Reduction of Carboxylic Acids by *Nocardi*a
Aldehyde Oxidoreductase Requires a Phosphopantetheinylated Enzyme*

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Aldehyde oxidoreductase (carboxylic acid reductase (Car)) catalyzes the magnesium-, ATP-, and NADPH-dependent reduction of carboxylic acids to their corresponding aldehydes. Heterologous expression of the *car* gene in *Escherichia coli* afforded purified recombinant enzyme with a specific activity nearly 50-fold lower than that of purified native *Nocardi*a sp. enzyme. The 5-fold increase in specific activity obtained by incubating purified recombinant Car with CoA and *Nocardi*a cell-free extracts indicated that post-translational phosphopantetheinylation of Car is required for maximum enzyme activity. *Nocardi*a phosphopantetheine transferase (PPTase) expressed in *E. coli* was isolated and characterized. When incubated with [3H]acetyl-CoA and *Nocardi*a PPTase, the labeled acetylphosphopantetheine moiety was incorporated into recombinant Car. Coexpression of *Nocardi*a Car and PPTase in *E. coli* gave a reductase with nearly 20-fold higher specific activity. Site-directed mutagenesis in which Ser689 was replaced with Ala resulted in an inactive Car mutant. The results show that Car expressed in *Escherichia coli* is an apoenzyme that is converted to a holoenzyme by post-translational modification via phosphopantetheinylation. Doubly recombinant resting *E. coli* cells efficiently reduce vanillic acid to vanillin.

Microbial whole cell reductions of aromatic carboxylic acids, usually to their corresponding alcohols, have been observed with a number of microorganisms, including *Actinomyces* sp. (1), *Clostridium thermoaceticum* (2), *Aspergillus niger* (3, 4), *Corynespora melonis* (5), *Coriolus* sp. (3), *Neurospora* sp. (5), and *Nocardi*a sp. (6, 7). We previously isolated and purified carboxylic acid reductase (Car)2 from *Nocardi*a sp. NRRL 5646; this enzyme was identified as an ~128-kDa monomeric protein that requires Mg2+, ATP, and NADPH in a process by which bound carboxylic acids are first converted to acyl adenylate intermediates and subsequently reduced to aldehydes by NADPH (Scheme 1) (8, 9). We recently identified and sequenced the first bacterial gene encoding Car from *Nocardi*a sp. NRRL 5646 (10). The enzyme (originally named aryl-aldehyde oxidoreductase) exhibits broad substrate acceptance, including many aromatic carboxylic acids as well as a very wide range of aliphatic carboxylic acid substrates (11). The cloning and expression of this gene in *Escherichia coli* provided us with new opportunities to explore the mechanism of carboxylic acid reduction by this enzyme.

Phosphopantetheine transferases (PPTases) (12) catalyze post-translational modifications of proteins by covalently attaching the 4-phosphopantetheine (Ppant) moiety of CoA usually to a conserved serine residue of an apoprotein (Scheme 2). The role of PPTases has been well documented in fatty acid and polyketide biosynthesis and in non-ribosomal peptide synthesis (12–14). By phosphopantetheinylation, PPTases convert inactive apoproteins to active holoproteins that participate in the biosynthesis of fatty acids and in the biosynthesis of polyketides such as bleomycin (14) and rifamycin (15), bacterial acyl oligosaccharides (16), and acylated proteins (17). PPTases have been classified according to their specificity for given proteins: (a) for acyl carrier proteins involved in fatty acid and polyketide biosynthesis; (b) for aryl carrier proteins and peptide carrier proteins involved in non-ribosomal peptide and siderophore biosynthesis; (c) a PPTase that exists as an integrated domain of fatty-acid synthases; and (d) a subclass of PPTase that is related to the *Bacillus subtilis* PPTase Sp, but shows higher catalytic efficiencies with carrier protein involved in primary metabolism (18).

We were initially puzzled to observe that purified recombinant Car (rCar) from *E. coli* BL21-CodonPlus(DE3)-RP/PHAT305 shows a specific activity of 0.1 unit/mg of protein, nearly 50-fold lower than that obtained for purified *Nocardi*a sp. wild-type Car (10). BLAST analysis of *car* showed that Car contains an N-terminal domain (amino acids 90–544) with high homology to known AMP-binding proteins. The C-termini...
nal domain (amino acids 750–1094) has high homology to NADPH-binding proteins. During analysis of the deduced sequence of rCar, we observed the sequence LGGDSLSA (present between amino acids 661–693), similar to the consensus sequence (LGGXSA) for Ppant attachment sites in other proteins (12). Homology searches also showed that the serine at position 689 is conserved, and this residue was hypothesized as the position at which a Ppant moiety could be attached.

