THE ENZYMATIC OXIDATION OF ADENOSINE DIPHOSPHORIBOSE

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SUMMARY

Penicillium charlesii extracts contain UDP-galactose:NAD^+ 2-hexosyl oxidoreductase (1). ADP-ribose also serves as a substrate resulting in formation of NADH and an oxidized ADP-ribose derivative. Treatment of the oxidized product with $NaBH_4$ followed by hydrolysis at pH 2 and 100° releases xylose as well as ribose. We conclude that ADP-D-glycero-D-glycero-3-pentosulose (ADP-3-keto-ribose) is the product derived from ADP-ribose.

INTRODUCTION

Cell-free extracts of P. charlesii contain UDP-galactose:NAD^+ 2-hexosyl oxidoreductase which oxidizes UDP-galactose ($UDP\text{-}Gal_p$) to the 2-ketogalactopyranosyl ($UDP\text{-}2\text{-}keto\text{-}Gal_p$) intermediate (1). The role, if any, of this reaction in forming uridine 5'-(D-galactofuranosyl pyrophosphate)($UDP\text{-}Gal_f$) is under investigation in this laboratory. $UDP\text{-}Gal_f$ has been tentatively identified in extracts of P. charlesii (2). During the course of our investigation we noted that NAD^+ was reduced when ADP-ribose was substituted for $UDP\text{-}Gal_p$ in the reaction mixture. Kinetic studies suggest that a common oxidoreductase catalyzes the oxidation of both $UDP\text{-}Gal_p$ and ADP-ribose (3).

A ubiquitous NADase is known to catalyze the hydrolysis of NAD^+ to ADP-ribose and nicotinamide (4,5,6,7). The metabolism of ADP-ribose has not received much attention, although the synthesis of poly(ADP-ribose) from NAD^+ catalyzed by poly(ADP-ribose) synthetase from mammalian cell nuclei, has been investigated (8,9,10). Neither ADP-ribose, $NADP^+$, NADPH nor NADH serve as a substrate in the synthesis of poly(ADP-ribose). However, UDP-galactose 4'-epimerase containing bound NAD^+ reacts with ADP-ribose to form enzyme-bound NADH and ADP-ribonic acid (11,12).

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Our investigation was undertaken to characterize the product formed during oxidation of ADP-ribose catalyzed by the oxidoreductase from Penicillium extracts.

MATERIALS AND METHODS

a. Growth conditions of Penicillium and isolation of oxidoreductase. Penicillium charlesii G. Smith (ATCC 1877) was grown as a stationary culture (13) and ADP-ribose:NAD oxidoreductase was partially purified: Fungal mats (approximately 50 g) were washed with distilled H_2O, blotted dry, and were macerated at 4° in Tris-NaCl pH 8.3 buffer containing 10 mM dithiothreitol (DTT), and were fractionated up to the addition of $(NH_4)_2SO_4$ as described (1). The fraction precipitating between 55-65% saturated (at 4°) $(NH_4)_2SO_4$ was dissolved in 2-3 ml of 0.25 M Tris-HCl pH 8.3 buffer containing 0.125 M NaCl, 2 mM DTT and 1.7 mM NAD^+ and was stored at -15°. The enzyme was purified further by chromatography on Bio-Gel A-0.5m (100-200 mesh) at 4° followed by chromatography on Bio-Gel A-1.5m (100-200 mesh). The oxidoreductase was not purified additionally by chromatography on CM-Sephadex C-25 (medium), Sepharose 4B-ADP-ribose, Agarose-Hexane-NAD^+, Sepharose 4B-NAD^+, or Sepharose 4B-concanavalin A affinity columns. The enzyme preparation was stored at -15° until used.

