Purification and Characterization of NAD:Penicillamine ADP Transferase from Bacillus sphaericus

A NOVEL NAD-DEPENDENT ENZYME CATALYZING PHOSPHORAMIDE BOND FORMATION*

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A strain of Bacillus sphaericus isolated from a local soil sample has been found to use β,β-dimethyl-DL-cysteine (Dl-penicillamine) as the sole nitrogen source. Crude cell extract of the bacterium showed potent penicillamine-consuming activity only in the presence of NAD, which, however, was not used as an electron acceptor. Characterization of reaction products revealed that penicillamine was derivatized to a phosphoramidate adduct with the ADP moiety of NAD, whereas the nicotinamide-ribose group was released and hydrolyzed spontaneously to ribose and nicotinamide. The phosphoramidate product, ADP-penicillamine, caused potent product inhibition on the purified enzyme, and adenylic deaminase was found to be effective in converting the inhibitory product into inosine-diphosphate-penicillamine and thereby maintained the catalysis for several hours. The novel enzyme, termed as NAD:penicillamine ADP transferase, showed a single band on SDS-polyacrylamide gel electrophoresis with a mass of approximately 42 kDa. The native enzyme was monomeric. The enzyme showed high substrate specificity to NAD (Km = 13.0 mM) and L-penicillamine (Km = 6.5 mM); other nucleotides such as NADP, NAD(P)H, AMP, ADP, and ADP-ribose did not substitute for NAD, and l-valine, l-cysteine, l-homocysteine, l-cystine, l-leucine, and l-isoleucine did not serve as the substrate. Kinetic studies suggested an Ordered Bi Bi mechanism, with NAD as the first substrate to bind and ADP-l-penicillamine as the last product released. The novel NAD-dependent enzyme may catalyze the first step in penicillamine degradation in the strain of B. sphaericus.

Penicillamine, β,β-dimethyl-cysteine, is one of the most unusual amino acids because it is not catabolized by pyridoxal-5'-phosphate (PLP)-dependent enzymes. Although penicillamine has the obvious structural similarities to cysteine and valine, none of the enzymes that degrade Cys or Val can act on penicillamine. In fact, l-penicillamine can specifically inhibit various PLP-dependent enzymes, whose cofactor is compelled to form a thiadiazoline ring and is no longer bound on the enzyme (1–4). The driving force for this modification may derive from the unique structure of penicillamine, which bears a hard nucleophile (-NH₂) and a soft nucleophile (-SH) on the vicinal carbons. The β,β-dimethyl group may also help orienting the amino and thiol groups to the same direction and by that promote the thiazolidine ring formation. D-Penicillamine, in contrast to the l-isomer, had been considered not to inhibit PLP-dependent enzymes (5), but later it was shown that the d-isomer also causes PLP depletion in rat (6, 7). Myeloperoxidase is another class of enzyme that d-penicillamine can specifically bind and abolish its catalytic functions (8, 9). Despite these inhibitory effects, d-penicillamine has been extensively used in the treatment of Wilson's disease (10) and in cases of lead poisoning (11). The curative property is partly due to the ability of d-penicillamine to bind copper or lead as a stable chelate that is filterable by the kidney and more importantly due to the metabolic stability by which penicillamine is not degraded until excreted in urine. In fact, penicillamine has originally been found in urine specimens of penicillin-treated patients, suggesting that the β-lactam antibiotic was not degraded beyond penicillamine (12). Besides these effects on enzymes and metals, penicillamine can exert various biological effects such as decreasing chemotaxis of polymorphonuclear leukocytes (13), inhibiting DNA synthesis in T lymphocytes (14), and inhibiting transactivation of human immunodeficiency virus type 1 long terminal repeat (15). Mechanisms underneath these biological effects are not fully understood yet.

