NAD- AND FAD-3′-PYROPHOSPHATES – ENZYMIC SYNTHESIS AND INERTNESS

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1. Introduction

We have shown [1,2] that ATP:nucleotide pyrophosphotransferase (EC 2.7.6.4) purified from *Streptomyces adephospholyticus* [3,4] catalyses the transfer of the 5′-β,γ-pyrophosphoryl group from ATP, dATP and adenosine-5′-triphosphate-3′-diphosphate to the 3′-hydroxy site of a variety of nucleotidic compounds including: purine and pyrimidine 5′-ribomononucleotides; several 5′-diphosphonucleosidic coenzymes such as NAD, FAD, CDP-choline and ADP-glucose; short oligonucleotides; tRNA and $\overset{7}{m}$G-5′-ppp-5′-Am; and a cap structure found at the 5′-termini of eucaryotic mRNA. These products of 3′-pyrophosphorylation may be supposed to have lost or altered their initial biological activity. In the present experiments, we isolated and identified the transfer products formed from NAD and FAD, i.e., their 3′-pyrophosphoryl derivatives (at the adenosine moiety), then found these products to be devoid of the respective cofactor and inhibitory activities.

2. Materials and methods

ATP:nucleotide pyrophosphotransferase was purified as in [3,4], spec. act. 821. Nuclease P1 was a commercial product from *Penicillium citrinum* (Yamasa Shoyu Co., Choshi) [5]. Venom exonuclease VPH was purchased from Worthington Biochem. Corp. Yeast alcohol dehydrogenase was obtained from Boehringer Mannheim. Hog kidney D-amino acid oxidase was a Sigma product and was used after extensive dialysis against 1 M KBr–0.1 M sodium pyrophosphate–3 mM EDTA, pH 8.5, then sodium pyrophosphate–EDTA to remove bound FAD exactly by the method in [6].

The reaction products were analysed by Avicel thin-layer chromatography with isobutyric acid adjusted to pH 3.7 with 0.5 M NH_4OH as the solvent system. The chromatograms were usually scanned by Shimadzu CS 900 dual wavelength scanner at 270/310 nm, followed by spray for P_i and PP_i [7] if necessary. Scanning for venom exonuclease NAD-pyrophosphate digest was done at 237/290 nm, an isosbestic point of pApp and NMN [8] for the convenience of comparison of the peaks, followed by ammonia methylethylketone spray for the NMN moiety [9]. Flavin compounds were also detected by their yellow color and fluorescence under ultraviolet lamp. PP_i was determined colorimetrically [10].

3. Results and discussion

The synthetic reactions were carried out as in [2]. For NAD-3′-pyrophosphate, an 8 ml reaction mixture

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containing 17 mg dATP, 22 mg β-NAD^+, 300 units enzyme, 0.8 mg bovine serum albumin as an enzyme stabilizer, 10 mM $MgCl_2$, 2 mM $EDTANa_3$ and 0.1 M Tris–HCl buffer, pH 9.0 was incubated for 5.5 h at 37°C and chromatographed on a DEAE–Sephadex A-25 column (2 × 35 cm) with a 1 l linear gradient from 0.1–0.3 M LiCl buffered with 0.05 M Tris–HCl, pH 7.5. Among 4 peaks that appeared, the first, second and fourth peak emerging at 12%, 34% and 60% gradient were identified as NAD, pdA and dATP, respectively, by Avicel thin-layer chromatography (R_F 0.38, 0.50 and 0.26). The third peak at 53% and with R_F 0.18 was concentrated under reduced pressure and at bath temp. < 45°C and precipitated with 5 vol. ethanol in the cold, followed by ethanol wash and drying in vacuum (16 mg). For the structural characterization, the product was digested by nuclease P1 [5] and snake venom exonuclease separately, and the resultant products identified by comparison with authentic markers on chromatography (fig.1). The former gave only NAD^+ and PP_i. It is already known that the enzyme splits nucleoside-3′-pyrophosphate as well as 3′-monophosphate linkages to quantitatively liberate PP_i and P_i, respectively [2]. Colorimetric determination of PP_i [10] indicated equimolar formation of NAD^+ (19.0 nmol) and PP_i (18.3 nmol). In the latter digestion, the venom exonuclease split 5′-α,β pyrophosphate linkage [7,14] and so gave pApp (plus pAp) and NMN in equimolar amounts. The digestion was found virtually complete, giving no fluorescence on spraying for nicotinamide riboside group at the position of pApp where NAD-pyrophosphate if remaining undigested would appear to overlap. pAp should have come from the action of venom enzyme on NAD-3′-monophosphate, the intermediary degradation product, during storage and through incubation and also from degradation during incubation of pApp once formed by the enzyme. The results of these two different digestions allowed us to conclude that the product obtained is NAD-3′-pyrophosphate (at adenosine moiety). This new NAD derivative was tested for cofactor and inhibiting activities towards yeast alcohol dehydrogenase and the results were found to be negative for both (fig.2).

