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PURIFICATION AND PROPERTIES OF A NUCLEOSIDE DIPHOSPHOSUGAR : NAD$^+$ 2-HEXOSYL OXIDOREDUCTASE

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Summary

Penicillium charlesii contains a nucleoside diphosphosugar : NAD 2-hexosyl oxidoreductase that oxidizes UDPgalactose (UDP-Gal$_p$), ADPribose [1,2] and UDPglucose (UDP-Glc). Dithiothreitol, NAD and 0.25 M NaCl but not nucleoside diphosphates stabilize the enzyme activity. The enzyme was purified and separated on polyacrylamide disc gels by electrophoresis into one major and eight minor bands of protein. Oxidoreductase activity was located in the major and three of the minor bands of protein. Each of these proteins catalyze the oxidation of UDPGal$_p$, ADPribose and UDP-Glc.

Introduction

Penicillium charlesii cultures produce an exocellular glycopeptide (peptidophosphogalactomannan) which contains approx. 10 galactan chains composed of 5-*O*-β-D-galactofuranosyl residues [3]. We have shown previously that extracts of *P. charlesii* contain an oxidoreductase activity which oxidizes UDP-Gal$_p$ to UDP-2-keto-Gal$_p$ [1]. More recently we showed that ADPribose also serves as a substrate resulting in the formation of NADH and ADP-3-ketoribose (ADP-D-*glycero*-D-*glycero*-3-pentosulose) [2]. Treatment of this proposed intermediate with $NaBH_4$ followed by hydrolysis at pH 2 and 100°C released xylose

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Abbreviations: UDP-Gal$_p$, uridine-5-(α-D-galactopyranosyl pyrophosphate); UDP-Gal$_f$, uridine-5′-(α-D-galactofuranosyl pyrophosphate); UDP-Glc, uridine-5′-(α-D-glucopyranosyl pyrophosphate); ADPribose, adenosine-5′-(ribose-5-pyrophosphate); ADP-3-keto-ribose, adenosine-5′-(D-*glycero*-D-*glycero*-pentosulose-5-pyrophosphate); UDP-2-keto-Gal$_p$, uridine-5′-(D-*arabino*-2-hexosulopyranosyl pyrophosphate).

as well as ribose [2]. The data provide only indirect evidence that UDP-Gal_p and ADPribose oxidation are catalyzed by the same enzyme.

The role, if any, of UDPgalactose : NAD 2-hexosyl oxidoreductase-catalyzed reaction in the formation of UDP-Gal_f is under investigation in this laboratory. This paper reports the partial purification of the enzyme, conditions for stabilizing the enzyme and its relative activity toward UDP-Gal_p, UDP-Glc and ADP-ribose.

Materials and Methods

Materials. Dithiothreitol, ADPribose, UDP-Gal_p, UDP-Glc, NAD, concanavalin A and Tris · HCl (enzyme grade) were obtained from Sigma Chemical Co., St. Louis, Mo.; $(NH_4)_2SO_4$ (ultrapure) from Schwarz/Mann, Orangeburg, N.Y.; Agarose-hexane-nicotinamide adenine dinucleotide from P and L Biochemicals Inc., Milwaukee, Wisc.; Sepharose 4B from Pharmacia Fine Chemicals, Uppsala, Sweden; all other reagents were analytical grade.

The NAD used in the enzyme purification and in enzyme assays was purified free of ADPribose [4].

Cultivation of P. charlesii. P. charlesii G. Smith has been maintained in our laboratory for a number of years. Spores were transferred to Czapek-Dox medium [5] containing 2% Difco-Bacto agar and 5% glucose. The organism was cultured in modified Raulin-Thom medium [6] at 20°C under constant light for 66 h as a stationary culture.