We here report purification and characterization of a novel enzyme involved in penicillamine degradation in a strain of Bacillus sphaericus that can grow on Dl-penicillamine as the sole nitrogen source. The enzyme, specifically induced when the bacterial cells were incubated with D- or L-penicillamine, used NAD and penicillamine as the sole substrates. The enzyme activity has been assayed by NAD-dependent penicillamine consumption. The enzyme was purified to the homogeneity using nucleotide affinity ligands and characterized as a 42-kDa monomeric protein. The catalysis was irreversible, and penicillamine was derivatized to the phosphoramidate adduct, ADP-penicillamine (Scheme 1). The phosphoramidate product caused potent product inhibition, and a commercially available Aspergillus adenylic deaminase was effective in removing the inhibitory product. In the adenylic deaminase-coupled system, inosine-diphosphate-penicillamine (IDP-penicillamine) was identified as the deaminated form of the product. Kinetic studies on substrate binding interaction and the product inhibition by ADP-l-penicillamine suggested an Ordered Bi Bi mechanism with NAD as the first substrate to bind and ADP-l-penicillamine as the last product to be released. The enzyme, NAD:penicillamine ADP transferase, showed relaxed stereospecificity with respect to D- and L-penicillamine, and the l-isomer was a better substrate than the d-isomer. The stereospecificity preference in the enzyme reaction was consistent.

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§ The abbreviations used are: PLP, pyridoxal 5'-phosphate; NAD, nicotinamide adenine dinucleotide; HPLC, high performance liquid chromatography; IDP, inosine-diphosphate.
with the observation that the cells of B. sphaericus consumed L-penicillamine more rapidly than the d-isomer. The NAD-dependent modification catalysis may be the first step in the penicillamine degradation pathway in the strain of B. sphaericus.

**Experimental Procedures**

**General**—Ultraviolet and visible spectra were measured on Beckman DU-65 spectrophotometer, and molecular mass was determined by a quadrupole ion spray mass spectrometer API III (Perkin-Elmer, Canada). Protein was determined by the method of Bradford using bovine serum albumin as the standard (16). 1H and 31P NMR spectra were obtained at 500 and 80.9 MHz using Varian VXR-500 and VXR-200 spectrometers, respectively, with a Fourier transform accessory at ambient temperature.

**Screening of Bacteria**—Screening for penicillamine-catabolizing bacterium has been carried out by the enrichment culture on a medium containing 0.1% (w/v) DL-penicillamine, 0.5% glucose, 0.1% KH2PO4, 0.1% K2HPO4, 0.1% NaCl, 0.05% MgSO4·7H2O, and 0.05% yeast extract, pH 7.5. Microorganisms were grown at 30°C under aerobic conditions, and a bacterium has been isolated as a pale yellow colony formed on the medium containing 1.3% (w/v) agar.

**Enzyme Assays**—Enzyme activity was assayed by measuring the decrease of penicillamine (method 1) or NAD (method 2). In method 1, the reaction was initiated by adding 10 μl of enzyme solution to 1 ml of the assay mixture containing 30 mM L-penicillamine, 30 mM NAD, and 30 mM-Tris-HCl buffer, pH 7.5. After incubation at 37°C for 15 min, the reaction was stopped by adding 100 μl of 25% (w/v) trichloroacetic acid. Then an aliquot of 2 μl was spotted on 3MM chromatography paper (Whatman) and developed with butanol/acetic acid/28% ammonium/water (10:10:5:2). Penicillamine was visualized by spraying 0.5% ninhydrin in 50% aqueous acetic acid, and the paper was heated at 110°C for 10 min. The spot of penicillamine was cut out, extracted in 0.5 ml of 75% methanol containing 0.05% (w/v) CuSO4, and determined spectrophotometrically at 510 nm. In method 2, the amount of NAD remaining in the reaction mixture was determined by the method of Nussrallar et al. (17). The sample was diluted to contain less than 0.3 mM of NAD, and the 100-μl aliquot was mixed with 100 μl of ethanol and 700 μl of 0.1 M glycine/NaOH buffer, pH 10.0. Equine liver alcohol dehydrogenase (0.1 units/100 μl) was added to the solution, and the absorbance at 340 nm was monitored for 3 min. The slope of the time-dependent increase in the absorbance was used to estimate the concentration of NAD.

