Overexpression, Purification, and Mechanistic Study of UDP–*N*-Acetylenolpyruvylglucosamine Reductase†

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ABSTRACT: The recently isolated *Escherichia coli murB* gene (Pucci et al., 1992) has been cloned into an expression vector and the encoded UDP–*N*-acetylenolpyruvylglucosamine reductase (EC 1.1.1.158) was overproduced to about 10% of soluble cell protein. The encoded 38-kDa protein has been purified to near homogeneity. It was found to be a monomer and to contain stoichiometric amounts of bound FAD which is reducible in catalytic turnover. The enzyme utilizes the 4-*pro-S* hydrogen of NADPH to reduce the enolpyruvyl group of UDP–*N*-acetylglucosamine enolpyruvate to the lactyl ether in UDP–*N*-acetylmuramic acid. NMR analysis of products from ^2H_2O and 4*S*-[^{2}H]NADPH incubations establishes that a hydride from NADPH via E·FADH$_2$ is transferred to the β-methyl of the 3-*O*-lactyl moiety and a proton from solvent to the α-carbon of the lactyl moiety of UDP–*N*-acetylmuramic acid. A mechanism for this unusual enolether reduction in bacterial cell wall assembly is proposed.

The biosynthesis of the peptidoglycan layer in bacterial cell wall assembly is a multistage process unique to prokaryotes and the site of action of several therapeutically important classes of antibiotics such as β-lactams, bacitracin, and the vancomycin group of glycopeptides (Bugg & Walsh, 1993). Peptidoglycan assembly begins with a two-step sequence catalyzed by MurA and MurB converting UDP–*N*-acetylglucose into the 3-*O*-lactyl ether of UDP–*N*-acetylglucose known as UDP–*N*-acetylmuramic acid. The peptide chain of the peptidoglycan is then sequentially extended from the carboxyl terminus of this lactyl moiety by subsequent enzymes to form the UDPMurNAc[1] pentapeptide (Park, 1987). The lactyl group derives from phosphoenolpyruvate via the MurA-catalyzed enolpyruvylation of the 3-hydroxyl of the *N*-acetylglucose moiety of UDPGlcNAc (Zemell & Anwar, 1975). The enolpyruvyl ether is then reduced by the NADPH requiring UDP–*N*-acetylenolpyruvylglucosamine reductase (EC 1.1.1.158) to the UDP–*N*-acetylmuramic acid product (Scheme I) (Taku et al., 1970). The enolpyruvyl transferase, Mur A, is the site of action of the antibacterial drug fosfomycin (Kahan et al., 1974), but little is known about these two enzymes in part because they have not been purified in quantity or studied since their initial characterization two decades ago (Anwar & Vlaovic, 1979; Gunetileke & Anwar, 1968). Recently, we and Wanke et al. (1992) have reported the cloning, overproduction, and purification of a UDP–*N*-acetylglucosamine enolpyruvyl transferase which we have named *murZ* (Marquardt et al., 1992) to distinguish it from the *murA* gene which maps at a different locus in *Escherichia coli*. Both *murA* and *murZ* encode UDP–*N*-acetylglucosamine enolpyruvyl transferases. The availability of MurZ has allowed us to purify substantial quantities of UDP–*N*-acetylglucosamine enolpyruvate, the substrate for MurB. The *E. coli murB* gene was recently cloned and sequenced (Pucci et al., 1992), and we here describe the overproduction, purification, and mechanistic characterization of MurB. We have found that MurB is a flavoprotein reductase with bound FAD as a redox active intermediate in catalysis of the enol reduction. We also report studies with 4*H*-[^{2}H]NADPH and ^2H_2O that establish the regio- and stereochemistry of the biological reduction of this enolether.

MATERIALS AND METHODS

Materials. PCR was performed using a Perkin Elmer Cetus kit and DNA thermal cycler. UDPGlcNAc, PEP, NADPH, NADP$^+$, DTT, reduced glutathione, acetaldehyde-d_4, and ^2H_2O were obtained from Sigma. Protio-acetaldehyde was obtained from Aldrich. Fast Flow Q Sepharose gel and Butyl-Sepharose were from Pharmacia. Blue A gel and Centricon filters were obtained from Amicon (Danvers, MA). Glutathione reductase (EC 1.6.4.2) type IV from Baker's yeast and aldehyde dehydrogenase (EC 1.2.1.5) from Baker's yeast were obtained from Sigma. HPLC columns were from Bio-Rad.

PCR Cloning of MurB. Two primers were constructed corresponding to the 5′ and 3′ ends of the open reading frame identified as the *murB* gene (Pucci et al., 1992). The 5′ sequence was GTCGACGAATTCCTGCAGAAGGA-GATATACATATGAACCACTCCTTAAAACCCTGG. The 3′ sequence was GAGCTCCTAGGATCCTCTAGAAA-TTTCAGTGGCACGGTGTTATCC. The primers incorporated *Eco*RI and *Xba*I sites for cloning into the expression vector. The start codon (in bold) is 9 bases from the ribosome binding site (underlined) which has an optimized Shine–Dalgarno sequence. The *murB* gene was amplified from genomic DNA of the *E. coli* strain AB 1157 (ATCC) by PCR (30 cycles). PCR fragment was digested with *Eco*RI and *Xba*I and ligated into the expression vector, pKen (gift from G. Verdine, Harvard University), cut with the same enzymes

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[1] Abbreviations: UDPGlcNAc, uridine diphospho-*N*-acetylglucosamine; UDPGlcNAcEP, uridine diphospho-*N*-acetylglucosamine enolpyruvate; UDPMurNAc, uridine diphospho-*N*-acetylmuramic acid; PEP, phosphoenolpyruvate; NADPH, reduced β-nicotinamide adenine dinucleotide phosphate; IPTG, isopropyl thio-β-D-galactoside; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; FPLC, fast protein liquid chromatography; FAD, flavin adenine dinucleotide; PCR, polymerase chain reaction; NMR, nuclear magnetic resonance; COSY, ^{1}H–^{1}H correlation spectroscopy; DQF, double-quantum filtered; ^DV, V_{max} deuterium isotope effect.