Preparation of nucleoside diphosphosugar : NAD 2-hexosyl oxidoreductase. Washed fungal mats (45—55 g) and Al_2O_3 (1 : 1, w/w) were macerated with a pestle with two volumes of 0.05 M Tris · HCl/0.30 M NaCl (pH 8.3)/10 mM dithiothreitol and centrifuged at 27 000 $\times g$ for 30 min. This and succeeding procedures were performed at 4°C. Protamine sulfate (2 mg/ml) was added to the supernatant and after centrifugation at 27 000 $\times g$ $(NH_4)_2SO_4$ (351.1 mg/ml) was added to supernatant and the mixture again centrifuged. $(NH_4)_2SO_4$ (66.2 mg/ml) was added to the supernatant and the pellet obtained following centrifugation was dissolved in Tris · HCl/0.125 M NaCl (pH 8.3)/2 mM dithiothreitol/1.68 mM NAD. The enzyme preparation was stable at —15°C for at least 1 month. One unit of enzyme catalyzes the formation of 1.0 μmol NADH/min.

The enzyme was purified by gel filtration chromatography on Bio-Gel A-0.5 m and Bio-Gel A-1.5 m columns with 0.25 M Tris · HCl/0.125 M NaCl (pH 8.3/0.84 mM NAD/2.85 mM dithiothreitol (flow rate 1.5—2.0 ml/min) as eluant. The active fractions were concentrated on a DiaFlo ultrafiltration apparatus with a UM-10 membrane. Samples containing 0.5—1.5 mg protein/ml were stored at —15°C and were thawed only once.

Discontinuous polyacrylamide gel electrophoresis of enzyme preparations was performed according to Davis [7] after the samples were dialyzed at 4°C for 16 h against Tris · HCl/0.46 M sucrose (pH 6.7). Samples containing up to 300 μg were applied to each gel and were electrophoresed at 0—4°C. Following electrophoresis, the gels were stained with Coomassie Brilliant Blue R [8] and were destained in 50% methanol/9% glacial acetic acid. Some of the gels were scanned at 550 nm with a Gilford Linear Transport attached to a Gilford

spectrophotometer. Other gels were sliced into 1-mm sections with a Mickle Gel slicer. Each section was incubated at 4°C for 16 h with 0.80 ml Tris · HCl/0.25 M NaCl/0.84 mM NAD and the protein eluted from the gels was assayed for oxidoreductase activity in a system containing nucleoside diphosphosugar, 0.84 mM NAD and added buffer. The enzyme preparations were fractionated also by polyacrylamide gel electrophoresis in a system in which the electrophoresis buffers contained 0.84 mM NAD. All other aspects of the procedure were as above. The electrode buffer contained 2.85 mM dithiothreitol.

Proteins eluted from polyacrylamide gels were re-electrophoresed in 0.1% sodium dodecyl sulfate (SDS) and 5 mM dithiothreitol according to Laemmli [9] after treating the preparations with 2% SDS/5% dithiothreitol for 3 min at 84°C. The gels were fixed overnight in 50% trichloroacetic acid and were stained and destained as decsribed above.

Analytical methods. Protein was quantified by a procedure which employs tannic acid [10] to avoid interference from sulfhydryl-containing substances. Bovine serum albumin was used as a reference in the protein assays.

The extent of proteolysis was estimated fluorometrically following treating homogenates with dansyl chloride [11] and dialysis.

Results

Partial purification of nucleoside diphosphosugar : NAD oxidoreductase

The enzyme preparation (200 μg) obtained following the second addition of $(NH_4)_2SO_4$ was subjected to polyacrylamide gel electrophoresis. Approx. 50 bands of protein were observed. Gel filtration chromatography through Bio-Gel A-0.5 m and A-1.5 m columns effected considerable additional purification although oxidoreductase activity was eluted in many fractions. This apparent heterogeneity may have resulted from partial proteolysis of the native enzyme. The relative extent of proteolysis during 24 h at 0°C was determined by measuring the increase in dansyl-amino acyl residues [11]. A 100 μl sample of a fresh homogenate contained 4.1 fluorescent units (activation 350 nm; emission 510 nm), and a similar sample removed and derivatized after 24 h at 0°C contained 3.9 fluorescent units. These data show that little proteolysis occurred.