**Preparation of Crude Cell Extract—B. sphaericus** was grown on 4 liters of Luria-Bertani broth for 16 h at 37°C and then inoculated to 30 liters of the same medium in a 40-liter vessel fermentor equipped with a mechanical stirrer. Cells were grown under aerobic conditions at 37°C until an early stationary phase, harvested by centrifugation at 7,000 × g for 15 min and then washed twice with cold 20 mM Tris-HCl buffer, pH 7.5. The cells (wet weight, 120 g) were suspended in 2 liters of the Tris buffer, pH 7.5, and incubated at 30°C for 6 h, and then L-penicillamine (1% w/v) was added and incubated for another 6 h. Cells were suspended in 500 ml of the same buffer containing phenylmethylsulfonyl fluoride (0.1 mM) and disrupted by sonication for 20 min, and cell debris was removed by centrifugation. Ammonium sulfate was added to 80% of the saturation, and precipitates were dissolved in the buffer (50 mM, pH 7.5) and dialyzed twice against 2 liters of the same buffer. Insoluble materials were removed by centrifugation, and the supernatant solution was used as the crude cell extract of B. sphaericus.

**Enzyme Purification**—Enzyme purification was carried out at under 4°C, and 20 mM Tris-HCl buffer, pH 7.5, containing 0.01% 2-mercaptoethanol (buffer A) was used throughout the procedure. The crude cell extract was applied to DEAE Toyopearl 650 M column (50 × 30 cm) equilibrated with buffer A. After washing the column extensively with buffer A, the enzyme activity was eluted with a 0–0.5 M KCl linear gradient in the buffer. Active fractions were concentrated on Amicon YM-10 membrane and applied to a DyeMatrex Blue C column (15 mm × 14 cm), which was equilibrated with buffer A. The column was washed with 2 volumes of 1 mM NADH and 1 mM NAD in the same buffer, and the enzyme was eluted with a linear gradient of KCl (0–0.5 M) contained in buffer A. Active fractions were combined and concentrated on Centricut-mini (Kurabou, Japan). The enzyme was absorbed on the DyeMatrex Orange A column equilibrated with buffer A. The column was washed with 1 mM of NADH and 1 mM of NAD in the buffer, and the enzyme was eluted with a 0–0.5 M KCl linear gradient. Active fractions were concentrated, and applied to TSK-GEL 3000SW column (7.5 × 600 mm, TOOSOH, Japan) equilibrated with buffer A containing 0.1 mM KCl, and the active fractions were combined and concentrated on Centricut-mini.

**SDS-Polyacrylamide Gel Electrophoresis and Gel Filtration**—Molecular mass of the enzyme was determined by SDS-polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli (18). Purified enzyme was applied to a TSK-GEL 3000SW column in 0.1 M sodium phosphate buffer, pH 7.5, containing 0.1 M sodium sulfate to estimate the native molecular size. The following molecular markers were used to calibrate the column: myoglobin (17,000), chicken ovalbumin (44,000), bovine gamma globulin (158,000), and bovine thyroglobulin (670,000).

**Analytical HPLC—Substrate disappearance and product formation were monitored on reverse-phase high performance liquid chromatography (HPLC).** After protein was removed from the reaction mixture (1 ml) by adding 250 μl of 25% trichloroacetic acid and subsequent centrifugation, a 20-μl aliquot of the sample was injected onto reverse-phase HPLC system consisting of Waters 600E system controller, an on-line degasser, and Waters 484 Tunable Absorbance Detector. Reverse-phase separation was achieved on μBondapak C18 column (19 mm × 15 cm) using a linear gradient of 0–5% acetonitrile in 50 mM sodium acetate buffer, pH 3.5, at a flow rate of 5.0 ml/min. Eluate was monitored by the absorbance at 260 nm.