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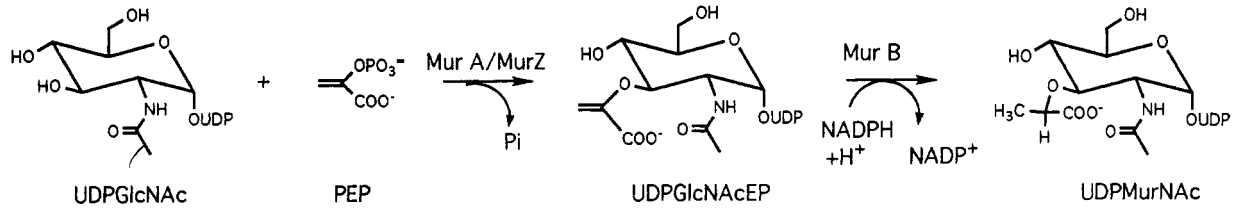
Scheme I: MurA/MurZ- and MurB-Catalyzed Reactions



to give plasmid pTEB004. Restriction digests of the resulting construct revealed an insertion of approximately 1 kb in the vector. The correct sequence of the cloned *murB* gene was confirmed by automated sequencing on an Applied Biosystems 373A automated DNA sequencer (part number 901497) using dye-labeled dideoxy nucleotides, Taq DNA polymerase, and double-stranded DNA template at the Dana Farber Cancer Institute Molecular Biology Core Research Facility.

Overexpression and Purification of MurB. A 1-L culture of cell line XA90 F′ *lacI*^{Q1} (MacFerrin et al., 1990) carrying the plasmid pTEB004 was grown to an OD_{595} of 0.5 and induced with IPTG to a final concentration of 1 mM. After 8 h, the cells were harvested by centrifugation and lysed with a French press at 20 000 psi in 50 mM Tris-HCl, pH 8.0, and 0.5 mM DTT (buffer A). The cell debris was pelleted, and the supernatant was treated with DNase (1 mg) and RNase (1 mg) with stirring on ice for 1.5 h and precipitated with 70% $(NH_4)_2SO_4$. This precipitate was then redissolved in buffer A and desalted on a G-25 column (36 × 3 cm). The dark yellow fractions were pooled and applied to a Fast Flow Q Sepharose column (11 × 3 cm). The protein was eluted with a linear gradient from 0 to 1 M KCl in buffer A. MurB eluted around 0.5 M KCl. Fractions were assayed for MurB activity as described below. Active fractions were pooled, and $(NH_4)_2SO_4$ was added to a final concentration of 1.5 M. The sample was applied to a Butyl-Sepharose column (15 × 2.5 cm) and eluted with a gradient from 1.5 M $(NH_4)_2SO_4$ in buffer A to 0 M salt in buffer A. Active fractions were determined by the assay described below. The enzyme eluted at 0.18 M $(NH_4)_2SO_4$ from the Butyl-Sepharose column. Pure fractions were pooled, dialyzed against buffer A, and stored at a concentration of 7.3 mg/mL at −20 °C. Protein concentration during the purification was determined by Bradford assay (Bradford, 1976). Protein concentration for purified enzyme was measured by A_{463} (see Results).

Synthesis of UDP-N-Acetylglucosamine Enolpyruvate. UDPGlcNAcEP was synthesized in milligram quantities in 4 mL of 0.5 M Tris-HCl, pH 7.8, with UDPGlcNAc (130 mg, 0.2 mmol) and PEP (82 mg, 0.4 mmol). MurZ (100 μL, 200 units) (Marquardt et al., 1992) was added and the reaction proceeded at 37 °C for 2 h. The product mixture was filtered through a Centricon-10 filter and purified by HPLC as described below to yield 67 mg (0.1 mmol, 50%) of UDP-GlcNAcEP.

Synthesis of UDP-N-Acetylmuramic acid. UDP-*N*-acetylmuramic acid was synthesized in milligram quantities by coupling the MurZ with the MurB reaction (Scheme I). In a typical reaction, UDPGlcNAc (52 mg, 80 μmol), NADPH (67 mg, 80 μmol), and phosphoenolpyruvate (33 mg, 160 μmol) were combined in 4 mL of 100 mM Tris-HCl, pH 8.0, and 0.5 mM DTT. Pure MurZ (50 μL, 100 units) and purified MurB (50 μL, 0.6 unit) were added to the reaction mixture. Progress of the reaction was assessed by monitoring the decrease in A_{340}. After 3 h the product mixture was filtered through a Centricon-10 filter and then purified by HPLC as described below to yield 30 mg (44 μmol, 55%) of UDP-MurNAc: ^{1}H-NMR (500 MHz, ^2H_2O) δ 8.00 (d, 1 H, *J* = 8.1 Hz, H6″), 5.99 (d, 1 H, *J* = 4.4 Hz, H1′), 5.98 (d, 1 H, *J* = 9.3 Hz, H5″), 5.66 (dd, 1 H, *J* = 2.2 and 4.6 Hz, H1), 4.38 (m, 2 H, H2′ and H3′), 4.32 (q, 1 H, *J* = 7.0 Hz, H9), 4.30 (m, 1 H, H4′), 4.24 (dm, 1 H, *J* = 11.6 Hz, H5′), 4.19 (dm, 1 H, *J* = 11.6 Hz, H5′), 3.93 (dm, 1 H, *J* = 10.0 Hz, H5), 3.87 (m, 1 H, H2), 3.83 (m, 2 H, H6), 3.79 (m, 1 H, H3), 3.62 (dd, 1 H, *J* = 9.3 and 9.6 Hz, H4), 2.07 (s, 3 H, Me8), 1.36 (d, 3 H, *J* = 7.0 Hz, Me11).

Purification of UDPGlcNAcEP and UDPMurNac. HPLC purification was carried out on a Bio-Rad HPLC reverse-phase Hi-Pore 318 column (250 × 21.5 mm) with 50 mM ammonium formate, pH 3.5, at a flow rate of 5 mL/min by monitoring at 254 nm (Mengin-Lecreulx et al., 1983). The elution times were as follows: 11.2 min PEP, 12.9 min UDPGlcNAc, 16.0 min UDPGlcNAcEP, 20.9 min UDP-MurNAc, and 35.2 min NADP$^+$ and NADPH. Peak fractions were pooled and lyophilized. The residue was taken up in H_2O and relyophilized two times to remove the ammonium formate.