To prepare FAD-3′-pyrophosphate, a 10 ml reaction mixture containing 21 mg dATP, 33 mg FAD, 350 units enzyme, 1 mg bovine serum albumin,

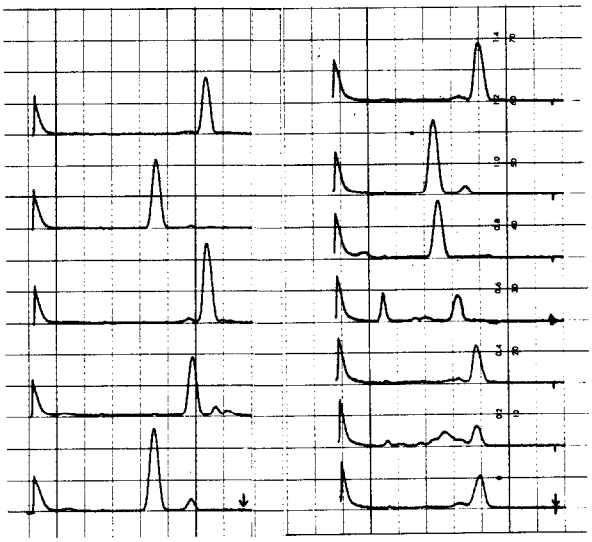

Fig.1. Identification of NAD-3′-pyrophosphate by nuclease P1 (left) and venom exonuclease digestion (right). A reaction mixture, 10 μl, containing 40 μg product, 4 μg nuclease P1 and 0.1 M ammonium acetate buffer, pH 5.0, was incubated at 37°C for 15 min. An aliquot was taken for analysis. From bottom to top: NAD^+, NADP, the product as obtained, and incubated with and without nuclease P1 added, respectively. NAD^+ and NADP are authentic markers. A small peak accompanying NAD^+ is contaminating NADP. Another reaction mixture, 10 μl, containing 40 μg product, 10 μg venom exonuclease, 0.1 M Tris acetate, pH 6.5 [11] and 15 mM $MgCl_2$ were reacted at 37°C for 5 h. From bottom to top: the product incubated without and with enzyme added, pApp, pAp plus adenosine, NMN, NAD and the product as obtained, respectively. pApp (Sanraku-Ocean Co., Tokyo) and NMN are authentic markers. pAp marker with adenosine was prepared by alkaline hydrolysis of pApA segregated from silkworm nuclease-digested poly(A) [12,13]. Arrows show the starting point; the left terminal peak corresponds to the solvent front.

10 mM $MgCl_2$, 2 mM $EDTANa_3$ and 0.2 M Tris–HCl, pH 9.0 was incubated for 5.5 h at 37°C. This and subsequent steps were carried out in a place as dark as possible. The mixture was chromatographed on a DEAE–Sephadex A-25 column (2 × 30 cm) with a 1.2 l linear gradient from 0.1–0.3 M LiCl buffered with 0.05 M Tris–HCl, pH 7.5. Unreacted FAD and pdA appeared together at 34%, unreacted dATP at 69% and one small and one big yellow-colored peak at 78% and 89%, respectively, of the gradient. The last peak was concentrated, precipitated and dried

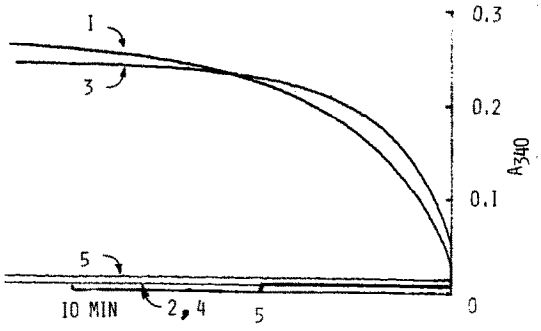

Fig.2. Inactivity of NAD-3′-pyrophosphate as cofactor or inhibitor of yeast alcohol dehydrogenase. The enzyme, 0.6 unit, and 0.03 ml ethanol were incubated with the addition of (1) 0.1 mg NAD, (2) 0.1 mg NAD-3′-pyrophosphate and (3) 0.1 mg NAD plus 0.3 mg NAD-3′-pyrophosphate separately in 3 ml 75 mM sodium pyrophosphate buffer, pH 8.7, containing 25 mg semicarbazide HCl [15]. (4) is (1) without enzyme. (5) is (3) without enzyme. The reactions were run at 25°C in photocells with 1 cm light-path and the increment at 340 nm was recorded.