**Purification of Enzyme Reaction Product—A reaction mixture (5 ml, pH 7.5) containing 30 mM NAD, 30 mM L-penicillamine, adenylyl deaminase (10 units; Sigma), and the purified enzyme (10 units) was incubated at 37°C for 6 h. Enzyme reaction product has been purified by four successive preparative HPLC systems. 1) Preparative HPLC was performed as described elsewhere “Analytical HPLC.” 2) HPLC employed Superdex Peptide HR column (10 × 300 mm) eluted with deionized water at a flow rate of 0.25 ml/min. 3) Preparative HPLC was again performed on μBondapak C18 column with 0–35% linear gradient of methanol in deionized water at a flow rate of 2.5 ml/min. 4) Ion exchange HPLC on a TSK-GEL DEAE-5PW column (7.5 × 75 mm) has been performed with a linear gradient of 10–300 mM NaH4CO3 buffer (pH 8.2). Eluate was monitored at 280 nm, and concentrated by lyophilization at each step of the preparative HPLC.

**Chemical Synthesis of ADP-L-Penicillamine—ADP-L-penicillamine has been chemically synthesized by the method described by Rossomondo et al. (20) with some modifications, and 31P NMR spectra of reaction mixture were obtained at each step of the synthesis.** To the aqueous solution of ADP (5 mmol/8 ml), which was adjusted to pH 8 with triethylamine, 1-ethyl-3-(3dimethylaminopropyl)-carbodiimide hydrochloride (500 mmol/2 ml water) was added dropwise and stirred at room temperature for 30 min. An aqueous solution of L-penicillamine (50 mmol/10 ml, pH 8, with triethylamine) was added to the mixture, and the solution was incubated at 50°C for 2 h. The reaction mixture was passed through a column (0.7 × 5 cm) of activated charcoal (Wako, Japan), and the column was thoroughly washed with deionized water. ADP-L-penicillamine was eluted with 50% aqueous ethanol, and was further purified by the preparative HPLC described above in 4). Purity of the synthetic ADP-t-penicillamine was verified by analytical reverse-phase HPLC.

**Kinetic Studies—Kinetic properties of NAD-penicillamine ADP transferase were examined using the purified enzyme, and the activity was assayed by method 1.** For kinetic studies, adenylyl deaminase was not included in the assay mixture. Initial velocities of penicillamine disappearance were measured during the first 15 min, where we observed linear time-dependent substrate disappearance. Data were obtained by varying the concentration of one substrate with the fixed concentrations of the other, and presented as double-reciprocal plots as initial velocity versus varied substrate concentrations. Product inhibition studies were performed by including chemically synthesized ADP-t-penicillamine in the assay mixture at several fixed concentrations and varying the concentration of either t-penicillamine or NAD in the presence of fixed concentration of the other substrate (30 mM t-penicillamine or 30 mM NAD). Double-reciprocal plots using initial velocity versus varied substrate concentrations were used to determine the mode of inhibition (21).
NAD:Penicillamine ADP Transferase

RESULTS

Penicillamine-catabolizing Bacterium—A penicillamine-catabolizing bacterium has been isolated from a local soil sample collected in our university campus. The bacterium was identified as a strain of *B. sphaericus* based on the following biological characteristics: the microorganism was a Gram-positive, spherical spore forming, catalase positive bacterium, and it can grow on media at pH 6.0 but not on media containing 10% (w/v) NaCl. Starch was not used as a carbon source.

Enzyme Induction—The bacterium did not show the ability to consume DL-penicillamine when grown on Luria-Bertani broth. The enzyme activity was most effectively induced when the cells were incubated in 20 mM Tris-HCl buffer, pH 7.5, at 30 °C for 6 h and then further incubated in the presence of 0.1% (w/v) DL-penicillamine for another 6 h. Cells treated with 0.1% DL-penicillamine consumed L-penicillamine at the rate of 0.3 μmol/min/g of wet cells at 30 °C. The rate of D-penicillamine uptake was less than 0.1 μmol/min under the same conditions. The enzyme was induced by D- or L-penicillamine, and in either case cells consumed L-penicillamine more rapidly than the D-isomer. L-Valine, L-cysteine, L-cystine, L-homocysteine, L-leucine, and L-isoleucine did not induce the activity in the bacterial cells.