We therefore considered the possibility that wild-type Nocardia sp. Car is actually a holoenzyme produced by post-translational phosphopantetheinylation of apo-Car to afford maximum enzyme activity. A 4′-Ppant prosthetic group in active Car could serve as a “swinging arm” that would react with acyl-AMP intermediates at the N-terminal adenylation domain to form a covalently linked thioester that “swings” to the C-terminal reductase domain for reduction by NADPH, subsequent aldehyde release, thiol regeneration, and a new catalytic cycle. This arrangement of the Car protein would reflect a sequential catalytic mechanism wherein the N-terminal domain catalyzes substrate activation by formation of an initial acyl-AMP intermediate, and the C-terminal portion then catalyzes the reduction by NADPH of an intermediate thioester formed from acyl-AMP to finish a catalytic cycle (Fig. 1). The 50-fold difference in specific activity between rCar and wild-type Car was attributed largely of apo-Car versus fully active wild-type Car as a phosphopantetheinylated holoenzyme. We report the cloning and identification of an Sfp-type PPTase from Nocardia sp. NRRL 5646, present evidence that rCar phosphopantetheinylation is required for maximum activity, and demonstrate the expression of a rCar of high specific activity with an E. coli clone containing both the car and npt (Nocardia phosphopantetheine transferase) genes.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Materials**—LB medium contained 1% (w/v) Bacto-Tryptone, 0.5% Bacto yeast extract, and 1% NaCl. Iowa medium contained 2% (w/v) glucose, 0.5% yeast extract, 0.5% soybean flour, 0.5% NaCl, and 0.5% K₂HPO₄ and was adjusted to pH 7.2 with 6 N HCl. Antibiotics were added where appropriate to the following final concentrations: 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. Solid medium was prepared by the addition of 1.5% (w/v) Difco agar. Isopropyl β-D-thiogalactopyranoside and ampicillin stock solu-
Nocardia sp. NRRL 5646 Carboxylic Acid Reductase

tions were sterilized by 0.22-μm filtration. Chloramphenicol stock solutions were prepared in 95% ethanol. Protein concentrations were measured by the Bradford protein assay (19) with bovine serum albumin as a standard. Gel analysis of proteins was carried out on SDS–4–20% polyacrylamide gel (Bio-Rad). Talon® resin was purchased from Clontech. All other materials were purchased from Sigma unless specified otherwise. Buffer A contained 50 mM K2HPO4 (pH 7.5), 300 mM NaCl, and 10% (v/v) glycerol. Buffer B contained 50 mM K2HPO4 (pH 7.5), 300 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM β-mercaptoethanol, and 10% (v/v) glycerol. Buffer C contained 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1 mM dithiothreitol (DTT), 1 mM EDTA, and 10% (v/v) glycerol.

Nocardia sp. strain NRRL 5646 is held in the College of Pharmacy Culture Collection of the University of Iowa and is grown and maintained on slants of Sabouraud dextrose agar. DNA manipulations were performed by standard protocols (20). Competent cells of E. coli BL21- CodonPlus®(DE3)-RP and JM109 were obtained from Stratagene (La Jolla, CA) and Promega Corp. (Madison, WI), respectively.

Restriction endonucleases were obtained from New England Biolabs and Promega Corp., respectively. Plasmids pUC19 and pGEM-T were purchased from New England Biolabs and Biotechnology (Madison, WI). Fast-Link® T4 DNA ligase was obtained from Epicentre (Madison, WI). Pfu Turbo and Pfu Ultra DNA polymerases (Stratagene) were used for PCR following the manufacturer's protocol. DNA sequencing was performed by Integrated DNA Technologies (Coralville, IA). DNA fragments from restriction digests or PCR amplifications were synthesized by Integrated DNA Technologies (Coralville, IA). Fast-Link® T4 DNA ligase was obtained from Epicentre (Madison, WI).

DNA miniprep spin kit. Southern blotting, labeling of DNA probes with digoxigenin, hybridization, and detection were performed by standard protocols (20). Competent cells of E. coli BL21-CodonPlus®(DE3)-RP and JM109 were obtained from Stratagene (La Jolla, CA) and Promega Corp. (Madison, WI), respectively. Restriction endonucleases were obtained from New England Biolabs (Beverly, MA). Fast-Link® T4 DNA ligase was obtained from Epicentre Biotechnologies (Madison, WI). Pfu Turbo and Pfu Ultra DNA polymerases (Stratagene) were used for PCR following the manufacturer's protocol. DNA sequencing was performed by Integrated DNA Technologies (Coralville, IA). DNA fragments from restriction digests or PCR amplifications were purified using a DNA Clean & Concentrator™ kit (Zymo Research). DNA from agarose gels was recovered using a Zymoclean™ gel DNA recovery kit (DNA Clean & Concentrator kit). Plasmid DNA was isolated and purified using a Qiagen DNA miniprep spin kit. Southern blotting, labeling of DNA probes with digoxigenin, hybridization, and detection were performed according to the protocols of Roche Diagnostics. Plasmids pUC19 and pGEM-T were purchased from New England Biolabs and Promega Corp., respectively. E. coli MV1190/pUC8-sfp was a generous gift from Dr. Peter Zuber (21). Construction of plasmid pHAT305 expressing the car gene and isolation of Nocardia sp. NRRL 5646 genomic DNA have been described previously (10).

