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Residues C123 and D58 of the 2-Methylisocitrate Lyase (PrpB) Enzyme of *Salmonella enterica* Are Essential for Catalysis

T. L. Grimek,[1] H. Holden,[2] I. Rayment,[2] and J. C. Escalante-Semerena[1]*

Departments of Bacteriology[1] and Biochemistry,[2] University of Wisconsin—Madison, Madison, Wisconsin

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The *prpB* gene of *Salmonella enterica* serovar Typhimurium LT2 encodes a protein with 2-methylisocitrate (2-MIC) lyase activity, which cleaves 2-MIC into pyruvate and succinate during the conversion of propionate to pyruvate via the 2-methylcitric acid cycle. This paper reports the isolation and kinetic characterization of wild-type and five mutant PrpB proteins. Wild-type PrpB protein had a molecular mass of approximately 32 kDa per subunit, and the biologically active enzyme was comprised of four subunits. Optimal 2-MIC lyase activity was measured at pH 7.5 and 50°C, and the reaction required Mg^{2+} ions; equimolar concentrations of Mn^{2+} ions were a poor substitute for Mg^{2+} (28% specific activity). Dithiothreitol (DTT) or reduced glutathione (GSH) was required for optimal activity; the role of DTT or GSH was apparently not to reduce disulfide bonds, since the disulfide-specific reducing agent Tris(2-carboxyethyl)phosphine hydrochloride failed to substitute for DTT or GSH. The K_m of PrpB for 2-MIC was measured at 19 μM, with a k_{cat} of 105 s^{-1}. Mutations in the *prpB* gene were introduced by site-directed mutagenesis based on the active-site residues deemed important for catalysis in the closely related phosphoenolpyruvate mutase and isocitrate lyase enzymes. Residues D58, K121, C123, and H125 of PrpB were changed to alanine, and residue R122 was changed to lysine. Nondenaturing polyacrylamide gel electrophoresis indicated that all mutant PrpB proteins retained the same oligomeric state of the wild-type enzyme, which is known to form tetramers. The $PrpB^{K121A}$, $PrpB^{H125A}$, and $PrpB^{R122K}$ mutant proteins formed enzymes that had 1,050-, 750-, and 2-fold decreases in k_{cat} for 2-MIC lyase activity, respectively. The $PrpB^{D58A}$ and $PrpB^{C123A}$ proteins formed tetramers that displayed no detectable 2-MIC lyase activity indicating that both of these residues are essential for catalysis. Based on the proposed mechanism of the closely related isocitrate lyases, PrpB residue C123 is proposed to serve as the active site base, and residue D58 is critical for the coordination of a required Mg^{2+} ion.

Propionate catabolism in *Salmonella enterica* serovar Typhimurium LT2 (herein referred to as serovar Typhimurium) occurs via the 2-methylcitric acid (2-MC) cycle (Fig. 1) (18). The 2-MC cycle of propionate metabolism was first identified in the yeast *Yarrowia lipolytica* and several years later was found to exist in prokaryotes (8, 18, 36, 37). The prokaryotic enzymes of this pathway have been characterized. PrpE is the propionyl coenzyme A synthetase (14, 17), PrpC is the 2-MC synthase (18, 39), PrpD is the 2-MC dehydratase (8, 15), and PrpB is the 2-methylisocitrate (2-MIC) lyase (7, 18). This work focuses on the serovar Typhimurium PrpB enzyme, which catalyzes the cleavage of 2-MIC into pyruvate and succinate (15).