b. Methods used in characterizing the products derived from ADP-ribose and UDP-Gal_p. A reaction mixture containing NAD^+ (0.84 mM), ADP-ribose (0.47 mM) and enzyme preparation (14 µg protein) was incubated in 0.35 M Tris-HCl pH 8.3 containing 0.175 M NaCl and 2 mM DTT in a total volume of 1.0 ml. The course of the reaction was followed at 340 nm. After 120 min 0.1 µmole of sodium pyruvate and 100 µg of lactic dehydrogenase were added and the reaction continued until 340 min with a second addition of sodium pyruvate at 220 min. Approximately 0.27 µmole of NADH was formed during the course of the reaction. A small quantity of NaB^3H_4 (specific activity 130 mCi/mmole) was added to the reaction mixture. After 20 min incubation at 25°, 5 ml of 0.05 M $NaBH_4$ was added and the mixture reacted an additional 70 min. The solution was acidified with glacial acetic acid which stopped the reaction and destroyed any unreacted $NaBH_4$. The products were fractionated by chromatography on DEAE-cellulose in the phosphate form using a gradient of phosphate concentrations as eluent (Fig. 1). The fractions were monitored by measuring the absorbance at 280 nm and the ^3H in each 5 ml fraction. This chromatographic procedure cleanly separates NADH and NAD^+ from ADP-ribose. The products eluting in the position of ADP-ribose were adjusted to pH 2 with HCl and were held i) at 96° for 10 min, or ii) 100° for 16 hr followed by neutralization of the sample. Samples were chromatographed in the descending direction on Whatman 1 MM paper prewashed with acetic acid (system A) and on Whatman (DE 81) DEAE-cellulose paper (system B) in a solvent composed of ethyl acetate:pyridine:0.005 M H_3BO_3 (3:2:1 v/v). Samples containing ^3H were located in lanes separated by a 1.9 cm spacer region to eliminate the crossover of radioactivity between lanes. Appropriate reference substances were run on all chromatograms. No heat was used in drying the samples applied to the chromatograms. Following chromatography, 2.5 cm lanes were cut from the chromatogram and each lane was sectioned into a series of 1 x 2.5 cm strips. The ^3H in each section was estimated as described later.

UDP-Gal_p (0.47 mM) was substituted for ADP-ribose and the reaction mixture was treated as described above. Treatment at pH 2 and 96° for 10 min was sufficient to release the aldose(s) from the nucleotide.

c. Analytical procedures. Aqueous samples (5-200 µl) containing radioactivity were added to 10 ml of fluid from a solution containing 110 ml of toluene, 880 ml methylcellosolve, 130 ml absolute ethanol and 11.6 g of 2,5-

diphenyloxazole. The radioactivity was quantified in a Beckman LS-230 liquid scintillation spectrometer. Radioactivity on paper chromatograms was quantified by placing 1 x 2.5 cm paper strips in 10 ml of fluid from a solution containing 1000 toluene, 6 g of 2,5-diphenyloxazole and 50 mg of 1,4-di-[2-(5-phenyloxazolyl)]benzene. Non-radioactive aldoses and alditols were visualized on paper by treating the appropriate lanes of the chromatograms with alkaline $AgNO_3$ (14). Nucleotides were visualized on paper chromatograms with the aid of a short wavelength ultraviolet lamp. Enzymic activity was routinely measured at ambient temperature using a Beckman Kintrac VII spectrophotometer attached to a 10 in recorder. Protein was quantified by an assay employing tannic acid (15) using bovine serum albumin as the reference protein. This assay is quantitative over the range of 5 to 100 μg of protein and can be used in the presence of sulfhydryl-containing substances.

d. Purification of NAD^+ and ADP-ribose. NAD^+ was purified from contaminating quantities of NADH and ADP-ribose by the procedure of Dalziel (16). When necessary ADP-ribose was purified in a similar manner.

RESULTS AND DISCUSSION

We have shown previously that incubation of UDP-Gal_p, NAD^+ and partially purified UDP-galactose:NAD^+ 2-hexosyl oxidoreductase at pH 8.3 followed by i) treating the reaction mixture with NaB^3H_4 and ii) hydrolysis in dilute acid results in the formation of [2-^{3}H]galactose. An experiment was conducted to determine if talose, the 2-epimer of galactose, was formed as a result of treating the oxidized UDP-Gal_p derivative with $NaBH_4$. UDP-Gal_p (0.47 mM), NAD^+ (0.84 mM) were incubated with the enzyme preparation with the addition of pyruvate and lactic acid dehydrogenase, followed by treatment with NaB^3H_4, fractionation on DEAE-cellulose, acid hydrolysis of appropriate fractions and paper chromatography as described in the methods section. A control containing only UDP-[U-^{14}C]galactose was treated in a similar manner. Tritium was incorporated into a substance that migrated as talose as well as into galactose (Fig. 2). These results support our previous conclusions that UDP-Gal_p is oxidized to the 2-keto derivative in a reaction in which NAD^+ is the electron acceptor.

During the course of these investigations we observed that ADP-ribose substituted for UDP-Gal_p as a substrate. We previously proposed UDP-2-keto-galactofuranoside, (uridine 5'-(D-arabino-2-hexosulopyranosyl pyrophosphate)) as a feasible intermediate in the biosynthesis of galactofuranosyl-containing substances (1). Therefore it is of interest to determine if oxidation of ADP-ribose occurs on one of its furan rings.