Penicillamine Consumption in Crude Cell Extract—Penicillamine-consuming activity in the cell extract has diminished when small molecules were removed by dialysis. To identify the molecules required for the activity, we have incubated the cell extract with 30 mM NAD, NADP, NADPH, PLP, FAD, and FMN, respectively, with 30 mM DL-penicillamine, and penicillamine disappearance was measured by ninhydrin. Among the compounds tested, only NAD promoted the penicillamine-consuming activity in the cell extract. Penicillamine and 30 mM NAD at pH 7.5, and the decrease of *B. sphaericus* cell extract of deaminase (10 units) was incubated with 30 mM DL-penicillamine and 30 mM NAD at pH 7.5, and the decrease of amino group (Fig. 1A, inset). Despite the competent enzyme activity induced in the cell extract, our preliminary attempts to identify the enzyme reaction products were not successful due to the small yield of products. It seemed likely that crude cell extract of *B. sphaericus* contained enzymes for the succeeding pathway, and therefore the products were not accumulated.

Enzyme Purification and Characterization—The NAD-dependent penicillamine-consuming enzyme was purified to the homogeneity with a yield of 7.7% in the four-step procedure summarized in Table I. Nucleotide affinity ligands, DyeMatrex Blue B and DyeMatrex Orange A were efficient means for this enzyme. Other affinity ligands such as DyeMatrex Blue A, Red A, and Green A were not effective for this enzyme. The purified enzyme showed the specific activity of 850 units/mg of protein. Gel filtration chromatography on TSK-GEL 3000SW column was used to estimate the molecular weight of the native enzyme as 42,000, and SDS-polyacrylamide gel electrophoresis analysis suggested that the denatured protein has the molecular mass of 42 kDa (Fig. 2). The purified enzyme did not have chromophore groups detectable in the ultraviolet and visible regions. The enzyme showed high substrate specificity to penicillamine and NAD. Analogous amino acids such as L-valine, L-cysteine, L-cystine, L-homocysteine, L-leucine, and L-isoleucine did not serve as a substrate. D-Penicillamine was a poor substrate with the *Km* value of 200 mM. Nucleotides such as NADH, NADP, NADPH, ATP, ADP, AMP, ADP-ribose, nicotinamide mononucleotide, and nicotinamide did not substitute for NAD. Nucleotide triphosphates such as GTP, CTP, and TTP were also inert as the substrate. The effect of pH was examined using 50 mM potassium phosphate buffer (pH 5.5–7.5) and 50 mM Tris-HCl buffer (pH 7.0–8.5). The optimum pH was in a narrow range around 7.5; at pH 6.5 and 8.0 the activity decreased to 25 and 40% of the optimum activity, respectively. Although the choice of buffer was not as significant as the pH values, the activity with the phosphate buffer was about 85% of the activity with Tris buffer at the optimal pH 7.5. The optimum temperature for the catalysis was 37 °C.

Preparation of Enzyme Reaction Product—Potent product inhibition has been observed when the purified enzyme (12 μg; 10 units) was incubated for more than 1 h with 30 mM L-penicillamine and 30 mM NAD at pH 7.5. The rate of substrate disappearance decreased in 1 h, and only 7% of L-penicillamine was used in 6 h (Fig. 1B). Supplementing enzyme to the mixture did not restore the activity. We tested ADP, ADP-ribose, AMP, and nicotinamide mononucleotide to see whether they could be inhibitory to the purified enzyme. ADP was found to act as a potent inhibitor (IC50 = 20 μM), whereas other compounds had no effects. To circumvent the product inhibition we tested adenylate deaminase and adenylate kinase to see if either of them could restore the catalysis. Adenylate kinase had no effects, but in the presence of adenylate deaminase (10 units), penicillamine consumption continued for 6 h, and the concentration of L-penicillamine has decreased to 6 mM (Fig. 1B). The reaction mixture was analyzed on the analytical reverse-phase HPLC system, and the eluate was monitored by the absorbance at 260 nm. Besides the remaining substrate NAD (24.0 min), nicotinamide (27.1 min), and an unknown compound (16.3 min) were detected in the reaction mixture. The unknown product was purified by the four-step preparative HPLC system and lyophilized to give 75 mg of white powder. 1H NMR (500 MHz: D2O): 8 1.33 (3H, s), 1.43 (3H, s), 3.62 (1H, s), 4.02 (2H, m), 4.38 (2H, m), 6.03 (1H, d, J = 5.9 Hz), 8.10 (1H, s), 8.37 (1H, s). 31P NMR (80.9 MHz: D2O): δ - 8.9 (d, J = 21 Hz), 0.79 (dt, J = 21 Hz, 9.8 Hz).