Comparisons of the amino acid sequence of the PrpB protein to other protein sequences identified it as a homolog of carboxyphosphonoenolpyruvate (CPEP) mutase, phosphoenolpyruvate (PEP) mutase, and isocitrate lyases (ICL) (16). The mutases are anabolic enzymes used for the production of secondary metabolites and the only enzymes known to create a carbon-phosphorus bond (13, 27), while the lyases are catabolic enzymes (18, 24, 43). Even though the reactions catalyzed by these enzymes are different, they are all proposed to proceed via mechanisms that stabilize enol(ate) intermediates (32). Based on the similarities shared by these enzymes, it has been proposed that these proteins represent a new α/β-barrel superfamily (19). The α/β-barrel proteins are known to be a very diverse group of enzymes that may catalyze different reactions by binding substrate differently inside the barrel. The evolutionary link between the mutases and the lyases is strengthened by the fact that the key active-site residues required for PEP mutase catalysis are invariant in the lyases (19). Although the PrpB enzyme catalyzes a reaction similar to that catalyzed by ICL, the PrpB enzyme shares more sequence identity with CPEP and PEP mutases (36 and 30% end-to-end identity, respectively) than with ICL (ca. 20% identity).

Structure-function analyses of PEP mutase have shed valuable insights into how this enzyme and probably how ICL and 2-MIC lyase (2-MICL) enzymes may work. Early studies of the PEP mutase mechanism entertained the possibility that residue D58 could serve as a phosphoryl group acceptor during a reaction involving a modified enzyme intermediate (20). Recently reported evidence, however, ruled out a phosphoryl transfer mechanism directly involving residue D58, and proposed that PEP mutase catalyzes the phosphoryl transfer via a dissociative mechanism with residues R159 and H190 residues holding the phosporyl-metaphosphate-phosphonyl group stationary during the reaction (23). Mechanisms of catalysis have also been proposed for the ICL proteins based on three-dimensional structures and data from active-site mutant proteins (5, 6, 34). Based on ICL structures with bound inhibitors and previous inhibition experiments using 3-bromopyruvate and iodoacetate, it was concluded that a cysteinyl residue was most likely the active-site residue required for isocitrate cleavage (5, 6, 21, 26, 34). It is interesting that an equivalently positioned

* Corresponding author. Mailing address: Department of Bacteriology, University of Wisconsin, 1710 University Ave., Madison, WI 53726-4087. Phone: (608) 262-7379. Fax: (608) 265-7909. E-mail: escalante@bact.wisc.edu.