Enzymatic Assays. A typical MurB assay was performed in a volume of 500 μL with concentrations of the reactants as follows: 100 μM UDPGlcNAcEP (purified by HPLC), 150 μM NADPH, 20 mM KCl, 50 mM Tris-HCl, pH 8.0, and 0.5 mM DTT (Anwar & Vlaovic, 1979). All assays were run at room temperature. Reaction mixture was prepared daily. Activity of the enzyme was measured by monitoring the decrease in absorbance at 340 nm. To determine specific activities, an extinction coefficient of 6220 M^{-1} cm^{-1} for NADPH at 340 nm was used.

4S-[^{2}H]NADPH and 4R-[^{2}H]NADPH Synthesis and Purification. 4*S*-[^{2}H]NADPH was prepared by dissolving NADP$^+$ (9 mg, 12 μmol), reduced glutathione (18.4 mg, 60 μmol), DTT (61.7 mg, 400 μmol), and triethanolamine (9 mg, 50 μmol) in ^2H_2O and lyophilizing the mixture two times (Ploux et al., 1988). The residue was taken up in a final volume of 1 mL of ^2H_2O. The pD was adjusted to 8.9 with 40% NaOD. Glutathione reductase (2 μL, 5.5 units) was added to the mixture which was stirred at room temperature for 4 h. The reaction was monitored by following the increase in A_{340}. Protio-NADPH for isotope effect measurements was prepared in a similar manner by using H_2O (final pH 8.5) in place of ^2H_2O.

The 4*S*-[^{2}H]-NADPH and protio-NADPH samples were purified on a FPLC MonoQ column connected to a HPLC system (Orr & Blanchard, 1984) with elution by a 0–1 M KCl gradient in 20 mM triethanolamine. Fractions were collected and analyzed for purity by A_{260}/A_{340} ratio. Fractions with a ratio of 2.5 or less were considered to be pure. For isotope effect experiments, fractions with equivalent A_{260}/A_{340} ratios and concentrations were used.

4*R*-[^{2}H]NADPH was prepared with NADP$^+$ (9 mg, 12 μmol), acetaldehyde-d_4 (3 μL, 60 μmol), and DTT (2 mg, 1 μmol) taken up in 1 mL of 0.1M KP_i, pH 7.0 (Yuan & Hammes, 1984). Aldehyde dehydrogenase (5 units) was added and the reaction mixture was stirred at room temper-

ature for 4 h. The reaction was quenched with 5 μL of chloroform. Protio-NADPH was prepared using protio-acetaldehyde. Purification of the 4*R* products were carried out by HPLC as above.

Synthesis of UDP-N-Acetylmuramic Acid Using MurB in ^2H_2O. All components of the reaction mixture were taken up in ^2H_2O and lyophilized twice before final resuspension in ^2H_2O. UDPGlcNAcEP (18 mg, 26.5 μmol) and NADPH (31 mg, 37 μmol) with MurB (50 μL, 0.604 unit) in 3 mL of 50 mM Tris-DCl in ^2H_2O, pD 8.2, 0.5 mM DTT, and 20 mM KCl were stirred at room temperature overnight. The product was filtered through a Centricon-10 filter to remove the enzyme and then purified by HPLC as described above. The final yield was 5.7 mg (8.4 μmol, 32%) of product.

Synthesis of UDP-N-Acetylmuramic Acid Using MurB with 4S-[^2H]NADPH. A larger quantity of 4*S*-[^{2}H]NADPH was prepared as above with $NADP^+$ (26.7 mg, 36 μmol), reduced glutathione (55.2 mg, 180 μmol), DTT (185 mg, 1.2 mmol), triethanolamine (27 mg, 150 μmol), and glutathione reductase (6 μL, 16.5 units). 4S-[^{2}H]NADPH fractions were collected and analyzed for purity by the A_{260}/A_{340} ratio and combined to a volume of 5.3 mL. UDPGlcNAcEP (17 mg, 25 μmol), KCl (14.2 mg, 0.2 mmol), and DTT (1 mg, 6.5 μmol) were added to the combined fractions. Stirring was continued for 90 min at room temperature until the A_{340} stopped decreasing. The product mixture was stored at −70 °C overnight. The product was filtered through a Centricon-10 filter to remove the enzyme and then purified by HPLC as described above. The final yield was 2.7 mg (4.0 μmol, 16%) of product.

NMR Methods. ^{1}H-NMR spectra were measured on a 500-MHz Varian VXR 500 FT NMR spectrometer. Compounds were dissolved in ^2H_2O, filtered over a small magnet through cotton wool, and kept under N_2. Sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 (final concentration of 1 mM) was added for referencing. The residual water signal was suppressed by low-power presaturation during the relaxation delay of 1.0 s. The 90° proton pulse was 9.4 μs; the acquisition time was 0.128 s, and the spectral width was 4000 Hz. For 1D proton spectra, the number of transients was 128 and spectra were processed without apodization for accurate integrations. COSY (Piantini et al., 1982) spectra included 256 experiments of 8 scans and 1024 points each. The 2D spectra were processed with zero-filling to 1K in *F*1 and sine-bell multiplication in each dimension. COSY spectra (Figure 6) were acquired in the absolute value mode. The assignments of UDP-*N*-acetylmuramic acid protons were made from a DQF-COSY (Rance et al., 1983; Shaka & Freeman, 1983) spectrum acquired with parameters as above with the exception of 512 experiments.

Mass Spectrometry. Samples prepared for mass spectrometry analysis were the same as used in the NMR experiments described above. Samples were exchanged twice in H_2O and lyophilized. Fast atom bombardment mass spectra (FAB) were obtained at the Harvard University Chemistry Mass Spectrometry Facility.