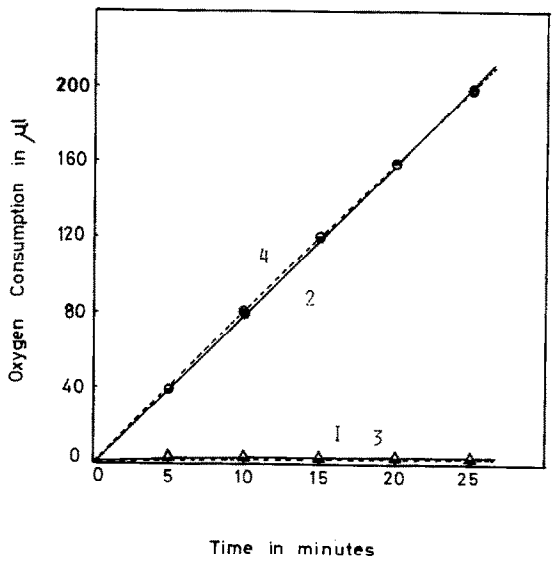

Fig.3. Inactivity of FAD-3′-pyrophosphate as cofactor or inhibitor of D-amino acid oxidase. FAD-freed apoenzyme of hog kidney D-amino acid oxidase, 0.7 unit and 0.2 ml D,L-alanine were reacted in 2.45 ml 0.05 M sodium pyrophosphate buffer, pH 8.3 containing (1) none as control, (2) 10 µl 2 mM FAD, (3) 10 µl 2 mM FAD-3′-pyrophosphate and (4) 10 µl 2 mM FAD plus 30 µl 2 mM FAD-3′-pyrophosphate, separately. The reactions were conducted in Warburg manometric apparatus thermostated at 35°C. O_2 uptake was measured at time intervals.

with ethanol (6 mg). The structural characterization of the product, FAD-3′-pyrophosphate (at adenosine moiety) was achieved by its quantitative conversion to FAD and PP_i by digestion with nuclease P1 and equimolar formation of FMN and pApp by venom exonuclease just as in the case of NAD-3′-pyrophosphate mentioned above (figures not shown). The low yield of FAD-3′-pyrophosphate may be due to various reasons: lower acceptor activity of FAD in comparison with NAD^+ [2]; higher solubility in alcohol; elevated chemical instability and photosensitivity of the 3′-pyrophosphate; and its very hygroscopic and sticky characteristics. FAD-3′-pyrophosphate thus obtained was checked manometrically for cofactor and inhibitory activities using FAD-freed apoenzyme of D-amino acid oxidase. The results were totally negative for both (fig.3) as in the case of NAD-3′-pyrophosphate.

It is interesting that these results are coincident with those in [16] with NAD monophosphate (at nicotinamide riboside-2′(3′)-site) and NAD diphosphate (at both adenosine- and nicotinamide riboside-2′(3′)-sites). These compounds were prepared from NAD^+ and *p*-nitrophenyl phosphate by the phosphotransfer action of *Proteus mirabilis* enzyme and found that these derivatives are inactive both as cofactors and as inhibitors of various kinds of NAD^+- and NADP-specific dehydrogenase systems tested. Combined together, their and our results indicate the importance of both adenosine- and nicotinamide riboside-2′(3′)-hydroxy groups in the enzyme actions.

It is not known, and therefore would be interesting to know, whether or not these 5′-pyrophosphate-driven 3′-pyrophosphorylation reactions on NAD^+, FAD and other nucleotides are actually operative in vivo and play any important role in the physiology of microorganisms and other life forms, like guanosine-3′,5′-polyphosphates in stringent control [17] and in selective gene expression [18], together with the metabolic fates of these apparently inactivated, namely, idling coenzymes and other nucleotides concerned [1,2].

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