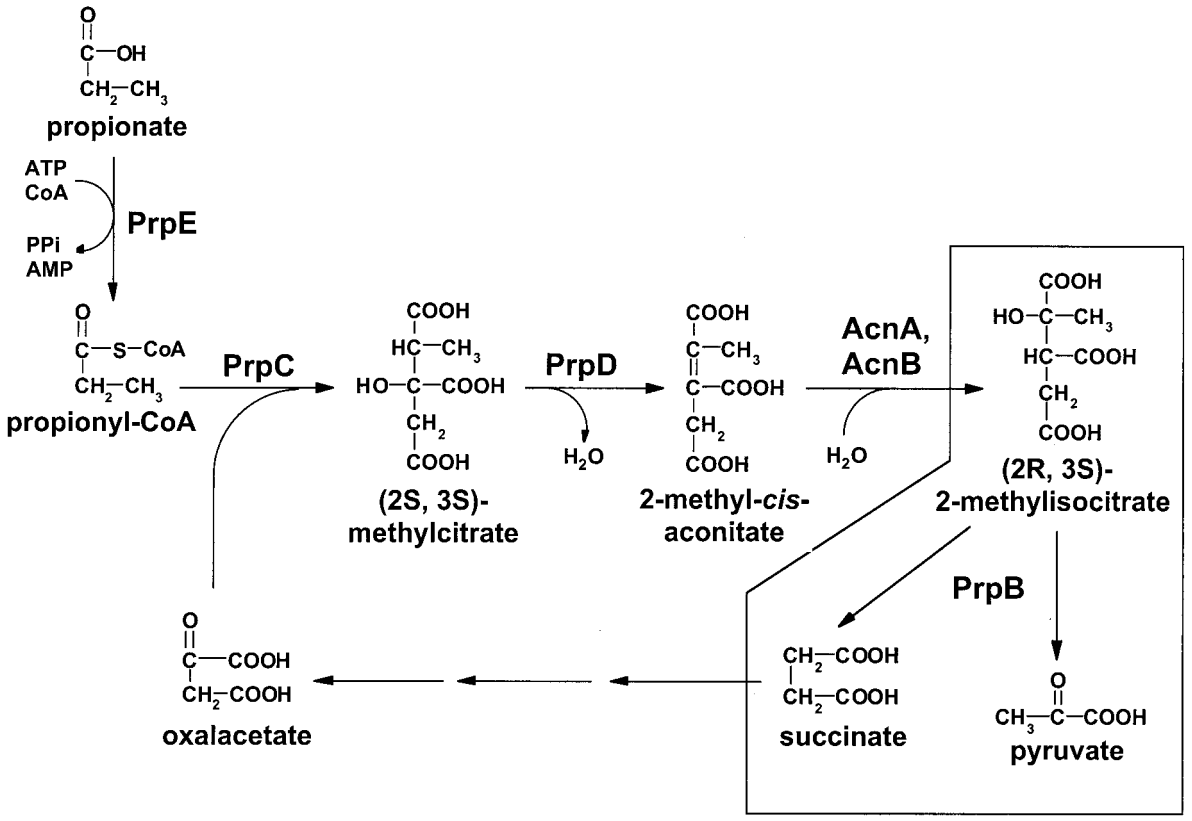

FIG. 1. 2-MC cycle of serovar Typhimurium. The boxed area of the scheme highlights the reaction catalyzed by the 2-MICL (PrpB) enzyme, the focus of the present work.

cysteinyl residue is not conserved in PEP mutase, yet is conserved in CPEP mutase and the lyases (Fig. 2). Crystal structures of ICL from three different organisms have revealed an electronegative depression which is responsible for the binding of a Mg^{2+} ion in each of the structures; this negatively charged region is created by four carboxyl groups corresponding to residues D58, D85, D87 and E115 of serovar Typhimurium PrpB (5, 6, 34).

Recent studies compared 2-MICL activities of PrpB proteins from *Aspergillus nidulans* and *Escherichia coli* (7). However, these studies did not look into the roles of specific amino acid residues in the reaction mechanism of PrpB from these organisms. Work reported here was undertaken to investigate the importance of active-site residues of PrpB from serovar Typhimurium. This work reports the cloning, isolation, and biochemical characterization of the wild-type PrpB and five site-directed PrpB mutant proteins. The data obtained from the kinetic analysis of the purified enzymes indicate that residues C123 and D58 are crucial for PrpB 2-MICL activity. During the review of this paper, the three-dimensional crystal structure of apo-PrpB enzyme from *E. coli* was published (11). The lack of substrate in the active site of the enzyme, however, resulted in a disordered C123 residue; hence, its role in the reaction could not be established. However, the reported model postulated a role of residue D58 consistent with the conclusions of the work reported in this paper.

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S.T. PrpB     54  LGLPDLG  60  115 EDQVG-AKRCGHRP 127
E.c. PrpB     54  LGLPDLG  60  115 EDQVG-AKRCGHRP 127
S.h. CPEPmu   53  LGLPDLG  59  113 EDQVN-PKRCGHLE 125
M.e. PEPmu    55  LGVRDSN  61  115 EDKLF-PKTNSLHD 127
E.c. ICL     104  SMYPDQS 110  186 EDQLASVKKCGHMG 199
                   *  .         **::   *  .
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FIG. 2. PrpB homologs. Amino acid sequence alignments between serovar Typhimurium (*S*.T.) PrpB, *E. coli* K-12 (*E.c.*) PrpB, *Streptomyces hygroscopicus* CPEP mutase (*S.h.* CPEPmu), *M. edulis* PEP mutase (*M.e.* PEPmu) and *E. coli* K-12 ICL. Boxed residues correspond to those implicated in the PEP mutase or ICL reaction mechanisms as described in the text. Serovar Typhimurium PrpB protein sequence was compared with sequences deposited in GenBank using the BLAST algorithm (1, 2). Sequence alignments between serovar Typhimurium PrpB and other homologs were performed by ClustalW (40).

