AMP LIKE CONTAMINATION IN COMMERCIAL NAD PREPARATIONS

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Received 22 July 1971
Revised version received 11 August 1971

1. Introduction

In most investigations of the properties and requirements of enzymes, commercial coenzymes and substrates are used. These products are prepared by similar methods by different manufacturers and are well standarized. The results published can therefore in most cases be reproduced by other laboratories and few questions arise concerning their validity.

Recently we have been studying glutamate dehydrogenase from *Blastocladiella emersonii.* Previously it has been found [1–3] that this enzyme shows an unusual response curve when the oxidative deamination of glutamate is measured for increasing concentrations of NAD. Several explanations of the mechanisms underlying this observation have been put forward [1, 4]. The results presented in this paper indicate that the peculiar response curve is due to small amounts of an AMP-like substance in the commercial NAD preparations. Since many enzymes are affected by very small concentrations of purine nucleotides the question arises to what extent such impurities may lead to artefacts in enzyme studies.

2. Materials and methods

Glutamate, AMP, NAD (grade III and grade V), and 5′-adenylic acid deaminase (grade IV) were obtained from Sigma Chemical Co., St. Louis Mo. NAD (grade I) was also purchased from Boehringer and Soehne, GmbH, Mannheim, Germany. Glutamate dehydrogenase was prepared from *Blastocladiella emersonii*, as described by LéJohn and Jackson [1].

The enzyme activity was measured from the rate of increase in absorption at 340 nm upon reduction of NAD. The reaction mixture (in a total volume of 3 ml) contained in addition to the enzyme: 33 mM glutamate, 0.2 M Tris-chloride buffer, pH 8.5, and NAD and AMP as indicated. Protein concentration was determined by the method of Lowry et al. [5]. The specific activities of the different enzyme preparations were similar to the value obtained by LéJohn and Jackson [1–3].

3. Results and discussion

In fig. 1 is shown the effect of the NAD concentration on the catalytic activity of glutamate dehydrogenase from *Bl. emersonii.* The activity of this enzyme is stimulated by AMP and ADP while ATP inhibits the enzyme [1]. Cyclic AMP, GMP or TMP have no effect on the activity (unpublished experiments). In agreement with previous results a normal hyperbolic curve was obtained when the enzyme is assayed in the presence of AMP. In the absence of added AMP a plateau is observed at low NAD concentrations, while for higher concentrations the activity increases strongly [1–3]. The lengths of the plateau, as well as the final slope of the response curve varied slightly for different batches of NAD. If the NAD stock solution is treated with 5′-adenylic acid deaminase, the plateau is considerably extended and the slope of the final curve is reduced. Extensive treatment of the NAD solution with the deaminase abolishes nearly completely the activity at pH 8.5. Since 5′-adenylic

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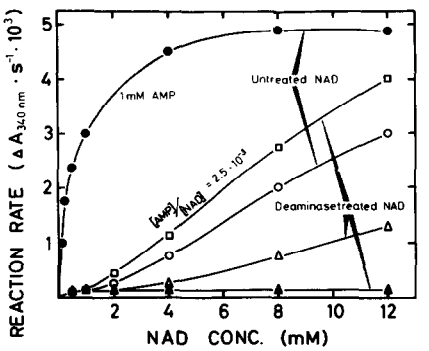

Fig. 1. Effect of NAD concentration on the oxidative deamination of glutamate. The reaction rate was measured with untreated NAD in the absence of added AMP (○—○) and in the presence of 1 mM AMP in the assay mixture (●—●), as well as with NAD pretreated with 5′-adenylic acid deaminase for 12 min (△—△) and for 30 min (▲—▲). The NAD solution which had been deaminase-treated for 30 min was also used for activity measurements after addition of 0.25% AMP (on a molar basis) to the NAD solution (□—□). The deaminase treatment was carried out in 0.01 M citrate buffer, pH 6.5 at 30°. The concentration of 5′-adenylic acid deaminase was 1 μg protein/ml. After incubation the pH was adjusted to pH 8.5 to stop the reaction. The concentration of glutamate dehydrogenase was 2.8 ng/ml during the assay.