Flavin Identification. The flavin moiety was detected in MurB by taking the optical spectrum of the purified enzyme solution (0.7 mg/mL). The flavin was released from MurB by boiling the enzyme solution for 5 min (100 °C). This was followed by centrifugation at room temperature for 5 min to remove the denatured protein. An optical spectrum of the supernatant was taken for comparison to the native spectrum. The flavin was identified as FAD by HPLC analysis (Hausinger et al., 1986) with a gradient in 5 mM ammonium acetate, pH 6.0, from 5% methanol to 70% methanol on a Bio-Rad analytical C-18 column. The extinction coefficient for the FAD bound to MurB was determined by comparing the λ_{max} at A_{463} of the purified enzyme with the A_{450} of the flavin released from the enzyme.

Determination of Molecular Weight for MurB. Molecular weight determination of MurB was accomplished using a Bio-Rad Sec-125 HPLC gel filtration column. Isocratic conditions of 5 mM Tris-HCl, pH 8.0, and 200 mM NaCl gave separation of bovine serum albumin (6.6 min), ovalbumin (7.2 min), and myglobin (8.8 min) standards. The retention time for MurB was 7.6 min, consistent with an apparent molecular weight of 35 000 and indicating that MurB eluted as a monomer.

RESULTS

Production and Characterization of UDPGlcNAcEP and UDPMurNAc. Study of the unusual enol ether reduction catalyzed by the MurB enzyme in bacterial peptidoglycan biosynthesis (Scheme I) required quantities of substrate UDPGlcNAcEP to assay MurB during its isolation. This entailed the preparation of 67 mg of UDPGlcNAcEP by large-scale incubations of UDPGlcNAc and phosphoenolpyruvate with pure MurZ (Marquardt et al., 1992). Analogously, UDPMurNAc was synthesized by coupling MurZ and MurB to produce 30 mg of product. UDPGlcNAcEP and UDP-MurNAc were separable by HPLC, and this enabled the MurB purification studies and the NMR experiments described below. The structures of UDPGlcNAcEP and UDPMurNAc were confirmed by ^{1}H-NMR and mass spectrometry analysis. UDP-*N*-acetylmuramic acid was produced by MurB in H_2O, and the protons were assigned from the DQF-COSY. The ^{1}H assignments from the 1D spectrum are listed in Materials and Methods, and the numbering scheme used for UDP-MurNAc is indicated in Figure 1. A ^{1}H-NMR assignment of enzymatically generated UDPGlcNAc, the substrate for MurA, has recently been published (Heidlas et al., 1992) and was of value in corroborating the assignments of UDPMur-NAc. This is the first report of NMR characterization of this substance perhaps because it has not been readily prepared in quantity until the overproduction of MurZ (Marquardt et al., 1992) and MurB described here. Negative ion mass spectrometry of UDPGlcNAcEP and UDPMurNAc gave molecular ion peaks (M − 1) at 676 and 678, respectively. These data are in agreement with the theoretical molecular weight of 677 g/mol for UDPGlcNAcEP and 679 g/mol for UDPMurNAc.

Overproduction and Purification of E. coli MurB. On the basis of the recently published DNA sequence of the *E. coli murB* (Pucci et al., 1992), an overproducing construct was prepared to express the encoded UDPGlcNAc enolpyruvate reductase from the *srp* promoter controlled by the *lac* repressor (Schreiber & Verdine, 1991). Substantial expression was achieved to yield soluble enzyme that could be assayed using the UDPGlcNAcEP produced above, permitting ready purification as shown in Figure 2 by G-25, Q Sepharose, and Butyl-Sepharose (Table I). From 1 L of bacterial growth, 10.4 mg of protein purified 6.8-fold was obtained with a specific activity of 31.4 μmol/(min·mg), over 9-fold higher than the value for the enzyme previously purified from *E. coli* whole cell extracts (Anwar & Vlaovic, 1979). The K_m values for the substrate and nicotinamide coenzyme by fitting the data to a hyperbolic plot were 12.5 μM for UDPGlcNAcEP at saturating NADPH (180 μM) and 19.9 μM for NADPH at saturating UDPGlcNAcEP (150 μM). Given a MW of 37 854 from the sequence, MurB shows a k_{cat} of 1300 min^{-1}. The

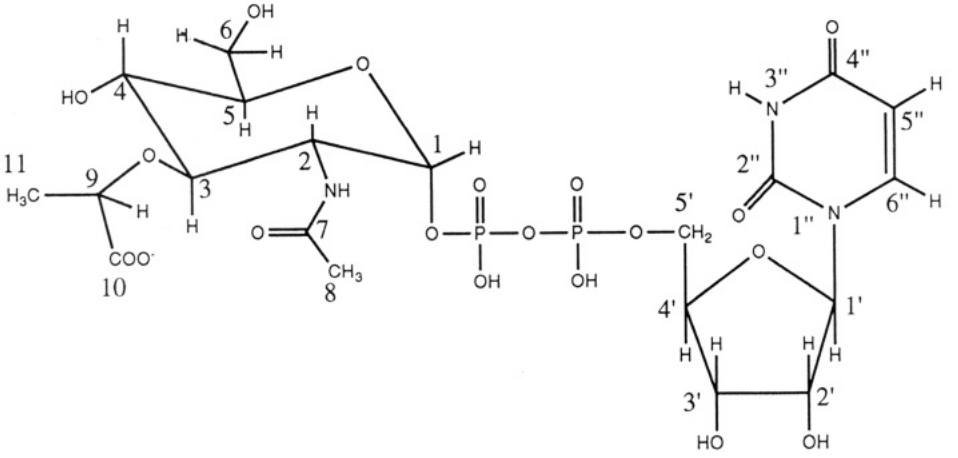


FIGURE 1: Structure of UDP–*N*-acetylmuramic acid.

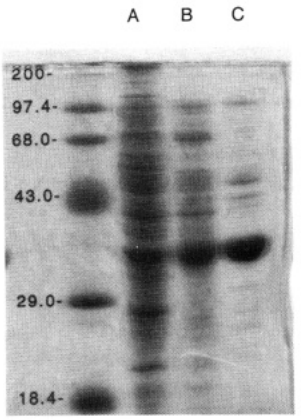


FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel (12.5%) stained with Comassie Blue (0.1%) showing the purification of overexpressed MurB. Molecular mass standards in kilodaltons: (A) G-25 pool, 153 μg of protein; (B) Fast Flow Q pool, 59 μg of protein; (C) Butyl-Sepharose pool, 37 μg of protein.