MATERIALS AND METHODS

Chemicals and culture media. Chemicals and rabbit muscle lactate dehydrogenase (LDH) were obtained from Sigma Chemical (St. Louis, Mo.). Restriction enzymes, dithiothreitol (DTT), and deoxyribonucleoside triphosphates were purchased from Promega (Madison, Wis.); primers were synthesized by IDTDNA (Coralville, Iowa); NaCl, $MgCl_2$, 2-(4-morpholino)-ethanesulfonic acid (MES), HEPES, *N*-[2-hydroxyethylpiperazine-*N*′-[3-propanesulfonic acid] (EPPS), kanamycin (KAN), and ampicillin (AMP) were acquired from Fisher Biotech (Itasca, Ill.); Tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) was purchased from Pierce Chemical Co. (Rockford, Ill.); His-bind resin and thrombin cleavage kits were obtained from Novagen (Madison, Wis.); Luria-Bertani (LB) medium was purchased from Difco (Detroit, Mich.); *threo*-2-MIC was a kind gift from

TABLE 1. Strains and plasmids used in this study[a]

Strain or plasmid	Genotype	Reference or source[b]
Strains		
JR501	*hsdSA29 hsdSB121 hsdL6 metA22 metE551 trpC2 ilv-452 rpsL120 xyl-404 galE719 H1-b H2-en,n,x* [cured of *Fels2*(−)] *fla-66 nml*	41
TR6583[c]	*metE205 ara-9*	K. Sanderson via J. Roth
Derivatives of TR6583		
JE3946	*prpB195 zai-6386*::Tn*10d*(Tc)[d]	16
JE3961	*prpB210 zai-6386*::Tn*10d*(Tc)	16
JE4016	pPRP21 *bla*$^+$	16
JE4175	pBAD30 *bla*$^+$	Laboratory collection
JE4182	pGP1-2 *kan*$^+$	Laboratory collection
JE5256	*prpB121*::Tn*10d*(Tc) pGP1-2 *kan*$^+$	
Plasmids		
pGP1-2	Plasmid carrying the T7 *rpo*$^+$ RNA polymerase, *kan*$^+$	35
pBAD30	P_{araBAD} expression vector, *bla*$^+$	12
pET15b	Plasmid for constructing N-terminal hexahistidine-tagged proteins	Novagen
pPRP21	*prpB*$^+$ in pBAD30 *bla*$^+$	16
pPRP65	*prpB*$^+$ in pET15b *bla*$^+$	
pPRP75	Hexahistidine-tagged *prpB*$^+$ in pBAD30 *bla*$^+$	
pPRP76	Hexahistidine-tagged *prpB*$^+$ D58A in pBAD30 *bla*$^+$	
pPRP131	Hexahistidine-tagged *prpB*$^+$ R122K in pBAD30 *bla*$^+$	
pPRP78	Hexahistidine-tagged *prpB*$^+$ H125A in pBAD30 *bla*$^+$	
pPRP79	Hexahistidine-tagged *prpB*$^+$ C123A in pBAD30 *bla*$^+$	
pPRP80	Hexahistidine-tagged *prpB*$^+$ K121A in pBAD30 *bla*$^+$	
pPRP81	Hexahistidine-tagged *prpB*$^+$ D58A in pET15b *bla*$^+$	
pPRP132	Hexahistidine-tagged *prpB*$^+$ R122K in pET15b *bla*$^+$	
pPRP84	Hexahistidine-tagged *prpB*$^+$ H125A in pET15b *bla*$^+$	
pPRP85	Hexahistidine-tagged *prpB*$^+$ C123A in pET15b *bla*$^+$	
pPRP86	Hexahistidine-tagged *prpB*$^+$ K121A in pET15b *bla*$^+$	

[a] All serovar Typhimurium strains are derivatives of the *S. enterica* serovar Typhimurium LT2 strain.
[b] Unless otherwise stated, strains and plasmids were constructed during the course of this work.
[c] Formerly SA2979.
[d] Tn*10*d(Tc) is an abbreviation of Tn*10*Δ*16*Δ*17* (44).