Further purification of the enzyme was attempted on various media (CM-Sephadex C-25, DEAE-cellulose, agarose-hexane-nicotinamide adenine dinucleotide, concanavalin A-Sepharose 4B, blue dextran-Sepharose 4B, peptidophosphogalactomannan-Sepharose 4B and ADPribose-Sepharose 4B). Enzyme activity was either not adsorbed or could not be removed from the adsorbant.

Stabilization of enzyme activity

Stability of enzyme activity was measured as a function of time of incubation in the presence of NAD, UDP-Gal_p or ADPribose (Fig. 1) and only NAD protected against inactivation at 25°C. Enzyme activity decreased when either UDP-Gal_p or ADPribose was substituted for NAD. The influence of NAD concentration on enzyme activity after storage for 84 h at 4°C (pH 8.3) shows that maximum stabilization occurs above 0.8 mM NAD (Fig. 2). The shape of the activity versus NAD concentration curve suggests that more than 1 mol of NAD

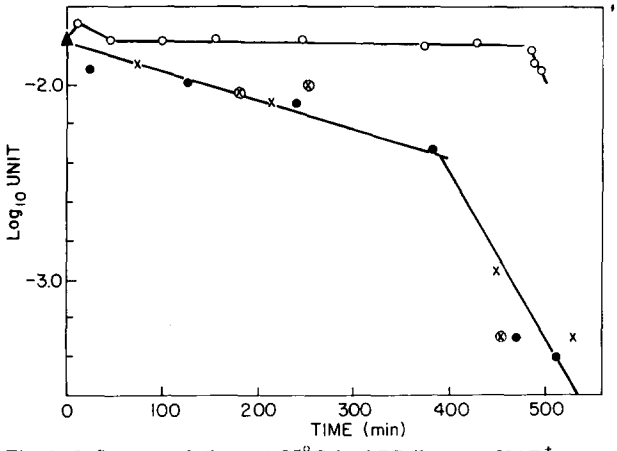


Fig. 1. Influence of time at 25°C in ADPribose or NAD^+ on nucleoside diphosphosugar oxidoreductase activity. Protein (17 μg) precipitated by $(NH_4)_2SO_4$ was held the indicated time at 25°C in 0.5 M Tris · HCl/0.25 M NaCl (pH 8.3)/2.85 mM dithiothreitol and either 5 mM sodium phosphate (⊗———⊗), 0.47 mM ADPribose (○———○), 0.84 mM NAD^+ (●———●), or no additions (X———X).At the end of the incubation period the standard assay was carried out. The initial activity of the preparation prior to incubation is shown (△).

is bound per mol of enzyme. Nearly 75% of the enzyme activity was lost during storage for 84 h in 0.84 mM NAD.

Examination of enzyme activity in dithiothreitol, Tris · HCl and NaCl showed that 2 mM dithiothreitol and ionic strength of 0.2—0.5 M stabilized the enzyme.

Polyacrylamide gel electrophoresis

The homogeneity of the enzyme preparation obtained from Bio-Gel A-1.5 m was examined by polyacrylamide gel electrophoresis. Omitting NAD from the