Table I: Purification of Overexpressed UDP–*N*-Acetylenolpyruvylglucosamine Reductase (MurB) from *E. coli*

purification step	total protein (mg)	sp act.[a] (units/mg)	total activity (units)	yield (%)	purification (*x*-fold)
G–25 pool	325	4.61	1498	100	1
Fast Flow Q Sepharose	105	13.1	1376	92	2.8
Butyl-Sepharose	10.4	31.4	327	22	6.8

[a] One unit = 1 μmol of NADPH oxidized/(min·mg).

7-fold purification to near homogeneity here compares to 2118-fold purification from wild-type levels reported previously and permits characterization of the enzyme at stoichiometric levels. Dye columns have been useful as pseudoaffinity columns for many nicotinamide-utilizing and FAD-containing enzymes. With this in mind, we attempted purification by Blue A and Red A columns, but we found repeatedly that about half of active enzyme was found in the flow-through fractions. While 2–3-fold purification was obtained for the fraction of MurB that bound to such columns, the hydrophobic chromatography step is a preferred route for purification at present.

MurB Is a Flavoprotein. During the MurB purification, a yellow color was observed to copurify with the enzymatic activity. The optical spectra of purified MurB (Figure 3, curve A) clearly revealed the presence of a tightly bound flavin cofactor. This coenzyme was FAD as determined by heat denaturation of the purified enzyme and HPLC analysis of the soluble cofactor (Hausinger et al., 1986). The extinction

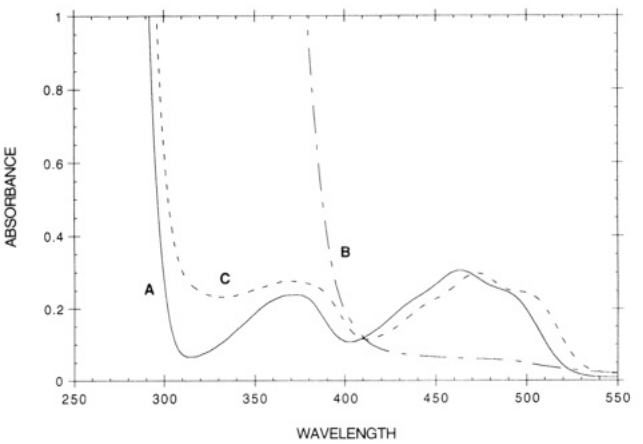


FIGURE 3: Presence of flavin in MurB and its involvement in the reduction of the enolether. (A) Spectrum of MurB. Protein concentration of 0.99 mg/mL was based on protein determination and flavin absorbance at 463 nm using an extinction coefficient of 11 700 M^{-1} cm^{-1} as discussed in Materials and Methods. This corresponds to a flavoenzyme concentration of 2.6×10^{-5} M. (B) Reduction of Enz·FAD by the addition of NADPH (1.0 mM). (C) Reoxidation of Enz·$FADH_2$ by the addition of UDPGlcNAcEP (1.1 mM).

coefficient of bound FAD was 11 700 M^{-1} cm^{-1} at 463 nm found by comparing the absorbance of purified enzyme and released FAD in the supernatant. The loading of MurB active sites by FAD was 100% using a protein concentration as determined by the Bradford assay. At this juncture, it is likely that the A_{463} value is an accurate index of pure holoprotein concentration. Purified MurB has an A_{280}/A_{463} ratio of 9:1 in the range of many other flavoenzymes.

When NADPH was added to samples of purified MurB, the yellow color was immediately bleached, consistent with the expectation that NADPH would reduce the bound FAD to $FADH_2$. The reduction is validated by the quantitative loss of oxidized FAD absorbance at 463 nm (Figure 3, curve B). On addition of the cosubstrate UDPGlcNAcEP (Figure 3, curve C), the E·$FADH_2$ was reoxidized, establishing bound FAD as a conduit of electrons from NADPH to the enolic site of UDPGlcNAcEP undergoing reduction.

NADPH Chirality: Reduction with 4S-[^{2}H]NADPH. To assess the stereospecificity of MurB for the two prochiral hydrogens at C_4 of NADPH, samples of 4*R*-[^{2}H]NADPH and 4*S*-[^{2}H]NADPH were generated and incubated with purified MurB under V_{max} conditions. The 4*R*-[^{2}H]NADPH was oxidized at the same rate as the protio-NADPH, but the 4*S*-[^{2}H]NADPH exhibited a $^D V$ of 2.5 compared to the protio-

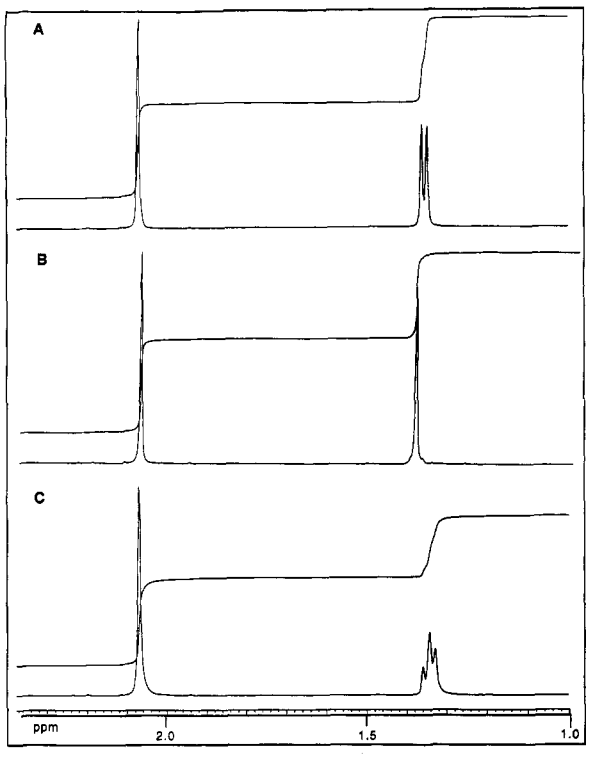


FIGURE 4: NMR spectra of products from the reduction of UDPGlcNAcEP by MurB (1.0–2.3 ppm). (A) UDPMurNAc from H_2O and protio-NADPH. (B) UDPMurNAc from ^2H_2O and protio-NADPH. (C) UDPMurNAc from H_2O and 4*S*-[^{2}H]NADPH.