W. W. Cleland (31). The genotypes of strains and plasmids used in this work are shown in Table 1.

Construction of plasmids. (i) Plasmid pPRP65. A 1-kb *Nde*I-*Bam*HI fragment from plasmid pPRP19 (16) containing the *prpB* gene was ligated into the cloning vector pET-15b (Novagen) cut with the same enzymes. The resulting plasmid, pPRP65 *bla*$^+$, contained a *prpB* gene that encoded an N-terminally tagged hexahistidine (H_6PrpB) fusion protein.

(ii) Plasmid pPRP75. A 1-kb *Xba*I fragment from plasmid pPRP65 was ligated into the arabinose-inducible vector pBAD30 (12) and transformed into the restriction-deficient modification-proficient serovar Typhimurium strain JR501 (41) as described (33). The resultant colonies were screened for the *prpB* gene in the correct orientation and the resulting plasmid was named pPRP75.

(iii) Plasmids pPRP76, pPRP78-80, and pPRP131. Site-directed mutagenesis was performed via the three-primer PCR mutagenesis (25) using plasmid pPRP65 as a template. Primers were designed flanking the T7 promoter and T7 terminator sites of plasmid pPRP65; primer site 5′-GAATTGTGAGCGGATA AC-3′ for the T7 promoter region, and primer site 5′-AGCCAACTAAGCTTC CTTTCGG-3′ for the T7 terminator region. Mutagenic primers contained 5′ phosphorylation modifications; sequences of the primers used are available upon request. PCR amplification products were purified using the QIAquick PCR Purification kit (Qiagen) and cut with the restriction enzyme *Xba*I. The restriction fragments were cleaned and ligated with plasmid pBAD30 cut with *Xba*I. Ligation products were introduced into electrocompetent cells of strain JR501 via electroporation and these cells were transferred onto LB plates supplemented with AMP (100 μg/ml). Colonies were screened for vector containing insert in the correct orientation. Each plasmid was sequenced using pBAD30 primers (pBAD30-3′, 5′-GGCTGAAAATCTTCTCTCAT-3′; pBAD30-5′, 5′-C AACTCTCTACTGTTTCTTT-3′) to determine whether the correct mutation was incorporated into the *prpB* gene and that no other mutations were introduced during amplification. PCR sequencing reactions were prepared using Big Dye (Biotechnology Center, University of Wisconsin—Madison). Reactions were purified in AutoSeq G-50 columns (Pharmacia Biotech), dried in a SpeedVac concentrator (Savant), and sequenced at the Biotechnology Center (University of Wisconsin—Madison). Plasmids were named pPRP76 (*prpB*^{D58A}), pPRP78 (*prpB*^{H125A}), pPRP79 (*prpB*^{C123A}), pPRP80 (*prpB*^{K121A}), and pPRP131 (*prpB*^{R122K}) (Table 1). Plasmids from strain JR501 were introduced into desired genetic backgrounds by transformation (29).

(iv) Plasmids pPRP82, pPRP84-86, and pPRP132. Plasmids pPRP76, pPRP78-80, and pPRP131 were cut with *Nco*I-*Hin*dIII and ligated into plasmid pET15b cut with the same enzymes to yield plasmids pPRP82, pPRP84-86, and pPRP132, respectively. *prpB* alleles in plasmid pET15b were used to overproduce mutant PrpB proteins.