Cultivation Methods—E. coli MV1190/pUC8-sfp was cultured according to a published procedure (13). Nocardia sp. NRRL 5646 cultures were grown by a two-stage incubation protocol in 200 ml of Iowa medium in 1-liter DeLong culture flasks (8). Frozen glycerol stocks of E. coli BL21-CodonPlus®(DE3)-RP harboring plasmid pHAT305, pPV1.184, pPV2.83, or pPV2.51 were streaked onto LB agar plates with ampicillin and chloramphenicol to obtain single colonies. Single colonies were inoculated into 20 ml of LB medium containing ampicillin and chloramphenicol in 125-ml stainless steel capped DeLong flasks. Cultures were incubated with shaking at 250 rpm on gyratory shakers at 37 °C. A 1% inoculum derived from 8-h stage I cultures was used to initiate fresh LB cultures (200 ml) with antibiotics in a 1-liter DeLong flask. These cultures were incubated at 37 °C for 16 h with shaking at 250 rpm on a gyratory shaker. E. coli BL21-CodonPlus®(DE3)-RP/pPV2.51 was cultured for 12 h (stage I) and for 14 h (stage II). E. coli cells were harvested at 5000 × g for 6 min at 4 °C.

Analytical Methods—Standard solutions were prepared by dissolving weighed amounts of standards in 0.1 M Na2HPO4 (pH 7) containing acetonitrile (1:1, v/v/v). Aliquots of 0.5 ml were removed from the biotransformation samples and mixed with 0.5 ml of acetonitrile. Acetonitrile-diluted samples were vortexed for 30 s, allowed to stand at room temperature for 30 min, and microcentrifuged at 20,000 × g for 3 min, and the supernatant was filtered through a 0.22-μm polyvinylidene difluoride syringe filter. Samples of 1–2 μl were injected for HPLC analysis. A Shimadzu HPLC system was used with mobile phases consisting of CH3CN/H2O/HCOOH (20:80:1, v/v/v). Quantitation of standards and samples was achieved by isocratically eluting them over an Econosil HPLC column (C18, 5 μm, 150 × 3.2 mm; Alltech) at a flow rate of 0.4 ml/min. The HPLC retention volumes and detection wavelengths for standards were as follows: vanillyl alcohol, 1.7 ml and 277 nm, respectively; vanillic acid, 2.5 ml and 260 nm, respectively; and vanillin, 4.1 ml and 284 nm, respectively.

Car—Car or rCar activity was measured by adding enzyme samples (10–50 μl) to solutions containing 50 mM Tris (pH 7.5), 1 mM EDTA, 10 mM MgCl2, 1 mM DTT, 10% (v/v) glycerol, 1 mM ATP, 0.2 mM NADPH, and 5 mM benzoate in a final volume of 1.4 ml. The rate of change in absorbance at 340 nm as NADPH was oxidized to NADP* was used to assess the specific activities of the Car and rCar preparations.

PPTase Assay—An indirect assay was developed to measure PPTase. Nocardia sp. NRRL 5646 cell-free extracts (CFE) were prepared by resuspending cell paste (5 g) in cold 50 mM Tris-HCl (pH 7.5) containing 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 10% (v/v) glycerol (25 ml). The cell suspension was disrupted with a Branson Sonifier 450 cell disrupter at 450 watts with 50% intermittent duty cycle for 10 min. Cell debris was removed by centrifugation at 26,000 × g for 40 min at 4 °C. The supernatant (30 ml) was concentrated by ultrafiltration in an Amicon 50-ml concentrator (PM-10 membrane) to give a 17-ml (5–6 mg/ml) crude protein solution and used further for assay. The CFE (320–400 μg) from Nocardia sp. NRRL 5646 or from E. coli MV1190/pUC8-sfp were incubated with purified rCar (256 μg, 2 nmol) in the presence of CoA (1 mM) as a cofactor for 1 h at 28 °C in a final volume of 100 μl of buffer C. Controls were (a) Nocardia CFE + rCar, (b) Car + CoA, (c) Nocardia CFE + CoA, (d) rCar alone, and (e) Nocardia CFE alone. Car activity in these mixtures was then measured by the addition of 50-μl samples to buffer C containing 1 mM ATP, 0.2 mM NADPH, and 5 mM benzoate in a final volume of 1.4 ml. The rate of change in absorbance at 340 nm was measured. In this assay, enhancement of rCar activity was attributed to the conversion of apo- to holo-enzyme by PPTase.