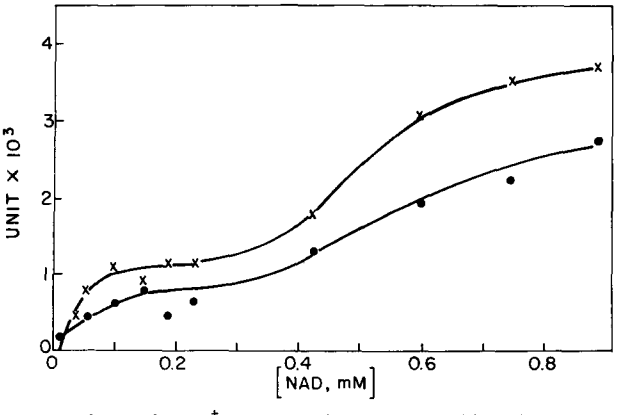


Fig. 2. Effect of NAD^+ concentration of the stability of nucleoside diphosphosugar 2-hexosyl oxidoreductase activity. Protein (35 μg) from Bio-Gel A-1.5 m was incubated for 84 h at 4°C in 0.35 M Tris · HCl/0.17 M NaCl (pH 8.3)/2 mM dithiothreitol and the indicated concentration of NAD^+. Enzymic activity was measured in a reaction mixture containing 0.84 mM NAD^+ and either 0.98 mM UDP-Gal_p (X———X) or 0.47 mM ADPribose (○———○). Prior to incubation for 84 h the enzyme contained about 0.015 units of activity.

electrophoresis buffer system resulted in the smearing of proteins in two areas of the gel (not shown) which is in contrast to the resolution of proteins into one major band and eight minor bands in the system containing 0.84 mM NAD The distribution of protein on the gels, with NAD in the buffers, was independent of protein concentration (Fig. 3). The major band of protein (band B) contained about 75% of the protein added to the gel. The protein in band B was eluted from the gel and again electrophoresed to determined if the distribution of protein resulted from dissociation of a major protein into several species. There was only a trace of band D protein and band B protein which indicates minimal dissociation occurred.

In separate experiments the enzyme preparation was fractionated by electrophoresis in NAD as described above and the gels were sliced and examined for enzyme activity. Oxidoreductase activity was observed in bands A, B, C and D (Figs. 4a—4c) using ADPribose, UDP-Gal_p and UDP-Glc_p, respectively, as electron donor. Band D contained the largest specific activity and the greatest total activity. From 40 to 80% of the enzyme activity applied to the gel was recovered. Omitting NAD from the buffers resulted in complete loss of oxidoreductase activity.

The enzyme is stabilized partially by 0.15 mM NAD (Fig. 2). The preparation was fractionated by polyacrylamide gel electrophoresis in 0.15 mM NAD. The distribution of protein along the gel (not shown) was essentially as in Fig. 4, and four bands of protein (A, B, C and D) containing oxidoreductase activity with each of the three electron donors was obtained (not shown). Band D contained the greatest activity. However, only 17% of the oxidoreductase activity was recovered in this system.

Proteins extracted from bands B and D were denatured in SDS and treated with dithiothreitol to reduce dithiol cross linkages. Proteins were electrophoresed in a polyacrylamide system [9] in which they migrate according to their molecular weight; the smallest proteins migrate most rapidly. Proteins from

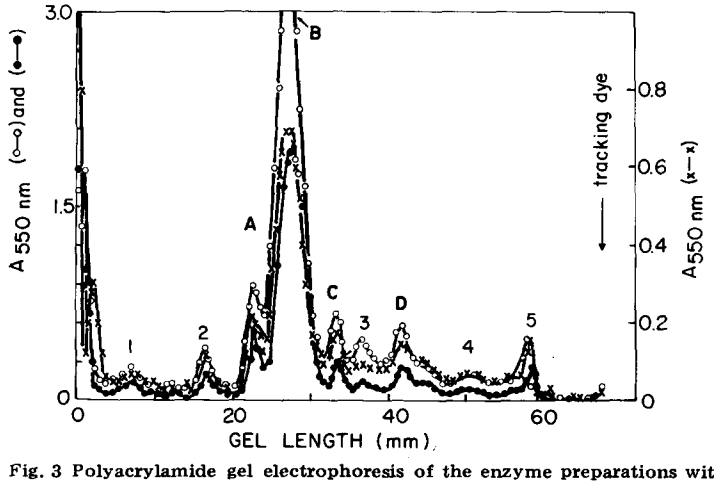


Fig. 3 Polyacrylamide gel electrophoresis of the enzyme preparations with NAD^+ in the electrophoresis buffers. Enzyme preparation from Bio-Gel A-1.5 m (220 μg, o———o; 150 μg, o———o; 50 μg, x———x) was fractionated by polyacrylamide gel electrophoresis with 0.84 mM NAD^+ in the buffer. The gels were stained with Coomassie blue, destained, and the absorbance at 550 nm recorded as the gels were scanned.

