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Nicotinamide-Adenine Dinucleotide Nucleosidase of *Mycobacterium butyricum*

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NAD-ase was extracted and purified from *Mycobacterium butyricum* by repeated ammonium sulfate fractionation and DEAE-cellulose ion-exchanger chromatography. Enzymological studies were carried out.

Many works have been done on NAD-ase*, an enzyme which hydrolyzes NAD* at the nicotinamide-ribose linkage, but very little was known about this enzyme of tubercle bacilli. Kern and Natale[1] observed that an extract of *Mycobacterium butyricum* contained rather heat-stable enzyme as well as a heat-labile inhibitor of this enzyme. Except this report, only scattered data, whereby the absence of NAD-ase activity was reported by human or bovine type of tubercle bacilli, can be found in literature[2,3].

On the other hand, NAD-ase exchange reaction, whereby isonicotinoyl hydrazide can replace nicotinamide in the NAD molecule[3–5], was postulated by Oka *et al.*[6] and Eda *et al.*[7] as a reaction providing a possible explanation for the anti-tuberculous activity of isonicotinoyl hydrazide (isoniazid).

Considering the importance of isoniazid in tuberculosis treatment, it is of significance to investigate mycobacterial NAD-ase in more details. The purpose of this paper is to describe the purification method and to report some properties of *Mycobacterium butyricum* NAD-ase.

MATERIALS AND METHODS

NAD (β-type) of 98 per cent purity was obtained from the Sigma Chemicals Company.

Mycobacterium butyricum was grown on Sauton synthetic medium, which was composed of 4.0 g of asparagine, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of K_2HPO_4, 2.0 g of citric acid, 0.05 g of ferri-ammonium citrate, 60 ml of glycerol per liter and adjusted to pH 7.0. After incubation at 37°C for 5 or 7 days, the cells were harvested on filter paper and washed thoroughly with distilled water. The purification procedures are described below.

*The following abbreviations will be used: NAD, nicotinamide-adenine dinucleotide; NAD-ase, nicotinamide-adenine dinucleotide nucleosidase; DEAE cellulose, diethylaminoethyl cellulose.

NAD-ase activity was measured in a reaction mixture containing 0.1 ml of NAD (10 μmoles per ml), enzyme preparation, 0.4 ml of M/15 phosphate buffer at pH 7.2, water to 1.0 ml of the total volume. The reaction mixture was incubated at 38°C for 30 or 60 min and the reaction was terminated by the addition of 5.0 ml of 1.0 M potassium cyanide. Cyanide was added to a control tube at zero time. From the change of extinction value at 325 mμ before and after the incubation, the amount of NAD decomposed was determined. A unit of enzyme is that amount which causes the hydrolysis of 0.01 μmole of NAD per hour under the conditions. Protein content of preparation was determined from extinction value at 260 mμ and 280 mμ according to the following equation: Protein mg per ml $= 1.45 \times E_{280} - 0.74 \times E_{260}$.

RESULTS

I. Procedure for NAD-ase purification

Step 1. Preparation of crude extract. Washed cells were suspended in five volumes of distilled water with minimal grinding in a mortar and the suspension in an Erlenmeyer flask was placed in a boiling water bath for 5 min and immediately cooled down in water. After being cooled and centrifuged at 10 000 r.p.m. for 30 min, the precipitated cell pellet was homogenized with original volume of distilled water. The suspension was placed in a Kubota's sonic disintegrator for 30 min. The resulting turbid solution was centrifuged for 30 min at 10 000 r.p.m. The supernatant was saved and the precipitated pellet was subjected once more to sonic oscillation and centrifugation as before. The combined supernatant served as the starting material.

Step 2. First ammonium sulfate fractionation. To each 100 ml of the above solution 17.6 g of solid $(NH_4)_2SO_4$ were added. After standing for three or four hours and centrifuged at 10 000 r.p.m. for 30 min, the precipitate was discarded and to each 100 ml of the supernatant 19.8 g of solid $(NH_4)_2SO_4$ were added. After three or four hours the solution was centrifuged at 10 000 r.p.m. for 30 min and the supernatant was discarded. The precipitate was dissolved in a minimal quantity of distilled water and dialyzed overnight against distilled water.

Step 3. Second ammonium sulfate fractionation. To each 100 ml of the above solution 27.7 g of solid $(NH_4)_2SO_4$ were added. After three or four hours the solution was centrifuged at 10 000 r.p.m. for 30 min and the precipitate was discarded. To each 100 ml of the supernatant 9.9 g of solid $(NH_4)_2SO_4$ were added. After standing for three or four hours and centrifuged for 30 min at 10 000 r.p.m., the supernatant was discarded. The precipitate was dissolved in a minimal amount of distilled water and dialyzed against distilled water overnight.

Step 4. DEAE-cellulose ion-exchanger chromatography. The above solution was subjected to the column of DEAE-cellulose ion-exchanger buffered with M/40 phosphate at pH 7.2. The column was washed with the same buffer to elute out inactive proteins and then the active material was eluted with M/15 phosphate buffer at pH 7.2.