Construction of pPV1.184—The 1.5-kb P sac fragment was amplified from plasmid pUC8-sfp (21) using primers SFP-FW and SFP-RV containing KpnI terminal restriction sites (see Table 1). PCR was performed using Pfu Turbo DNA polymerase for a total of 30 cycles as follows: 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.5 min. The 1.5-kb PCR product was purified, digested with KpnI, purified on a 0.7% agarose gel, and...
This study

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ASM1-FW/ASM1-RV, ASM2-FW/ASM2-RV, ASM3-FW/ASM3-RV, and ASM4-FW/ASM4-RV to create the car mutants ASM1 (S689A), ASM2 (S691A), ASM3 (S694A), and ASM4 (S696A), respectively. The PCR products were treated with DpnI and transformed into *E. coli* JM109. Transformants were selected, and the introduced mutation was confirmed by sequencing. Plasmids carrying the desired mutations were transformed into *E. coli* BL21-CodonPlus(R) (DE3)-RP competent cells, and the resulting colonies were used for enzyme analysis.

Overexpression and Purification of rCar—Cells expressing rCar were resuspended in buffer B and homogenized by two passages through a French press (16,000 p.s.i), and the homogenate was clarified by centrifugation at 26,000 × g. The clarified supernatant was loaded onto a column of Talon® resin (2.5 x 20 cm, 50-ml bed volume) equilibrated with buffer A and washed with buffer A (100 ml). rCar was eluted using a linear gradient of 0–50 mm imidazole in buffer A in a total volume of 100 ml. Fractions were assayed for benzoic acid reduction activity, and the active fractions were pooled and concentrated by ultrafiltration in an Amicon 200-ml concentrator (YM-30 membrane). The concentrate was diluted in 50 ml of buffer C. The solution was once again concentrated by ultrafiltration (YM-30 membrane) and again diluted with 50 ml of buffer C. This preparation was loaded onto a DEAE-cellulose column (2.5 x 20 cm, 50-ml bed volume) pre-equilibrated with buffer C. The column was washed with 100 ml of buffer C, and the bound protein was eluted using a linear gradient of 0–1 M NaCl in buffer C with a total of 150 ml of eluant. Fractions were assayed for rCar activity, and the active fractions were pooled and concentrated.

**Purification of Sfp and Npt**—The *B. subtilis* PPTase Sfp was purified from *E. coli* MV1190/pUC8-sfp according to a pub-

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**TABLE 1**

| Oligonucleotide | Sequence (5’ to 3’) | Source |
|-----------------|---------------------|--------|
| SFP-FW          | CGGTGTTACCTTATAGTACGG | This study |
| SFP-RV          | CGGTGTTACCTTATAGTACGG | This study |
| NPT-1           | CGGATCTCAGGTGCTCTCACTAAGCATT | This study |
| NPT-2           | GGTACCGGAGAACACAGCTT | This study |
| NPT-3           | CAGGATCTCAGGTGCTCTCACTAAGCATT | This study |
| NPT-4           | CGGATCTCAGGTGCTCTCACTAAGCATT | This study |
| NPT-5           | CGGATCTCAGGTGCTCTCACTAAGCATT | This study |
| ASM1-FW         | ACCGACCTGGGCGGATGCT | This study |
| ASM1-RV         | AACGACCTGGGCGGATGCT | This study |
| ASM2-FW         | GTTTACCTTATAGTACGG | This study |
| ASM2-RV         | GTTTACCTTATAGTACGG | This study |
| ASM3-FW         | GTTTACCTTATAGTACGG | This study |
| ASM3-RV         | GTTTACCTTATAGTACGG | This study |
| ASM4-FW         | GGCAGCTTACCTTATAGTACGG | This study |
| ASM4-RV         | GGCAGCTTACCTTATAGTACGG | This study |
| NPTORF-FW       | CAATAGCTTATAGTACGG | This study |
| NPTORF-RV       | CAATAGCTTATAGTACGG | This study |
| PUC191-FW       | CTATAGCTTATAGTACGG | This study |
| PUC191-RV       | CTATAGCTTATAGTACGG | This study |
| PPT1FW          | CAGGATCTCAGGTGCTCTCACTAAGCATT | This study |
| PPT1RV          | CAGGATCTCAGGTGCTCTCACTAAGCATT | This study |
| S71-F           | GAATTCACCGTCCGAAACATC | This study |
| S71-R           | GAATTCACCGTCCGAAACATC | This study |
| S72-F           | CACGCTTACCTTATAGTACGG | This study |
| S72-R           | CACGCTTACCTTATAGTACGG | This study |
| S73-F           | CACGCTTACCTTATAGTACGG | This study |
| S73-R           | CACGCTTACCTTATAGTACGG | This study |
| NPT-6           | GTTTACCTTATAGTACGG | This study |
| NPT-7           | GTTTACCTTATAGTACGG | This study |
| T7              | GTTTACCTTATAGTACGG | University of Iowa DNA Facility |
| SP6             | GTTTACCTTATAGTACGG | University of Iowa DNA Facility |
| M13 reverse     | GTTTACCTTATAGTACGG | University of Iowa DNA Facility |

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ligated into plasmid pHAT305 (previously digested with KpnI and treated with calf intestinal alkaline phosphatase) to give plasmid pPV1.184. The *lac* sfp gene was transcribed in the same direction as the *lac* car gene.