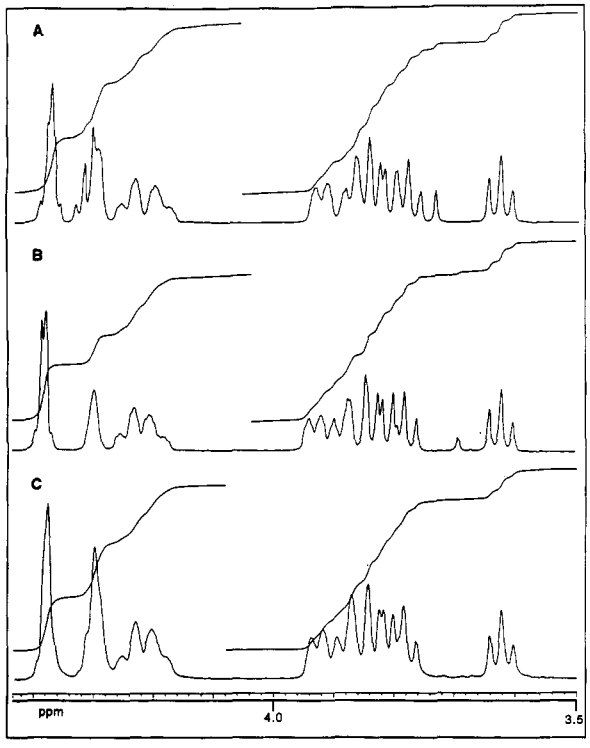


FIGURE 5: NMR spectra of products from the reduction of UDPGlcNAcEP by MurB (3.5–4.4 ppm). (A) UDPMurNAc from H_2O and protio-NADPH. (B) UDPMurNAc from ^2H_2O and protio-NADPH. (C) UDPMurNAc from H_2O and 4*S*-[^{2}H]NADPH.

NADPH. The 4*S*-[^{2}H] removal is partly rate-determining and the V_{max} isotope effect establishes the chirality of NADPH recognition and hydride transfer as 4*S* which is validated by the NMR product analysis below.

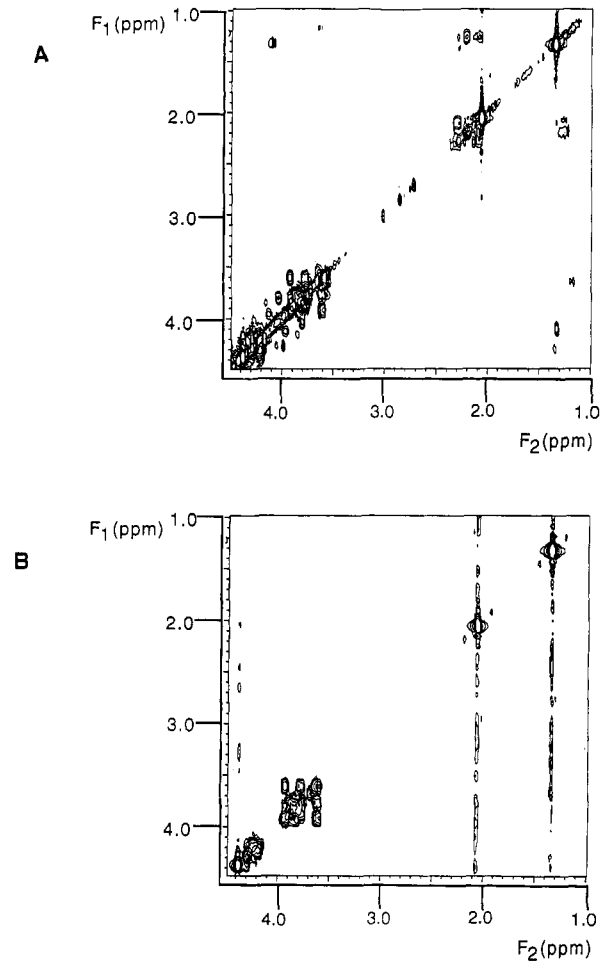


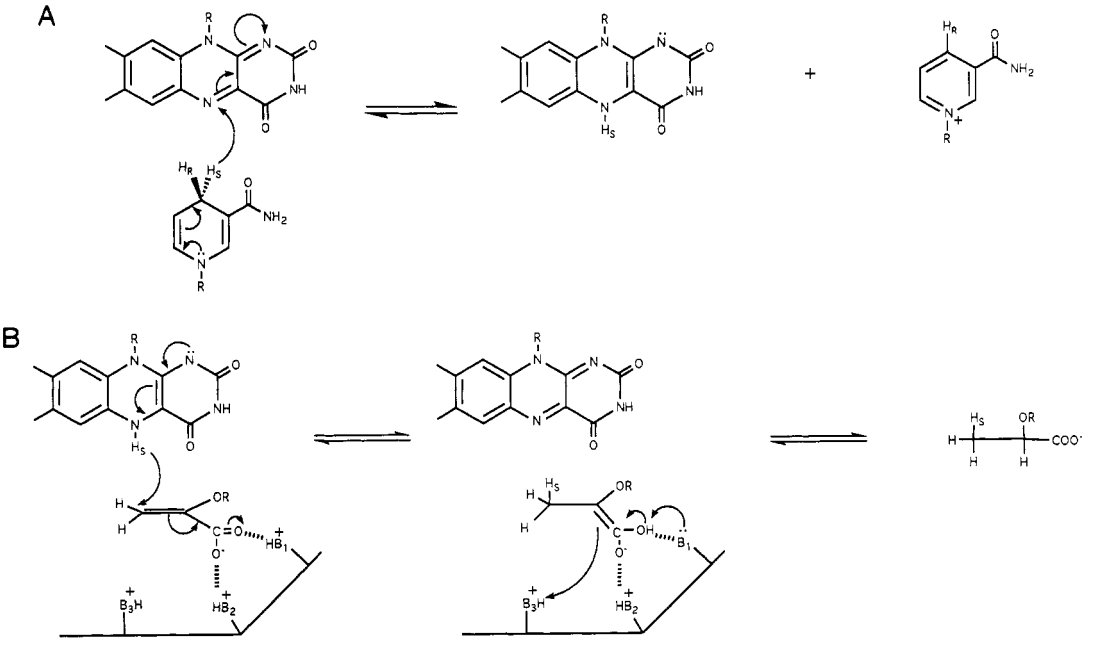
FIGURE 6: 2D absolute value COSYs. (A) UDPMurNAc from H_2O and protio-NADPH. (B) UDPMurNAc from ^2H_2O and protio-NADPH.