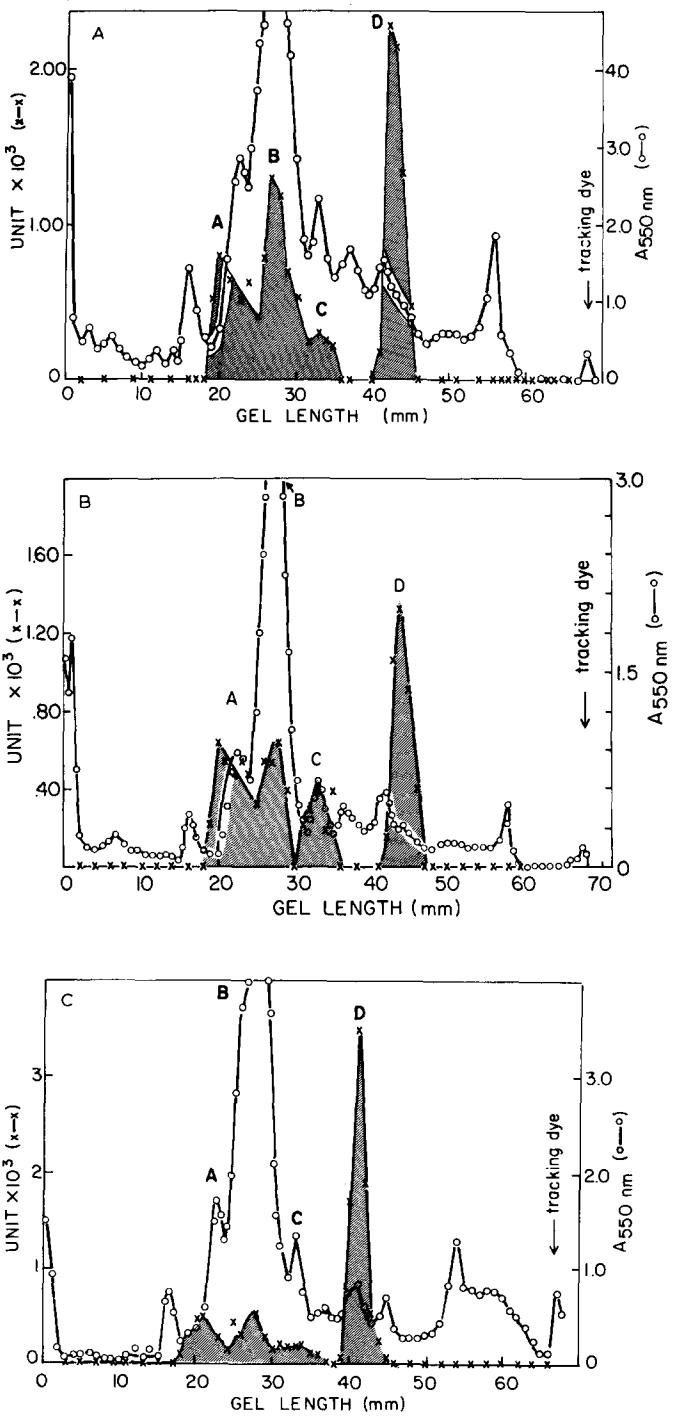


Fig. 4. Fractionation by polyacrylamide gel electrophoresis of the enzyme preparation. Six samples of enzyme preparation from Biol-Gel A-1.5 m were each fractionated in the system described in Fig. 3, in a series of three experiments. One gel was stained for protein and the other gel was cut into 1-mm sections and each section was assayed for oxidoreductase activity with 0.84 mM NAD^+ and (a) 2.35 mM ADP-ribose, (b) 1 mM UDP-Gal_p, or (c) 1 mM UDP-Glc.