**Construction of pGEM-T-S7**—20 μg of genomic DNA (1.75 μg/μl) from *Nocardia* sp. NRRL 5646 was digested with *Sma*I (200 units) and loaded onto a 1% agarose gel. DNA fragments of 5.1–5.4 kb were excised from the agarose gel, purified, and ligated into pGEM-T-Easy. The ligation mixture was transformed into *E. coli* JM109. The resulting transformants were screened by colony hybridization (20) using a 250-bp DNA probe that identified supernatant was loaded onto a column of Talon® resin (2.5 x 20 cm, 50-ml bed volume) equilibrated with buffer A and washed with buffer A (100 ml). rCar was eluted using a linear gradient of 0–50 mm imidazole in buffer A in a total volume of 100 ml. Fractions were assayed for benzoic acid reduction activity, and the active fractions were pooled and concentrated by ultrafiltration in an Amicon 200-ml concentrator (YM-30 membrane). The concentrate was diluted in 50 ml of buffer C. The solution was once again concentrated by ultrafiltration (YM-30 membrane) and again diluted with 50 ml of buffer C. This preparation was loaded onto a DEAE-cellulose column (2.5 x 20 cm, 50-ml bed volume) pre-equilibrated with buffer C. The column was washed with 100 ml of buffer C, and the bound protein was eluted using a linear gradient of 0–1 M NaCl in buffer C with a total of 150 ml of eluant. Fractions were assayed for rCar activity, and the active fractions were pooled and concentrated.

Overexpression and Purification of rCar—Cells expressing rCar were resuspended in buffer B and homogenized by two passages through a French press (16,000 p.s.i), and the homogenate was clarified by centrifugation at 26,000 × g. The clarified supernatant was loaded onto a column of Talon® resin (2.5 x 20 cm, 50-ml bed volume) equilibrated with buffer A and washed with buffer A (100 ml). rCar was eluted using a linear gradient of 0–50 mm imidazole in buffer A in a total volume of 100 ml. Fractions were assayed for benzoic acid reduction activity, and the active fractions were pooled and concentrated by ultrafiltration in an Amicon 200-ml concentrator (YM-30 membrane). The concentrate was diluted in 50 ml of buffer C. The solution was once again concentrated by ultrafiltration (YM-30 membrane) and again diluted with 50 ml of buffer C. This preparation was loaded onto a DEAE-cellulose column (2.5 x 20 cm, 50-ml bed volume) pre-equilibrated with buffer C. The column was washed with 100 ml of buffer C, and the bound protein was eluted using a linear gradient of 0–1 M NaCl in buffer C with a total of 150 ml of eluant. Fractions were assayed for rCar activity, and the active fractions were pooled and concentrated.

**Purification of Sfp and Npt**—The *B. subtilis* PPTase Sfp was purified from *E. coli* MV1190/pUC8-sfp according to a pub-
lished procedure (13). Npt was purified from *E. coli* BL21-CodonPlus(®)(DE3)-RP/pPV2.51. For Npt, harvested cells (26 g) were resuspended in buffer C (100 ml) and homogenized by two passages through a French press (16,000 p.s.i.), and the homogenate was clarified by centrifugation at 26,000 × *g* to give 115 ml of CFE (2875 mg of protein). Crystalline ammonium sulfate was added to 35% saturation of the extract, which was stirred for 1 h at 4 °C. The resulting precipitate was pelleted by centrifugation at 10,000 × *g*. The 35% supernatant was brought to 55% ammonium sulfate saturation and stirred for 1 h at 4 °C to give a 55% ammonium sulfate precipitate, which was collected by centrifugation at 10,000 × *g* (865 mg of total protein). The 55% precipitate (~433 mg of protein) was dissolved in 5 ml of buffer C and loaded onto a Sephacryl S-100 column (2.5 × 20 cm, 50-ml bed volume) equilibrated with buffer C. The proteins were eluted with the same buffer at a flow rate of 0.35 ml/min. Fractions were assayed for their ability to enhance rCar activity (PPTase indirect assay), and the active fractions were pooled and concentrated to 15 ml (PM-10 membrane). This fraction was then loaded onto a reactive green 19 column (2.5 × 20 cm, 50-ml bed volume) equilibrated with buffer C. The proteins were eluted with the same buffer at a flow rate of 0.35 ml/min. Fractions were assayed for their ability to enhance rCar activity (PPTase indirect assay), and the active fractions were pooled and concentrated to 15 ml (PM-10 membrane). The proteins were then loaded onto a reactive green 19 column (2.5 × 20 cm, 50-ml bed volume) equilibrated with buffer C. The active fractions were pooled and concentrated (PM-10 membrane). The protein mixture was electrophoresed on a 10% SDS-polyacrylamide gel. The active fractions were pooled and concentrated by ultrafiltration using an Amicon PM-10 membrane to 5 ml (45 mg of protein), and aliquots (100 µl) were flash-frozen at −80 °C. 