Regiospecificity of Enolic Substrate Reduction. The regiospecificity of the enolether reduction in UDPGlcNAcEP was investigated by 1D and 2D NMR.

(*a*) *Analysis of UDPMurNAc from MurB in ^2H_2O.* Incubation of UDPGlcNAc with MurZ (Marquardt et al., 1992) and MurB in the presence of NADPH in ^2H_2O yielded sufficient quantities of the UDP-*N*-acetylmuramic acid product for NMR analysis. The product was separated from NADPH, $NADP^+$, and enolpyruvyl substrate by HPLC. The deuterium content and location were determined from the 1D ^{1}H-NMR spectrum (processed without apodization) and confirmed by the COSY.

The spectra of the sample from the ^2H_2O incubation revealed three differences from the cognate sample from H_2O. (1) The splitting pattern of the 11 CH_3 in the 1D spectrum changed from a doublet in the authentic protio sample to a singlet, while it still integrated to 3 H as compared with the CH_3 of the *N*-acetyl moiety indicating that no deuterium was incorporated at this site (Figure 4B). (2) Integration at 4.3 ppm, where 4 CH and 9 CH overlap, showed a decrease from 2 H at that position to 1 H (Figure 5B). (3) The cross-peak in the 2D COSY between the 11 CH_3 and 9 CH at 1.36, 4.35 ppm was absent in the COSY (Figure 6B). The negative ion FAB mass spectrometry of this compound gave a molecular ion peak (M – 1) at 679 (100%), M – 2 peak at 678 (10.4%), and an isotope peak at 680 (34.5%). Deuterium in the product is incorporated only at one site on the basis of the 680 isotope peak which is comparable to the isotope peak of protio-UDPMurNAc at 679 (32.5%). These data suggest that the

Scheme II: (A) First Half-Reaction: Transfer of 4-*pro*-*S*-H from NADPH to Enz-FAD and (B) Second Half-Reaction: Reduction of the Vinylic Enol Ether by Enz·$FADH_2$



4-*proS*-H of NADPH which had been kinetically sequestered by the enzyme·$FADH_2$ intermediate was subsequently transferred to C_{11}. The location of deuterium at C_9 indicates that this proton is delivered by solvent or, more likely, a solvent-exchangeable enzymatic acid.

(*b*) *Analysis of UDPMurNAc from MurB and 4S-[²H]-NADPH.* A second NMR sample prepared from 4*S*-[²H]-NADPH in H_2O gave a spectrum which had two major changes from the fully protio-UDPMurNAc. (1) Integration of this peak at 1.36 ppm showed only 2 H compared to the 3 H of the *N*-acetyl moiety. (2) The 11 CH_3 splitting pattern is changed from a doublet to a multiplet, probably a doublet of doublets (Figure 4C). As anticipated for a $^2HCH_2$–CH coupling, the two remaining protons are diastereotopic and give an ABX splitting pattern. Analysis of the region from 3.5 to 4.4 ppm (Figure 5C) showed the same integration as the protio form with alteration of the splitting pattern at 4.32 ppm, suggesting that deuterium from the NADPH is incorporated only at the C_{11} site. The cross-peak between 11 CH_2–^2H and 9 CH at 1.36, 4.35 ppm was observed in the COSY (data not shown). Negative ion FAB mass spectrometry of this sample gave a molecular ion peak (M – 1) at 679 (100%), a secondary peak (M – 2) at 678 (38.2%), and an isotope peak at 680 (34.6%). The secondary peak at 678 could represent a fragmentation of the molecule at the C_{11}–^2H bond or the presence of some of the nondeuterated compound. Because the integration of the NMR indicates that there is full deuterium incorporation (±5%), the fragmentation explanation is favored. It is also apparent that the deuterium in the product is incorporated only at one site on the basis of the 680 isotope peak which is comparable to the 679 isotope peak of protio-UDPMurNAc.

DISCUSSION

On overexpression and purification of UDPGlcNAcEP reductase, MurB, to near homogeneity, we find it is an FAD-containing enzyme which validates the involvement of a flavin cofactor in this bacterial peptidoglycan biosynthetic transformation and resolving earlier uncertainty (Taku et al., 1970; Anwar & Vlaovic, 1979). A previous study describing the purification of small amounts of the enzyme from whole cell extracts of *E. coli* did not find associated FAD (Anwar & Vlaovic, 1979). Their reported specific activity was 9-fold lower and SDS–PAGE showed 21- and 14-kDa fragments suggesting, in retrospect, proteolytic cleavage of the 35-kDa polypeptide (Anwar & Vlaovic, 1979); therefore, FAD binding may have been weakened. The 1:1 stoichiometry of FAD to MurB apoprotein and FAD reduction by NADPH and reoxidation by the specific cosubstrate UDPGlcNAcEP clearly demonstrate the presence of FAD in MurB and its involvement in catalysis.

These facts suggest a sequence of two half-reactions involving NADPH generation of E·$FADH_2$ followed by two-electron transfer from E·$FADH_2$ for the vinylic enolether reduction. Precedent strongly suggests that NADPH transfers a hydride equivalent to N_5 of FAD on flavin coenzyme reduction (Manstein et al., 1988). Precedent also suggests that a mechanism with a hydride from N_5 of E·$FADH_2$ is reasonable for the vinylic reduction half-reaction, but recent data on the analogous acyl-CoA desaturation flavoenzymes have been interpreted in favor of one-electron transfers (Lenn et al., 1990) between flavin cofactor and olefinic substrate.