both bands B and D contained two relatively large polypeptides (L_1 and L_2) (Fig. 5). Band B also contained a small polypeptide (S). The data suggest that protein D is composed of L_1 and L_2 and that protein B is composed of L_1, L_2 and S.

Kinetic properties of nucleoside diphosphosugar : NAD 2-hexosyl oxidoreductase

Kinetic investigations were conducted with oxidoreductase from Bio-Gel A-1.5 m and proteins from band B or D. Double reciprocal initial velocity and NAD concentration plots were linear with a fixed concentration of either UDP-Gal_p (0.47 mM) or ADPribose (0.47 mM) (not shown). Half maximal velocity was obtained with approx. 0.03 mM NAD. In contrast, concave downward double reciprocal plots were obtained with variable concentrations of UDP-Gal_p or ADPribose (Fig. 6). The non-linear kinetics may result from multiple species of nucleoside diphosphosugar oxidoreductase each with different kinetic parameters. Therefore, the influence of UDP-Gal_p concentration on the initial velocity of the reaction was determined using protein(s) from band D. The double reciprocal plot was curvilinear (not shown) similar to that shown in Fig. 6.

The influence of preincubating the enzyme in 0.15 mM NAD on the kinetic properties of the oxidoreductase was examined. Enzyme preparation from Bio-Gel A-1.5 m stored in 0.15 mM NAD was assayed in 0.84 mM NAD and variable concentrations of UDP-Gal_p and ADPribose. The double reciprocal plots were linear (not shown) with either UDP-Gal_p or ADPribose as variable substrate. These data suggest that the non-linearity described above results from

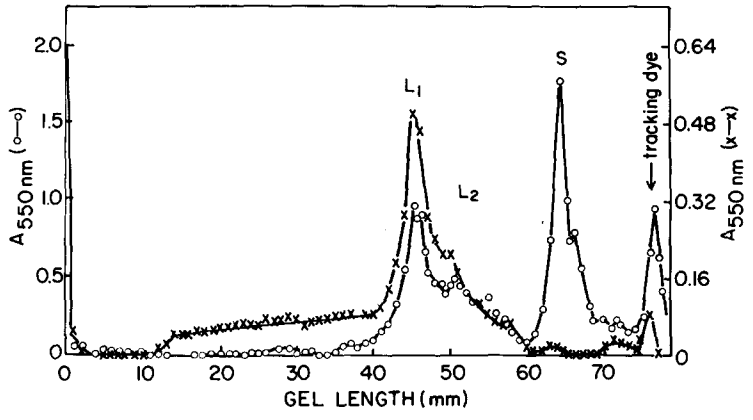


Fig. 5. Polyacrylamide gel electrophoresis of proteins B and D in a system containing SDS. Enzyme preparation from Bio-Gel A-1.5 m was fractionated as described in Fig. 3. The gels were sliced and proteins in bands B, C and D were eluted with buffered NAD^+, the eluates were pooled and concentrated. The small molecular weight substances were removed by dialysis against spacer buffer containing sucrose and each sample was held for 3 min at 84°C in 2% dodecyl sulfate and 5% dithiothreitol. The samples were electrophoresed in as system containing 0.1% SDS according to the modification of the Laemmli procedure [9]. Following electrophoresis the gels were stained and destained and the absorbance at 550 nm was scanned over the length of the gel. The Coomassie Blue-staining pattern for protein from B (o———o) and D (x———x) are shown.