**Labeling of Purified rCar with[^3H]Acetyl-CoA—**rCar was incubated with[^3H]Acetyl-CoA—rCar (25 µg, 0.21 nmol) was incubated with 18 µg of either Npt or Sfp and 7.5 µCi of[^3H]Acetyl-CoA (200 µCi/mmol, 37.5 nmol) in a final volume of 50 µl containing 50 mm Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM DTT, 1 mM EDTA, and 10% (v/v) glycerol. The reaction mixture was incubated for 1 h at 28 °C. The reaction was quenched with SDS-PAGE sample buffer without DTT (50 µl) and heated at 100 °C for 2 min. 50 µl of the above reaction mixture was electrophoresed on a 10% SDS-polyacrylamide gel. The gel was briefly stained with Coomassie Blue G-250 (BioRad), destained with water, and soaked in Fluoro-Hance (Research Products International Corp., Mount Prospect, IL) for 30 min. The gel was air-dried between a cellophane sheet and Saran Wrap™ for 2 days. The dried gels were then exposed to Hyperfilm™ MP (GE Healthcare) at −70 °C for 3 weeks.

**Whole Cell Carboxylic Acid Reduction—**Harvested *E. coli* cells were resuspended in 0.9% (w/v) NaCl and pelleted once again by centrifugation at 5000 × *g* for 6 min at 4 °C. Cells were then resuspended in 0.1 M Na₂HPO₄ (pH 7) to achieve wet cell weight concentrations of 0.5 g/ml. A sodium vanillate stock solution (50 mg/ml) was prepared by dissolving equimolar amounts of vanillic acid and NaHCO₃ in 0.1 M Na₂HPO₄ (pH 7). Reaction mixtures of 50 ml contained 0.4% glucose, 1.5 g of biocatalyst (wet cell weight), 200 mg of sodium vanillate, and 0.1 M Na₂HPO₄ (pH 7.0). Reactions were incubated at 28 °C with shaking at 220 rpm, and 1-ml samples were withdrawn at various time intervals for analysis. Reactions were quenched by diluting samples with equal volumes of acetonitrile, which were then equilibrated at room temperature for 30 min, followed by centrifugation in a Microfuge and HPLC analysis.

**RESULTS**

**Optimizing rCar Expression—**rCar was cloned and expressed in *E. coli* BL21-CodonPlus(®)(DE3)-RP/pHAT305. We found that cultures grown for 16 h without isopropyl β-D-thiogalactoside induction gave maximum levels of rCar with a specific activity of 0.3 ± 0.04 units/mg, 3-fold higher than rCar from induced cultures (10).

**Evidence for Car Phosphopantetheinylation—**PPTases (12) catalyze post-translational modifications of proteins by covalently transferring the Ppant moiety of CoA to a conserved serine residue as illustrated in Scheme 2. The presence of a possible phosphopantetheinylation site in the car ORF suggested that *Nocardia* sp. NRRL 5646 could contain a PPTase that converts apo-Car to fully active holo-Car.

**Table 2**

| Conditions | Specific activity units/mg |
|-----------|---------------------------|
| Nocardia CFE | 0.01 ± 0.006 |
| +CoA | 0.01 ± 0.003 |
| +rCar | 0.5 ± 0.06 |
| +rCar and CoA | 1.6 ± 0.1 |
| rCar | 0.3 ± 0.04 |
| +CoA | 0.3 ± 0.04 |
| +CoA and E. coli MV1190/pUC8-sfp CFE | 1.5 ± 0.03 |
| +CoA and Npt | 1.6 ± 0.06 |
| Car purified from BL21-CodonPlus(DE3)-RP/ pPV1.184 (car + sfp) | 2.1 ± 0.1 |

**Site-directed mutagenesis was used to replace the codon for Ser⁶⁹⁶ in the LGGDSLA phosphopantetheinylation signature sequence of *Nocardia* rCar with Ala. rCar Ser⁶⁹⁶, Ser⁶⁹⁴, and Ser⁶⁹⁸, near the signature site, were also replaced individually with alanines. CFE purified from rCar and the alanine mutants were analyzed directly for Car activity, and the results are expressed as relative specific activities in Table 3. The S689A mutant was completely inactive. Mutants S691A,
putative PPTases from Mycobacterium farcinica IFM 10152 (YP_120266) and was 50% identical to pI of 5.11. Residues with a calculated molecular mass of 24,286.2 Da and a bp, corresponding to a protein composed of 222 amino acid Bank (accession number 838316).

Recent changes caused by substituting alanine for serine.

The results show that Ser689 is essential for rCar activity, likely because of nonspecific protein structural changes caused by substituting alanine for serine.

