in the latter case may represent dissociation of NAD from glyceraldehyde-3-phosphate dehydrogenase followed by hydrolysis of the free coenzyme. When a mixture of free and bound NAD was used (Fig. 1), biphasic rates of destruction were clearly apparent. The NAD that was rapidly removed in the latter instance presumably represents free NAD, while the NAD that was destroyed at the slow rate would be the bound form. In order to obtain direct evidence concerning the actual fate of the NAD in the presence of NADase, reaction mixtures containing various combinations of bound and free NAD were subjected to gel-permeation chromatography to separate the bound and free forms.

The data in Table I show that incubation of glyceraldehyde-3-phosphate dehydrogenase with NADase resulted in little or no loss of NAD from the dehydrogenase. It can also be seen (Table I) that, upon supplementation with NAD, more NAD-binding sites on the dehydrogenase became filled and that these remained filled upon incubation with NADase. In contrast, 61% of the free NAD that was present during the NADase treatment was destroyed. The chromatographic separation on which these data are based is illustrated in Fig. 2. It is interesting to note that the rate of dissociation of NAD from glyceraldehyde-3-phosphate dehydrogenase appears to be sufficiently slow, relative to the rate of chromatographic separation, that it is possible to separate the free and bound forms of NAD with only a small amount of trailing between the peaks.

The results presented in this report are in complete accord with the earlier work of ASTRACHAN *et al.*[4]. However, the question remains as to whether enough NAD is protected from NADase by dehydrogenase binding *in vivo* for this to be a major mechanism for regulating NADase activity.

Rabbit skeletal muscle has been shown to contain 4.13 mg of glyceraldehyde-3-phosphate dehydrogenase[9] and 680 nmoles of NAD (ref. 10) per g of fresh tissue. Based on a molecular weight of 140 000 for glyceraldehyde-3-phosphate dehydrogenase[11] and a total of four NAD binding sites per enzyme molecule[12], a maximum of

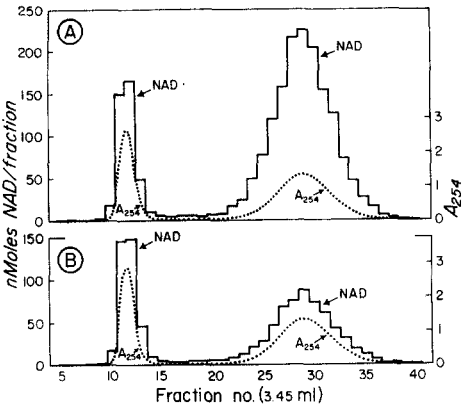

Fig. 2. Chromatographic analysis of free and bound NAD before and after NADase treatment. See legend to Table I. The supernatant fractions from reaction mixtures supplemented with NAD were chromatographed (A) before, and (B) after treatment with NADase. Duplicate samples (20 μl) were used for direct analysis of NAD. The effluent was monitored at 254 nm with a dual-beam analyzer (Instrumentation Specialties Co., Lincoln, Neb.).

118 nmoles of NAD can be bound to the dehydrogenase per g of tissue. This amount of bound NAD represents only 17.4% of the total NAD present, a figure that agrees reasonably well with the percentage of bound NAD (approximately 20%) recently found in crude extracts of rabbit muscle[13]. Since about 80% of the NAD in rabbit muscle is free, the binding of NAD to glyceraldehyde-3-phosphate dehydrogenase would only partially account for the protection of intracellular NAD from the hydrolytic action of NADase. The potential activity of NADase in most tissues is sufficient to destroy the intracellular NAD in a relatively brief time[14]. Therefore, it is likely that other mechanisms are also involved in controlling the activity of NADase.

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