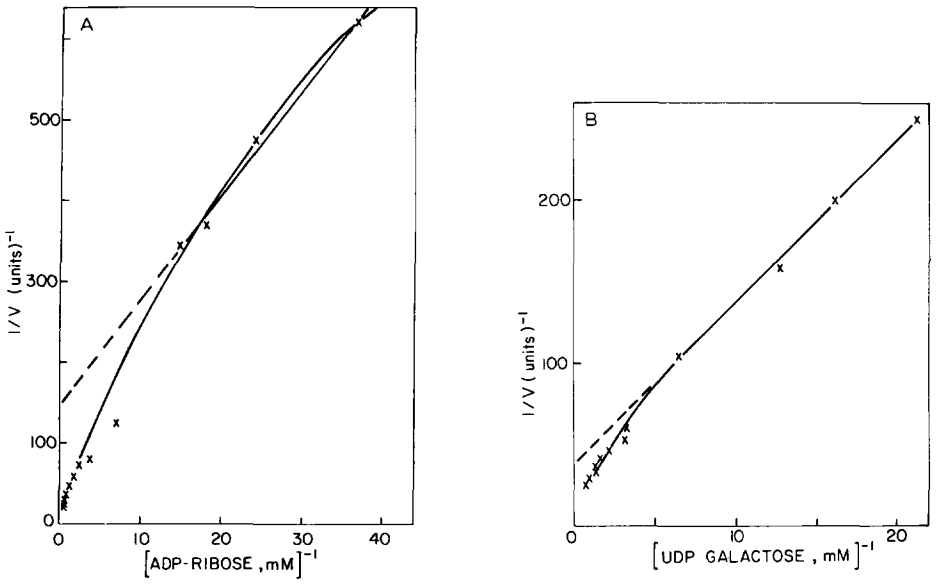


Fig. 6. Initial velocity of nucleoside diphosphosugar 2-hexosyl oxidoreductase as a function of (a) ADP-ribose concentration, and (b) UDP-gal$_p$ concentration. Enzyme preparation (33 μg, specific activity 0.34) from Bio-Gel A-1.5 m was assayed with 0.84 mM NAD^+ in Tris · HCl/0.25 M NaCl (pH 8.3)/2 mM 2-mercaptoethanol. The concentrations of ADPribose were from 0.027 to 6.1 mM, and that for UDP-Gal$_p$ were from 0.027 to 1.53 mM. All data points represent the average of two assays.

interaction between the oxidoreductase and NAD during storage and is not readily reversed by additional NAD.

NADH was shown to be potent competitive inhibitor of NAD and an apparent K_i for NADH of 3 μM was obtained. This value may not reflect the K_i for NADH as all enzyme preparations contained chromophoric material which was tightly bound to protein. The spectrum of this chromophore was essentially identical to that of NADH (not shown).

Discussion

A nucleotide diphosphosugar : NAD 2-hexosyl oxidoreductase that oxidizes the galactopyranosyl residue of UDP-Gal$_p$ at C(2) [1] or the ribose residue of ADPribose at C(3) [2] was partially purified and some of its properties were examined. This is a minor oxidoreductase in *P. charlesii* and as a result it is not possible to obtain a reliable estimate of its activity in crude homogenates due to (1) the occurrence of large concentrations of substrates which donate electrons to NAD, and (2) the occurrence of NAD glycohydrolase which degrades NAD to ADPribose and nicotinamide. The lability of the enzyme precludes removal of the substrates by dialysis or chromatography. Thus, the addition of NAD to the homogenates containing active NAD glycohydrolase provides both substrates for the oxidoreductase. A dependence of enzyme activity on added electron donor cannot be demonstrated prior to fractionation with $(NH_4)_2SO_4$. It was not possible to obtain active oxidoreductase preparations from DEAE-

cellulose without added NAD and the enzyme did not bind to the column with NAD in the buffer. In addition, the enzyme did not bind to CM-Sephadex C-25, nor did it bind to any of a series of potential affinity adsorbents.