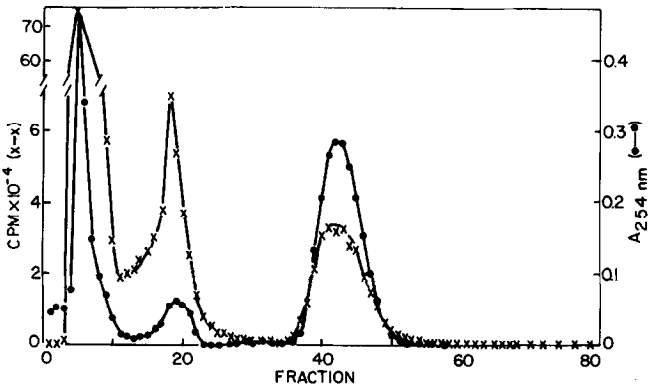

Fig. 1. DEAE-cellulose chromatography of the products of the reaction mixture following incubation of ADP-ribose and NAD^+ with the oxidoreductase. ADP-ribose and NAD^+ were incubated with the oxidoreductase, treated with NaB^3H_4, and immediately purified by DEAE-cellulose chromatography as described in the methods section. The figure shows the elution pattern obtained for ^3H (x—x) and for 254 nm absorbing substances (●—●). Reference compounds of NADH, NAD^+ and ADP-ribose elute in fractions 4-6, 14-20 and 30-44, respectively.

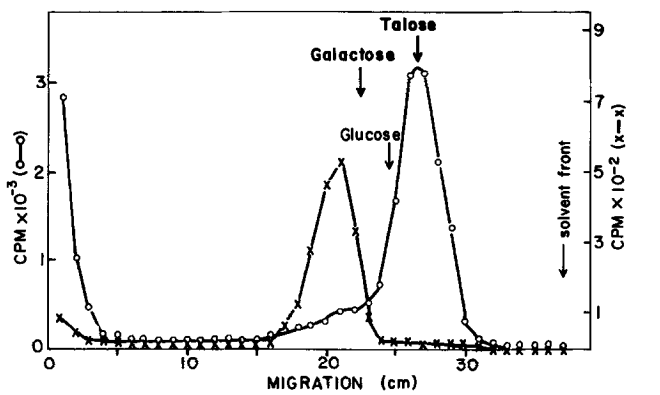

Fig. 2. Paper chromatography of a mixture of the acid hydrolysates of UDP-[U-^{14}C]-galactose and the product derived from UDP-galactose catalytically oxidized in NAD followed by reduction with NaB^3H_4. UDP-Gal_p was catalytically oxidized in NAD^+ with UDP-galactose:NAD^+ oxidoreductase. The reaction mixture was treated with NaB^3H_4 (specific activity, 130 mCi/mmole) and ^3H was incorporated into a substance which did not migrate when chromatographed in either solvent system A or B. The remainder of the reaction mixture was mixed with UDP-[U-^{14}C]-galactose and was treated at pH 2 and 96° for 10 min. The products were fractionated on paper chromatographic system A. The ^3H (●—●) and ^{14}C (x—x) in the chromatogram are plotted as a function of distance of migration. The position of reference aldoses on the chromatogram following chromatography are indicated by arrows. Mannose and glucose comigrate in this system.

Incubation of ADP-ribose (0.47 mM), NAD^+ (0.84 mM) with the partially purified oxidoreductase results in the reduction of about 10% of the NAD^+ as occurs when $UDP\text{-}Gal_p$ is a substrate. Addition of pyruvate and lactic acid dehydrogenase to the reaction mixture pulls the reaction toward the oxidized ADP-ribose derivative. The reaction was conducted for 340 min with two additions of pyruvate and lactic acid dehydrogenase as described in the methods section. Approximately 0.27 μmole of NADH was formed in 340 min. The reaction mixture was treated with NaB^3H_4 and the products were fractionated on DEAE-cellulose (Fig. 1). NAD^+ and ADP-ribose elute in fractions 14-20 and 30-44, respectively. Controls containing all components except ADP-ribose were carried out and ^3H was found in fractions 4-6 and 17-20 (not shown). The contents of fractions 40-44 from the products of the complete reaction mixture were subjected to paper chromatographic analysis. Tritium in the untreated sample and the sample treated at pH 2 for 10 min remained at the origin of the chromatogram.. Samples treated at pH 2 for 16 hr contained ^3H-labeled substances that migrate like xylose and xylitol in both chromatographic systems A and B (Fig 3a,b). An aliquot of the reaction mixture was treated for 20 min with NaB^3H_4 followed by a more extensive treatment with 0.05 M $NaBH_4$. The ratio of [^3H]-xylitol to [^3H]-ribitol was estimated (Fig. 3c) to be 10:1 and no [^3H]-aldoses were observed. A control containing only ADP-ribose was treated in a similar manner as the reaction mixture and only [^3H]-ribitol was found. In this chromatographic system aldoses migrate more rapidly than alditols. D-Arabinose and D-xylose comigrate but separate from D-ribose and D-lyxose which migrate more rapidly. Xylitol, arabinitol and ribitol are all well separated.