Characterization of the Enzyme Reaction Product—The en-
zyme reaction product did not react with ninhydrin, but acid-catalyzed hydrolysis of the product liberated penicillamine molecule, which was identified as a ninhydrin-positive spot on thin layer chromatography. Other chemical tests indicated the presence of thiol group (by platinum chloride-potassium iodide reagent), phosphate (by ammonium molybdate-perchloric acid reagent), and ribose (by orcinol-iron (III) chloride-sulfuric acid reagent) contained in the compound. $^1$H NMR showed the signals assigned to $\beta,\beta$-dimethyl group ($d_{1.33}$ and $d_{1.43}$) and $\alpha$-proton ($d_{3.62}$) of penicillamine, and the protons for ribose at $4.02$, $4.38$, and $6.03$ ppm. Singlet signals at $d_{7.8}$ and $d_{8.2}$ indicated the presence of a purine base, which was identified as inosine by the absorption maximum at $248.5$ nm. Fiske-SubaRow assay (22) showed that the product contained two phosphates for one inosine base, which was determined by the UV absorption at $248.5$ nm ($\varepsilon_{5122.2}$ mM$^{-1}$). These results suggest that the product may consist of penicillamine and IDP. Besides the result that the compound was ninhydrin-negative, several lines of evidence suggested that the amino group of penicillamine and IDP are bound by a phosphoramide linkage. First, alkaline phosphatase and 5'-nucleotidase failed to release inorganic phosphate from the product, suggesting that the $\beta$-phosphate of the IDP group was occupied. Secondly, the enzyme reaction product was stable in alkaline solution but labile in acidic conditions. Phosphoramide bond has been reported to be labile in acidic solution but stable in alkaline solution (23), whereas esters and acyl phosphate are unstable in alkaline solution (24). The enzyme reaction product has been decomposed to IDP and penicillamine under the acidic conditions, but it survived the alkaline conditions (Fig. 3). The decomposition reaction was reproducible but not reversible. The $^{31}$P NMR spectrum of the enzyme reaction product revealed two signals of the same peak intensity at $\delta = -8.9$ (d, $J = 21$ Hz) and $\delta 0.79$ (dt, $J = 21$, $9.8$ Hz) (Fig. 4). Quadrupole ion spray mass spectrometry showed that the product had the molecular mass of $558.5$ in the negative ion mode, and it was in good agreement with the calculated mass for IDP-penicillamine, $[M-H] = 558.3735$. Thus, the product was identified as IDP-penicillamine, the phosphoramide compound linked between the amino group of penicillamine and $\beta$-phosphate of IDP. Nicotinamide-ribose, the other product that would derive from NAD, was not detected in the reaction mixture, but nicotinamide has been identified in the reaction mixture. We have observed that nicotinamide-ribose prepared in situ by incubating nicotinamide mononucleotide with calf intestine alkaline phosphatase at pH 7.5 spontaneously decomposed to nicotinamide and ribose (data not shown).

**Chemical Synthesis of ADP-$\beta$-Penicillamine**—ADP-$\beta$-penicillamine has been chemically synthesized from ADP and $\beta$-penicillamine, and the reaction mixture was observed on $^{31}$P NMR spectrometry at each step of the synthesis. On proton-decouple...
amides in that the amide bond is formed on the pyrophosphate group and ATP as the substrate. The enzyme reaction product, IDP-penicillamine, has been prepared in the adenylate deaminase-coupled system and characterized by use of $^1$H and $^{31}$P NMR spectrometry, mass spectrometry, and other chemical tests. The phosphoramidate bond between IDP and penicillamine has been suggested by the result that the product was stable in alkaline solution but labile in acidic solution, in which the product irreversibly decomposed into IDP and penicillamine. The molecular mass determined by quadrupole ion spray mass spectrometry agreed with that of IDP-penicillamine. Proton-decouple $^{31}$P NMR spectrum showed two signals at $\delta = 8.9$ and $0.79$. The doublet signal at $\delta = 8.9$ was assigned to the $\alpha$-phosphate of IDP-penicillamine, representing the geminal homonuclear coupling with the $\beta$-phosphate by $21$ Hz. Although the double-triplet signal at $0.79$ has been assigned to the $\beta$-phosphate based on the comparison with $^{31}$P NMR spectra of chemically synthesized ADP-penicillamine, we could not give a clear explanation for the $9.8$-Hz splitting besides the geminal coupling by $21$ Hz. As one of likely reasons, we consider that this additional splitting may be representing the equilibrium of distinct conformational states caused by restricted pseudo-rotation on the $\beta$-phosphate.