Identification and Cloning of npt from Nocardia sp. NRRL 5646—Several peptidyl carrier protein-specific PPTases have been cloned from Streptomyces sp. (14). Because Nocardia sp. NRRL 5646 is also an actinomycete, we used reported procedures to identify and clone the Nocardia PPTase gene (14). BLAST analysis (22) followed by ClustalW (23) alignment were used to create a consensus sequence of 11 known or putative PPTases from actinomycetes, including the PPTase from the recently sequenced Nocardia farcinica strain IFM 10152 (24). PCR with NPT-5 and reverse primers NPT-2, NPT-3, and NPT-4 (designed based on conserved motifs) yielded a distinct 250-bp DNA fragment. The 250-bp DNA fragment was cloned into the pGEM-T vector, and five clones were selected and sequenced. Four of these clones afforded identical sequences (except for the difference arising from primer utilization) and were homologous to putative PPTase from N. farcinica IFM 10152 (YP_120266) (24). Using the PCR product as a probe, we identified a 5.2-kb DNA fragment from an SmaI genomic DNA library and a 3.2-kb DNA fragment from an HincII genomic DNA library, both of which contained the single complete PPTase gene.

Table 3

| Strain name                  | Relative specific activitya |
|-----------------------------|----------------------------|
| BL21-CodonPlus(DE3)-RP/pHAT305 (car) | 100                      |
| BL21-CodonPlus(DE3)-RP/pHAT305(S694A) | 0                        |
| BL21-CodonPlus(DE3)-RP/pHAT305(S691A) | 36                      |
| BL21-CodonPlus(DE3)-RP/pHAT305(S694A) | 50                      |
| BL21-CodonPlus(DE3)-RP/pHAT305(S691A) | 76                      |

a The specific activity of BL21-CodonPlus(DE3)-RP/pHAT305 (car) was measured at 17 × 10³ units/mg of protein. This value was considered to be 100%.

Within a polyketide synthase gene cluster. The biochemical role of Nocardia sp. NRRL 5646 PPTase remains uncertain.

Expression and Purification of Npt from E. coli—To determine whether npt codes for a PPTase required for Car activation, the npt gene locus was amplified from plasmid pGEM-T-S7 by PCR and cloned into the pUC19 expression vector to afford plasmid pPV2.51. Npt was purified from E. coli BL21-CodonPlus(DE3)-RP/pPV2.51 by a combination of (NH₄)₂SO₄ precipitation and reactive green 19-agarose affinity and Q-Sepharose chromatographies. The PPTase has a strong affinity for reactive green 19 resins and can be eluted by high salt concentrations. Npt was purified to 90% homogeneity. Upon SDS-PAGE, the purified enzyme migrated as a single band with an apparent molecular mass of 28,000 Da (calculated mass of 24,286.2 Da). This type of abnormal mobility of PPTase upon SDS-PAGE has been reported previously (14). Apo-rCar incubated with purified Npt and CoA (1 mM) for 1 h at 28°C reduced benzoyl acetate five times faster compared with rCar. Cloning of Pₐₜₙ npt along with car enhanced the in vivo activity of rCar. rCar purified from E. coli BL21-CodonPlus(DE3)-RP/pPV2.83 in which Pₐₜₙ car was expressed along with Pₐₜₙ npt had a specific activity of 2.2 ± 0.1 units/mg. The Kₘ values for benzoyl acetate, ATP, and NADPH were 869 ± 75, 75 ± 31, and 53 ± 86 μM, respectively, and were similar to those for the native Nocardia sp. enzyme. The Vₘₐₓ was 3.08 ± 0.06 μmol/min/mg of protein, ~3 times higher than that of the natural enzyme (0.902 ± 0.04 μmol/min/mg of protein). A total of 60–70 mg of rCar was obtained with a specific activity of 2.2 ± 0.1 units/mg from E. coli BL21-CodonPlus(DE3)-RP/pPV2.83.

In Vitro Labeling of rCar with [³H]Acetyl-CoA by Npt and Sfp—To demonstrate the post-translational modification of rCar by Npt and Sfp, we tested the covalent incorporation of [³H]acetylphosphopantetheine from [³H]acetyl-CoA into holo-rCar by autoradiography. In this experiment, rCar was labeled by both Npt and Sfp, as shown by the dark bands on the gels corresponding to rCar (Fig. 2, lanes 6 and 7). In addition, both Sfp and Npt appeared to be labeled, as shown by the dark bands corresponding to these proteins. The results indicate that the [³H]acetylphosphopantetheine moiety was transferred to the conserved serine of rCar by both Npt and Sfp. Based on the relative intensity of tagged rCar observed on the autoradiography film, Sfp appeared to more efficiently transfer-label from [³H]acetyl-CoA compared with Npt. Such relaxed substrate specificity for Sfp has been reported previously in the transfer of altered Pant moieties (13).