Step 5. Concentration with ammonium sulfate. To 100 ml of the above eluate 39.0 g of solid $(NH_4)_2SO_4$ were added. After three or four hours the solution was centrifuged at 10 000 r.p.m. for 30 min and the supernatant was discarded. The precipitate was dissolved in a minimal amount of M/45 phosphate buffer at pH 7.2 and dialyzed against the same buffer overnight.

A typical result of the purification procedures is summarized in Table 1.

Table 1. Purification of NAD-ase.

Preparation step	Volume ml	Total protein mg	Total units	Units per mg	Purity
Cell-suspension	105	5 722.5	250.95	0.0438	
Suspension of heated cells	110	4 994.0	26 011.70	5.209	
Crude extract	200	1 414.0	176 372.00	124.73	1
1st $(NH_4)_2SO_4$ fractionation	50	113.5	47 658.50	419.90	3.37
2nd $(NH_4)_2SO_4$ fractionation	40	52.8	35 956.40	680.99	5.46
DEAE-cellulose chromatography	80	1.680	24 272.80	14 448.10	115.84
$(NH_4)_2SO_4$ concentration	10	0.573	8 991.70	15 692.32	125.81

II. Properties of *Mycobacterium butyricum* NAD-ase

Ultra-violet absorption spectrum of the preparation after Step 5 had a single peak at 280 mμ due to the presence of aromatic amino acids. No positive evidence for the presence of any prosthetic group was obtained by this method.

Enzyme activity was maximal at pH 6.2, but it was essentially unchanged between pH 5.5 and 7.2. Approximately 70 per cent of maximal activity was retained at pH 8.0. Initial velocity of NAD hydrolysis was proportional to the amount of the enzyme used. Under the conditions the reaction proceeded until all NAD was consumed up completely. Apparent Michaelis constant was calculated from Lineweaver-Burk plot as $K_M = 1.88 \times 10^{-3}$ M.

Inhibition of the enzyme activity by some of pyridine carboxylic acid derivatives was tested. As shown in Table 2, the hydrolysis of NAD was inhibited

Table 2. Inhibition by Pyridine-carboxylic Acid Derivatives. Reaction mixtures consisted of 0.1 ml of NAD (1 μmole), 0.4 ml (for Exp. 1) or 0.3 ml (for Exp. 2) of the enzyme (0.0449 mg protein per ml), 0.4 ml of inhibitor in M/15 phosphate buffer at pH 7.2, water to 1.0 ml. Incubated at 38°C for 1 hour.

Exp. No.	Addition	E_{325}	Inhibition %
1	None	0.3619	0
	Nicotinamide 5×10^{-2}M	0.2436	32.7
	Nicotinic acid 5×10^{-2}M	0.2410	33.4
2	None	0.2757	0
	Isonicotinamide 5×10^{-2}M	0.2442	11.3
	Isonicotinic acid 1.25×10^{-2}M	0.2308	16.2

only in the presence of rather high concentration of inhibitor. Nicotinoyl and isonicotinoyl hydrazides were also tested as for their inhibition, but very intensive absorption at 325 mμ, which developed by the addition of cyanide to these hydrazides, interferred with the NAD determination and made it impossible to get any definite result.

DISCUSSION

As already reported by Kern and Natale[1], NAD-ase activity of *Mycobacterium butyricum* was only detectable after boiling of the cell-suspension or of the extract. Starting from the extract of heat-treated cells 150 times purification was achieved by the repeated ammonium sulfate fractionation and DEAE-cellulose ion-exchanger chromatography, while Kern and Natale had got 15 times purification starting from boiled extract of untreated cells.

The properties of the enzyme thus prepared were essentially identical to what was reported by Kern and Natale. But NAD hydrolysis by the enzyme preparation was inhibited in the presence of 5×10^{-2}M nicotinamide, though the lack of the inhibition by 10^{-2}M nicitinamide had been reported.

Studying the inhibition by isonicotinoyl hydrazide, the formation of isonicotinoyl hydrazide analogue of NAD was suggested by the developement of yellow color after addition of potassium cyanide, which is strong alkaline, to the reaction mixture containing isonicotinoyl hydrazide, NAD and NAD-ase. But by the control tube, which contained the same components, but where NAD was added after incubation and addition of cyanide, the yellow color developed in almost the same degree as by the experimental tube. The role of NAD-ase in this exchange reaction is now under investigation.

In the preliminary work for the extraction of NAD-ase from *Mycobacterium butyricum* cells, it was noticed that cell-free extract prepared by mechanical grinding of cells with sea-sand in mortar did not possess any NAD-hydrolyzing activity before or after boiling, but unheated extract thus prepared inhibited the NAD hydrolysis by purified *Mycobacterium butyricum* NAD-ase. This suggests that the enzyme and its inhibitor locate in different subcellular units but not as a complex.

The distribution of NAD-ase and its inhibitor in *Mycobacterium* and the specificity of the inhibitor will be published elsewhere.

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