Enzyme purification has been performed by following the activity of NAD-dependent t-penicillamine consumption. We could not obtain homogeneously purified enzyme by conventional procedure using DEAE Toyopearl, Butyl Toyopearl and Mono Q (data not shown). The enzyme has been successfully purified when affinity ligands DyeMatrex Blue B and Orange A were employed in the purification. Physicochemical properties, substrate specificity, and kinetic properties were examined using the purified enzyme. Because nicotinamide-riboside spontaneously hydrolyzed to nicotinamide and ribose, the NAD-dependent modification catalysis appeared to be an irreversible process. Therefore, the application of kinetic analysis was limited to initial velocity studies of the forward reaction in the absence and presence of the inhibitory product ADP-t-penicillamine. The substrate interaction studies and product inhibition kinetics were indicative of an Ordered Bi Bi mechanism.

Discussion

Amino acids, when utilized as a nitrogen source, are usually catabolized by PLP-dependent transamination or by NAD-dependent oxidative deamination. We have demonstrated in this paper that the penicillamine degradation in B. sphaericus may be initiated by a novel NAD-dependent modification catalysis that yields ADP-penicillamine, a phosphoramidate compound linked between $\beta$-phosphate of ADP and the amino group of penicillamine. The existence of biologically relevant phosphoramidate compounds has been documented for N-phospho-creatine formation from ATP and creatine, AMP phosphoramidate synthesis from ATP and ammonium in Mycobacterium avium (25), and the heptapeptide antibiotic microcin C7, which contains $O$-(3-aminopropanol)-AMP group linked to the $C$ terminus carbamidomethyl group (26). In addition to these phosphoramidate compounds, it has been demonstrated that Escherichia coli DNA ligase and T4 bacteriophage DNA ligase form a covalently bound enzyme-AMP intermediate in which a lysyl e-amino group and AMP are linked by phosphoramidate bond (27). E. coli ligase uses NAD as the substrate for the enzyme-AMP intermediate formation, whereas the bacteriophage enzyme uses ATP as the substrate. The enzyme reaction product elucidated in the present study differed from these known phosphoramidate compounds.

**Kinetic Studies**—Double-reciprocal plots gave converging lines when t-penicillamine was the variable substrate at fixed concentrations of NAD (Fig. 5A). The same pattern was also observed when NAD was the variable substrate using fixed concentrations of t-penicillamine (Fig. 5B). A replot of the intercepts against the reciprocal of the concentration of fixed substrate was extrapolated to the $x$ axis to reveal the $K_m$ values for each substrate (Fig. 5, A and B, right graphs). $K_m$ values for t-penicillamine and NAD were 6.5 and 13.0 mM, respectively. Product inhibition studies were performed by including chemically synthesized ADP-t-penicillamine in the assay mixture. When t-penicillamine was the variable substrate in the presence of 30 mM NAD, the double-reciprocal plot of the initial velocity data revealed a pattern consistent with noncompetitive inhibition (Fig. 6A). When NAD was the variable substrate, the double-reciprocal plot revealed a pattern consistent with competitive inhibition (Fig. 6B). As calculated from replots on the Dixon plot (Fig. 6B, inset), the $K_i$ value of ADP-t-penicillamine was 20 $\mu$M.