Sequencing mutant alleles of *prpB*. To determine the locations of *prpB* mutations in strains JE3946 and JE3961, overnight cultures were grown in LB medium and boiled in sterile water (1:10 dilutions) as templates. PCRs were prepared with 10 μl of template in a 100-μl reaction containing 50 pmol of each primer, a 0.2 mM concentration of each deoxyribonucleoside triphosphate, and 3 U of ENZY+ DNA polymerase (Enzypol Ltd.) according to the manufacturer's instructions. Reactions were performed in a GeneAmp PCR system 2400 (Perkin-Elmer, Norwalk, Conn.) using the following conditions: 30 cycles at 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min. Primers used were 5′-CTCGCT GCCTCTTTGCCTGGC-3′ (60 bp upstream of *prpB*) and 5′-GCATTGCCTG ATGGCGGCTTCG-3′ (23 bp downstream of *prpB*). Amplified DNA was purified using the QIAquick PCR purification kit (Qiagen). PCR sequencing reactions were carried out as described above.

Complementation analysis. Plasmids pBAD30, pPRP21, pPRP75-6, pPRP78-80, and pPRP131 were each introduced into mutant strain JE3946. These resulting strains were grown overnight in LB medium containing AMP (100 μg/ml). Four microliters of each overnight culture was inoculated into wells of a 96-well Falcon (Becton Dickinson) microtiter dish containing 200 μl of a no-carbon E (NCE) medium (3) with methionine (0.5 mM), propionate (30 mM), $MgSO_4$ (1 mM), glycerol (1 mM), AMP (50 μg/ml), and various amounts of L-(+)-arabinose (0, 50, 100, 250, or 500 μM). The density of the cultures in the wells was monitored at 650 nm with a SpectraMAX Plus spectrophotometer (Molecular

Devices). The plate chamber was maintained at 37°C. Absorbance measurements were taken every 15 min for 72 h with agitation (780 s) between reads.

Overproduction and purification of H_6PrpB and mutant proteins. Plasmids pPRP65, pPRP82, pPRP84-86, and pPRP132 were introduced into serovar Typhimurium strain JE5256 [*metE205 ara-9 prpB121*::Tn*10*d(Tc)/pGP1-2 *rpo*$^+$ *kan*$^+$]. The resulting strains were used to overproduce and purify PrpB and the PrpB variant proteins. Each strain was inoculated into 25 ml of LB broth containing AMP (100 μg/ml) and KAN (60 μg/ml) at 30°C. From the overnight cultures, 20 ml was inoculated into 2 liters of LB broth supplemented with AMP (100 μg/ml) and KAN (60 μg/ml). Cultures were grown in at 30°C to an A_{590} of 0.5 and transferred to a 42°C water bath for 1 h to induce synthesis of the T7 RNA polymerase. Cultures were incubated for 4 h at 37°C and harvested by centrifugation at 11,700 × *g* with a SS34 rotor for 10 min in a Sorvall RC-5B refrigerated centrifuge (DuPont). Cell pellets were resuspended in 40 ml of 20 mM Tris-HCl buffer, pH 7.9, containing 0.5 M NaCl and 5 mM imidazole. Cells were broken with a French pressure cell at ~10^4 kPa. Cellular debris was discarded after centrifugation at 39,000 × *g* for 45 min at 4°C. H_6PrpB and mutant proteins were isolated from crude supernatants by nickel-affinity chromatography according to the manufacturer's (Novagen) instructions. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (22) with 12% polyacrylamide gels and visualized by Coomassie blue R-250 staining (30). Fractions containing purified (>95%) H_6PrpB wild-type and mutant proteins were dialyzed overnight at 4°C against 1 liter of K^+ HEPES buffer (pH 7.5; 50 mM), EDTA (2.5 mM), and DTT (5 mM). Proteins were then dialyzed twice into 1 liter K^+ HEPES buffer, pH 7.5 (50 mM), and DTT (5 mM) for 2 h each. The hexahistidine tag was removed with the thrombin cleavage capture kit of Novagen according to the manufacturer's instructions. Resulting PrpB proteins had three extra amino acids at its N terminus (Gly, Ser, and His). Detagged proteins were dialyzed into 1 liter of K^+ HEPES buffer (pH 7.5; 50 mM) and DTT (5 mM) for 2 h. Glycerol (5% [vol/vol] final concentration) was added to each protein solution. The proteins were flash-frozen as drops in liquid nitrogen and stored at −85°C. The yield obtained under these conditions was ~25 mg of protein per liter of culture. No detectable loss of activity was observed after 6 months of storage.