In Vivo Evidence of Holo-rCar—In vivo reduction of vanilllic acid was evaluated using resting cells of E. coli BL21-CodonPlus(DE3)-RP/pHAT305 and E. coli BL21-CodonPlus(DE3)-RP/pPV2.83 (Scheme 3). With E. coli BL21-CodonPlus(DE3)-RP/pPV2.83, in which recombinant Npt was expressed along with rCar, vanillic acid was reduced to vanillin and vanillyl alcohol, with vanillin (80%) as the major product (Fig. 3A). Over the 10-h period, 80% of the vanillic acid was converted to vanillin and vanillyl alcohol. Car does not reduce aldehydes (9). Thus, it is presumed that E. coli contains an aldehyde reductase that reduces vanillin to vanillyl alcohol. In a similar 10-h period, E. coli BL21-CodonPlus(DE3)-RP/pHAT305 (expressing only rCar) reduced only 50% of the
vanillic acid starting material, with vanillyl alcohol being the major metabolite (Fig. 3B). With *E. coli* BL21-CodonPlus®(DE3)-RP/ppV2.83, in which rCar is presumed to be in the fully active, phosphopantetheinylated holo form, the rate of reduction of vanillic acid was much faster than that of vanillin to vanillyl alcohol by endogenous *E. coli* aldehyde dehydrogenase.

**DISCUSSION**

The identification of the *car* gene from *Nocardia* sp. NRRL 5646 and its heterologous expression provided an avenue to explore the direct reduction of carboxylic acids, a very unusual biochemical reaction. The moderate expression of *car* in *E. coli* BL21-CodonPlus®(DE3)-RP/ppHAT305 with an unusually low specific activity was initially confusing.

The Car sequence contains motifs for carboxylic acid adenylation and NADPH binding and a serine residue (Ser<sup>689</sup>) within the sequence LGG<sup>X</sup>S<sup>XX</sup>A, a consensus signature sequence for a Ppant attachment site (12). This analysis suggested that Car from wild-type *Nocardia* is a holoenzyme produced by post-translational phosphopantetheinylation of apo-Car to afford maximum enzyme activity.

In *vitro* incubation of rCar purified from BL21-CodonPlus®(DE3)-RP/ppHAT305 with *Nocardia* CFE and CoA provided evidence for the presence of a PPTase required for activating the enzyme. The rCar mutant S689A was completely inactive, suggesting that this serine within a phosphopantetheinylation signature sequence is essential for Car activity. The coexpression of a PPTase gene from *Nocardia* (npt) and *car* led to enhanced *in vivo* catalytic activity of rCar. Indeed, the reaction of <sup>3</sup>H-acetyl-CoA and rCar with either Npt (PPTase from *Nocardia*) or Sfp (PPTase from *Bacillus*) resulted in the transfer of label from acetyl-CoA to rCar. The results of this study support the hypothesis that, at least in part, rCar is an apoenzyme that requires phosphopantetheinylation for conversion to a fully active holoenzyme.
The newly cloned PPTase from Nocardia sp. is similar to the Sfp-type PPTase and is a monomer of 222 amino acids. This Nocardia PPTase appears to be the only enzyme of its type because a single gene was isolated from two different genomic libraries. PCR using degenerate primers based on consensus sequences gave a single DNA product. This PPTase is probably responsible for the activation of Car and other unknown proteins with phosphopantetheine attachment sites. Because a single gene was identified from two different genomic libraries, we speculate that the npt gene functions similarly to the sfp gene from Streptomyces verticillus (14) and to the sfp gene from B. subtilis (21) and that it has broad substrate specificity. The specific function of PPTase in Nocardia remains elusive.

Purified rCar from E. coli BL21-CodonPlus®(DE3)-RP/pPV2.83 had a specific activity of 2.2 units/mg, ~2.5-fold lower than that of wild-type Car. In vivo reduction of vanillic acid using resting cells of E. coli BL21-CodonPlus®(DE3)-RP/pHAT305 and E. coli BL21-CodonPlus®(DE3)-RP/pPV2.83 provided further evidence for the requirement of phosphopantetheinylation of Car. The 5-fold difference in the rate of reduction of vanillic acid can be attributed to the presence of a more catalytically active enzyme in E. coli BL21-CodonPlus®(DE3)-RP/pPV2.83.

These experiments provide important new mechanistic insights for the activation of Car. Additional problems potentially limiting maximum Car activity are requirements for NADPH and ATP. Requirements for NADPH can be alleviated by the incorporation of glucose-1-dehydrogenase, which will recycle the generated NADP⁺ to NADPH (25–27). Regeneration of ATP by cultures grown on glucose or glycerol (28) may provide insufficient intracellular levels of ATP for efficient biomacatalysis. Possible means of enhancing ATP availability being considered are recycling AMP to ATP via polyphosphate and adenylate kinase in an additionally modified biocatalyst (29, 30).

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