The *murB*-encoded gene product may now be classified as a 4-*pro*-*S*-NADPH-utilizing enzyme on the basis of both the V_{max} isotope effect data and NMR analysis of labeled products. The 4*S* deuteride equivalent ends up at the C_{11} methyl of the lactyl moiety of the product while a deuterium from solvent is incorporated at C_9. This regiochemistry suggests the mechanism depicted in Scheme II via the two indicated half-reactions. The second half-reaction postulates delivery of a hydride equivalent from N_5 of E·$FADH_2$ to the vinyl terminus of the enolpyruvate group yielding a carbanionic transition state or intermediate at C_9 which would then be protonated by a solvent equilibrated proton.

Viewed in the back direction, catalysis would involve abstraction of the C_9-H by an active site base exposing a C_9 carbanion, however transiently. The site α to the COO^- is the more acidic, and it seems a reasonable location for a

carbanionic transition state, relative to C_{11}. However, Gerlt and Gassman (1992) have recently pointed out the likely inadequacy of the kinetic acidity of protons α to carbonyls, including carboxylates, without some concerted protonation of the carboxylate. Thus, one may formulate the second half-reaction in Scheme II with stabilization of a C_9 carbanionic transition state by a protonated *aci* carboxylate tautomer to lower activation barriers. The C_9 center of the lactyl group is known to be of *D* configuration (Veyrières & Jeanloz, 1970), but the stereochemistry (*si* or *re*) of the vinylic ether reduction is not known.

In contrast with most other flavoproteins utilizing NADPH, the 4-*pro*-*S*-derived hydrogen does not exchange with solvent but is kinetically sequestered from bulk water and is transferred onto C_{11} of product before it can exchange. Tritium transfer from 4*R*,*S*-[^{3}H]NADPH to UDP-*N*-acetylmuramic acid was reported in the initial studies of MurB (Anwar & Vlaovic, 1979). This sequestration of migrating hydrogen is seen with two other flavoenzymes, the NADPH/$NADP^+$ transhydrogenase (Louie & Kaplan, 1970) and *N*-methylglutamate synthase (Jorns & Hersch, 1974). Transfer of this H_s at some stage in catalysis (either to or from N_5 of FAD) is substantially rate-limiting in overall catalysis given the ^DV of 2.5 observed with 4*S*-[^{2}H]NADPH.

With this new insight into the mechanism of vinylic enolether reduction and the identification of MurB as a flavoenzyme catalyst for reduction of this unusual enolether, it remains to be seen if MurB will be a new target for design of enzyme inhibitors of bacterial peptidoglycan biosynthesis.

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REFERENCES

Anwar, R. A., & Vlaovic, M. (1979) *Can. J. Biochem. 57*, 188–196.

Bradford, M. (1976) *Anal. Biochem. 72*, 248.

Bugg, T. D. H., & Walsh, C. T. (1993) *Nat. Prod. Rep.* (in press).

Gerlt, J. A., & Gassman, P. G. (1992) *J. Am. Chem. Soc. 114*, 5928–5934.

Gunetileke, K. G., & Anwar, R. A. (1968) *J. Biol. Chem. 243*, 5770–5778.

Hausinger, R. P., Honek, J. F., & Walsh, C. T. (1986) *Methods Enzymol. 122*, 199–209.

Heidlas, J. E., Lees, W. J., Pale, P., & Whitesides, G. M. (1992) *J. Org. Chem. 57*, 146–151.

Jorns, M. S., & Hersch, L. B. (1974) *J. Am. Chem. Soc. 96*, 4012–4014.

Kahan, F. M., Kahan, J. S., Cassidy, P. J., & Kropp, H. (1974) *Ann. N.Y. Acad. Sci. 235*, 364–386.

Lenn, N. D., Stankovich, M. T., & Liu, H. W. (1990) *Biochemistry 29*, 3709–3715.

Louie, D. D., & Kaplan, N. O. (1970) *J. Biol. Chem. 245*, 5691–5698.

MacFerrin, K. D., Terranova, M. P., Schreiber, S. L., & Verdine, G. L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1937–1941.

Manstein, D. J., Massey, V., Ghisla, S., & Pai, E. F. (1988) *Biochemistry 27*, 2300–2305.

Marquardt, J. L., Siegele, D. S., Kolter, R., & Walsh, C. T. (1992) *J. Bacteriol. 174*, 5748–5752.

Mengin-Lecreulx, D., Flouret, B., & van Heijenoort, J. (1983) *J. Bacteriol. 154*, 1284–1290.

Orr, G. A., & Blanchard, J. S. (1984) *Anal. Biochem. 142*, 232–234.

Park, J. T. (1987) in *Escherichia coli* and *Salmonella typhimurium* (Neidhardt, F. C., Ed.) pp 663–671, American Society for Microbiology, Washington.

Piantini, U., Sørensen, O. W., & Ernst, R. R. (1982) *J. Am. Chem. Soc. 104*, 6800–6801.

Ploux, O., Masamune, S., & Walsh, C. T. (1988) *Eur. J. Biochem. 174*, 177–182.

Pucci, M. J., Discotto, L. F., & Dougherty, T. J. (1992) *J. Bacteriol. 174*, 1690–1693.

Rance, M., Sørensen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 117*, 479–485.

Schreiber, S. L., & Verdine, G. L. (1991) *Tetrahedron 47*, 2543–2562.

Shaka, A. J., & Freeman, R. (1983) *J. Magn. Reson. 51*, 169–173.

Taku, A., Gunetileke, K. G., & Anwar, R. A. (1970) *J. Biol. Chem. 245*, 5012–5016.

Veyrières, A., & Jeanloz, R. W. (1970) *Biochemistry 9*, 4153–4159.

Wanke, C., Falchetto, R., & Amrhein, N. (1992) *FEBS Lett. 301*, 271–276.

Yuan, Z., & Hammes, G. G. (1984) *J. Biol. Chem. 259*, 6748–6751.

Zemell, R. I., & Anwar, R. A. (1975) *J. Biol. Chem. 250*, 4959–4964.