![Fig. 5. Initial velocity patterns with NAD and t-penicillamine as the substrates.](image)

![Fig. 6. Product inhibition by ADP-t-penicillamine.](image)
and product inhibition kinetics were used to determine the order of substrate binding and product release. NAD appeared to be the first substrate to bind and ADP-L-penicillamine is the last product to be released. According to the Dixon plot on the competitive inhibition, ADP-L-penicillamine showed much higher affinity to the enzyme ($K_m = 20 \mu M$) than the substrates, NAD ($K_m = 13.0 \text{mM}$) and L-penicillamine ($K_m = 6.5 \text{mM}$).

The high $K_m$ values for NAD and L-penicillamine have given rise to the question of whether they are the true substrates for this novel transferase. Although our efforts of substrate screening were within a limit of commercial availability, the purified enzyme showed exclusive substrate specificity among the compounds tested. L-Valine, L-cysteine, L-cystine, L-homocysteine, L-leucine, and l-isoleucine were inert substrates, and D-penicillamine served as a substrate with only 30-fold lower reactivity compared with the L-isomer. Thus, the enzyme appears to recognize the set of bulky substituents of $\beta$-$\beta$-dimethyl and thiol groups, which constitute the unique structure of penicillamine. The observation that the enzyme was specifically induced by L- or D-penicillamine provides more compelling evidence that the enzyme is biologically relevant to the penicillamine metabolism in the strain of B. sphaericus. As for the other substrate that donates ADP to penicillamine, we also tested various mononucleotides and dinucleotides, and only NAD served as the substrate. The observation that NADP, NADPH, and NADH did not substitute NAD also indicated the exclusive substrate specificity to NAD. Although we cannot completely rule out the possibility that an unidentified ADP donor with a smaller $K_m$ value might be synthesized in the bacterial cells, it would be more convenient for the cells to use NAD as the substrate for penicillamine modification. It is conceivable in this case that the high $K_m$ value helps to prevent unnecessary NAD consumption. It has been reported that enzymes such as glutamate dehydrogenase (28), alanine aminotransferase (29), and glycine aminotransferase (19) show very high substrate specificity, but their apparent $K_m$ values are high in a range of several millimolar levels. In such cases, however, the $K_m$ values are compensated by succeeding enzyme reactions that efficiently consume the product and thus draw the equilibrium to the forward reaction. The contribution of succeeding catalysis would be especially important for NAD: penicillamine ADP transferase because its phosphoramidine product ADP-L-penicillamine causes potent inhibition on the enzyme.

In the present study, we used a commercially available adenylyl deaminase to prevent the product inhibition by converting ADP-L-penicillamine to IDP-L-penicillamine. Although the enzyme was effective in removing the inhibitory product, we could not detect the corresponding activity in the cell of B. sphaericus. The penicillamine degradation pathway in the bacterium probably employs a different type of catalysis for removing ADP-L-penicillamine. This hypothesis may be substantiated by the observation that the thiol group slowly decreased in the cell extract (Fig. 1, inset). The catalysis of NAD:penicillamine ADP transferase leaves the thiol group of penicillamine unchanged, and therefore the time-dependent thiol disappearance strongly suggests that the thiol group of ADP-penicillamine would be removed in the succeeding pathway.

Although the novel NAD-dependent modification catalysis did not give us any clue on how penicillamine can be degraded and how the amino group of penicillamine is used as the nitrogen source by B. sphaericus, the enzyme reaction may be the first, rate-limiting step in the penicillamine degradation in the bacterial cell. Specific enzyme induction by D- or L-penicillamine and exclusive substrate specificity suggest that the enzyme would be involved in the penicillamine assimilation pathway. L-Penicillamine was a better substrate than the D-isomer, and this was consistent with the observation that the bacterial cells consumed L-penicillamine more rapidly than the D-isomer. Vigorous penicillamine consumption observed for crude cell extract, in contrast to the potent product inhibition on the purified enzyme, may be explained if the crude extract contained the enzymes that catalyze succeeding reactions. The succeeding enzyme reactions are still under our investigation on the premise that the thiol group should be the next target to be degraded. Considering the reaction sequence by which PLP-dependent enzymes are inactivated by penicillamine, modification of the amino group may also serve to protect PLP-dependent enzymes in the cell of B. sphaericus.

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