The oxidation of carbon 2 of the ribose moiety of ADP-ribose, followed by reduction of the carbonyl group at C-2 yields D-ribose and D-arabinose. In contrast, oxidation of carbon 3 followed by its reduction yields D-ribose and D-xylose, and carbon 4 oxidation and reduction yields D-ribose and D-lyxose. The evidence presented in this paper suggests that oxidation occurs at carbon 3. The observation that short term treatment of the ADP-ribose oxidation pro-

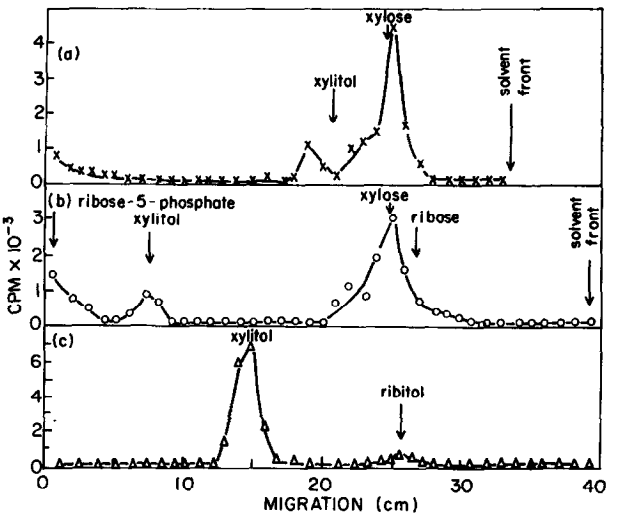

Fig. 3. Paper chromatography of products derived from ADP-ribose catalytically oxidized in NAD^+ followed by reduction with NaB^3H_4 and acid hydrolysis. ADP-ribose was incubated with NAD^+ and the enzyme preparation for 340 min as described in the methods section. Following treatment with NaB^3H_4 for 20 min the product derived from ADP-ribose was separated from NAD and NADH by chromatography on DEAE-cellulose. This product was treated at pH 2 for 16 hr at 100° and was chromatographed in either paper chromatographic system A (3a) or B (3b). In a separate experiment the reaction mixture was treated first with NaB^3H_4 for 20 min then with 0.05 M $NaBH_4$ for an additional 70 min and the products fractionated as described above using system B for paper chromatography. The position of migration of reference substances is shown in each of the panels.

duct with NaB^3H_4 followed by longer term treatment with $NaBH_4$ and hydrolysis in acid results in the formation of [^{3}H]-xylitol rather than [^{3}H]-xylose suggests that the ribose and not the ribosyl moiety of ADP-ribose is oxidized. ADP will not replace ADP-ribose as a substrate in this reaction. The disproportionate large quantity of [^{3}H]-xylitol formed over [^{3}H]-ribitol may result from a preferential approach of the tritide anion from the side opposite to the C-2 hydroxyl group of ADP-D-glycero-D-glycero-3-pentosulose (ADP-3-keto-ribose).

As mentioned above, UDP-galactose 4'-epimerase oxidizes ADP-ribose to ADP-ribonic acid (11,12). We obtained no evidence for ribonic acid in the oxidation products of ADP-ribose derived from the oxidoreductase catalyzed reaction. This provides additional evidence that the oxidoreductase is not UDP-galactose 4'-epimerase.

We note that the stereochemistry about carbons 1-3 of α-D-2-ketogalactofuranose are identical to that about carbons 4-2 or D-glycero-D-glycero-3-pentosulofuranose when the furanose ring is inverted. These results suggest that the enzyme preparation catalyzes the reversible oxidation-reduction of both pyranoside and furanoside rings. It will be of interest to determine if the oxidoreductase also catalyzes the reversible oxidation-reduction of UDP-Gal_f as well as UDP-Gal_p.

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