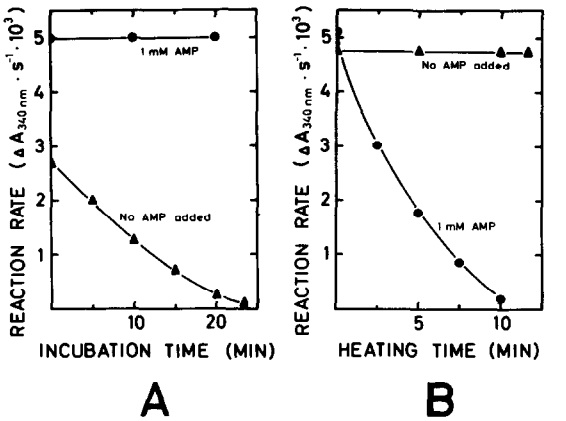

Fig. 2. Effect of deaminase treatment and heating on the coenzyme activity of NAD. (A) Preincubation of NAD with 5′-adenylic acid deaminase for different periods of time. Experimental conditions as in fig. 1. The catalytic activity was determined with 4 mM NAD in the absence of added AMP and with 1 mM NAD in the presence of AMP. (B) Heating of NAD for different periods of time. The NAD was dissolved in 0.025 M Tris-chloride buffer, pH 12.5, and kept on a boiling water bath for the times indicated. The catalytic activity was determined using 4 mM untreated NAD in addition to 4 mM heated NAD in the absence of AMP and with 1 mM NAD in the presence of AMP. The concentration of glutamate dehydrogenase was 5.6 ng/ml in both experiments.

acid deaminase is highly specific in its reaction [6] and IMP is unable to activate the enzyme [1], the results suggest that the peculiar biphasic curve obtained with untreated NAD is due to the presence of AMP as a contamination in the NAD preparation. This is further supported by the finding that addition of 0.25% AMP (on a molar basis) to the stock solution of NAD after the deaminase treatment restored the catalytic activity. In fact the curve obtained with increasing concentrations of deaminase-treated NAD after addition of AMP to the NAD solution had the same biphasic shape as found for untreated NAD assayed in the absence of added AMP.

The properties of commercial NAD is further studied in the results presented in fig. 2. In order to detect small changes in the concentration of the coenzyme, 1 mM NAD was used when the oxidative deamination of glutamate was measured in the presence of AMP (see fig. 1), whereas 4 mM or 8 mM NAD was used when the enzyme activity was determined in the absence of added AMP. When the stock solution of NAD was pretreated with 5′-adenylic acid deaminase for different lengths of time (fig. 2A), the reaction rate in the absence of AMP decreased in agreement with the results in fig. 1. The fact that the reaction rate measured in the presence of AMP is unaffected, clearly demonstrates that the deaminase treatment does not affect the NAD.

When the NAD was destroyed by heat treatment at high pH [7], the reaction rate in the presence of AMP decreased as expected (fig. 2B), while no effect was found on the rate measured in the absence of added AMP. In the latter experiment 4 mM untreated NAD was used in addition to 4 mM heated NAD. Since the activating effect of AMP is not affected by the heat treatment (unpublished experiments) the results support the view that the higher reaction rate observed with 8 mM NAD compared with 4 mM NAD (see also fig. 1) is due to AMP present in the NAD preparation.

Previously it has been found that commercial NAD may contain small amounts of ADP-ribose which inhibit certain enzymes [8, 9]. The present results indicate that AMP may also be present in NAD-prepara-

tions and that it may cause artefacts in the experimental results.

On the basis of the present data it is calculated that the contamination of AMP represents about 0.1% (by weight). The amount varied somewhat for the different batches and the grades of NAD used.

In the case of NADP we have likewise obtained evidence for purine nucleotide impurities. Thus, it was found that although this coenzyme does not function as coenzyme for the present enzyme, the addition of NADP from certain manufacturers greatly stimulated the enzyme activity, while NADP from other companies had no stimulating effect.

Acknowledgement

The work is supported in part by The Norwegian Research Council for the Science and Humanities. I wish to thank Prof. A. Pihl for valuable discussion. The able technical assistance of Miss Turid Holm is gratefully acknowledged. The stock culture of *Bl. emersonii* was kindly supplied by Prof. E.C. Cantino, Michigan State University, USA.

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