Polyacrylamide gel electrophoresis with NAD in the buffers resolved the proteins in the enzyme preparation into one major band (band B) and eight minor bands. Protein from the major band and each of three minor ones (bands A, C and D) each catalyzed the reduction of NAD with either UDP-Gal_p, UDP-Glc or ADPribose as electron donor. Protein from band D contained the greatest oxidoreductase activity and was of the greatest specific activity. Protein from band B, denatured and reduced with dithiothreitol in SDS, contained one small and two large polypeptides. Protein from band D contained only the large polypeptides. Re-electrophoresis of proteins from band B on polyacrylamide gels resulted in the release of a very small quantity of protein that migrated like protein from band D. These results suggest that the small polypeptide from band B is physically complexed with the large polypeptides and that the small polypeptide is not necessary for enzymic activity.

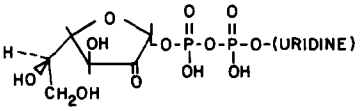
Although the data suggest a physical association between the polypeptides, it could also be interpreted as evidence for partial proteolysis resulting in multiple oxidoreductase species derived from the native enzyme. Storage of crude homogenates for 24 h at 0°C (pH 8.3) did not result in an increase in N-terminal amino acyl residues. Thus, it is unlikely that the multiple oxidoreductase species resulted from proteolysis as all operations were carried out at 0—4°C and the $(NH_4)_2SO_4$ fractionation was carried out within 4 h of macerating the mycelia.

Preliminary kinetic investigations were conducted on oxidoreductase from Bio-Gel A-1.5 m and that from band D to determine if the kinetics of the more highly purified preparation (band D) was similar to that obtained from Bio-Gel. The oxidoreductase activity of both protein preparations showed negative cooperativity [12] using 0.84 mM NAD and variable UDP-Gal_p concentrations. In contrast, no modulation was observed when the enzyme was stored in 0.15 mM NAD and assayed in the usual fashion. The evidence suggests that the interaction between oxidoreductase and NAD during storage determines some of the kinetic properties of the enzyme, and that the small polypeptide is of little or no importance in this interaction.

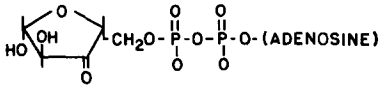
In addition to the kinetic evidence the complex relationship (Fig. 2) between enzyme stability and NAD^+ concentration also suggest that NAD binds to the enzyme in some fashion to increase its stability. Thus, the stability of the enzyme appears to be poised by the concentrations of NAD, dithiol-reducing agents and by the ionic strength of the medium. The importance of this balance is not understood currently.

The enzymically active protein(s) oxidize either galactopyranosyl, glucopyranosyl or ribofuranosyl moieties. The data show that the oxidoreductase does not show a large degree of specificity toward either the sugar or the base of the nucleoside diphosphosugars tested; that is UDP-Gal_p, UDP-Glc_p and ADPribose all served as electron donors. However, it is of particular interest to note that the structure of the 2-keto-galactofuranosyl moiety, a proposed intermediate in the conversion of galactopyranosyl to galactofuranosyl residues [1], and that of 3-keto-arabinofuranose moiety of the nucleoside diphosphosugars have strik-

ing similarities (Fig. 7). The 3-keto-arabinofuranose (3-keto-ribofuranose) is the



Uridine 5'-(2-ketogalactofuranosyl pyrophosphate)



Adenosine 5'-diphospho-3-ketoribofuranose

Fig. 7.

oxidation product of ADPribose. The observation that the oxidoreductase from the D protein band oxidizes either the galactopyranosyl or the ribofuranose rings is suggestive that this enzyme functions in the conversion of galactopyranosyl to galactofuranosyl moieties as intermediates. The activity of the most highly purified preparations using UDP-Glc as the electron donor is unexplained. Currently the position of oxidation of UDP-GLc is unknown.

Acknowledgments

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