2-MICL assay. Protein concentrations were determined by the method of Bradford (4) using a standard curve generated with bovine serum albumin (Sigma). Lyophilized 2-MIC was dissolved in 0.6 M NaOH and heated at 90°C for 45 min to saponify the lactone ring. Saponified 2-MIC was diluted into 50 mM HEPES buffer, pH 7.5, before use in assays. The reaction mixture (final volume,1 ml) for the 2-MICL assay contained K^+ HEPES buffer (pH 7.5; 50 mM), $MgCl_2$ (2.5 mM), DTT (5 mM), rabbit muscle LDH (1 U), NADH (250 μM), PrpB protein, and various concentrations of 2-MIC. Detagged PrpB protein was used in all assays unless specified otherwise. The progress of the reaction was monitored as a decrease in absorbance at 340 nm in a computer-controlled Perkin-Elmer Lambda 40 UV/VIS spectrophotometer (Perkin-Elmer). The assay cuvettes were maintained at 37°C with a circulating water bath. The extinction coefficient for NADH at 37°C was taken to be 6,110 mM^{-1} cm^{-1} (10). Inactive PrpB mutant proteins were tested for 2-MIC cleavage over 24 h. For this purpose, PrpB protein (up to 100 μg) was added to K^+ HEPES buffer (pH 7.5; 50 mM), $MgCl_2$ (2.5 mM), and DTT (5 mM) in sealed vials, the headspace was flushed with N_2 for 15 min, and these vials were incubated at 30°C for 24 h. Activity was assayed by adding rabbit muscle LDH (1 U) and NADH (250 μM) and monitored for a decrease in absorbance at 340 nm as described above.

Optimization of assay conditions. For coupled assays, the amount of LDH in the reaction mixture was maintained at an activity level of at least 10-fold excess over that of the PrpB protein.

pH. The average of at least three independent reactions is reported using the following buffers: MES, pH 6.0 and 6.5; HEPES, pH 7.0, 7.5, and 8.0; and EPPS, pH 8.5.

Temperature. To determine the temperature profile of the reaction the assay mixture without enzyme was preincubated at the specified temperatures for 5 min and the reaction was started by the addition of PrpB protein. The stability of PrpB proteins at different temperatures was assessed by incubation at the specified temperature for 10 min and then assaying for 2-MICL activity at 37°C.

Metal requirement and ionic strength. Assays were conducted as described above with either variation of the concentration of $MgCl_2$ (0 to 5 mM), replacement of $MgCl_2$ with $MnCl_2$ (5 mM), or addition of NaCl (0 to 250 mM) to the assay mixture.

Kinetic analyses. Kinetic constants for PrpB activity were determined by varying the concentration of 2-MIC. Assay sets contained at least 10 points and each set was performed in quadruplicate. Initial velocity data were analyzed using the program GraFit (version 4.0; Erithacus Software Ltd., Staines, United Kingdom). The k_{cat} values were calculated from V_{max} and [E] according to the equation $k_{cat} = V_{max}/[E]$ where V_{max} is the maximal velocity (determined from the GraFit results) and [*E*] is the molar concentration of the PrpB tetramer based on protein concentrations as determined by the Bradford assay (4).

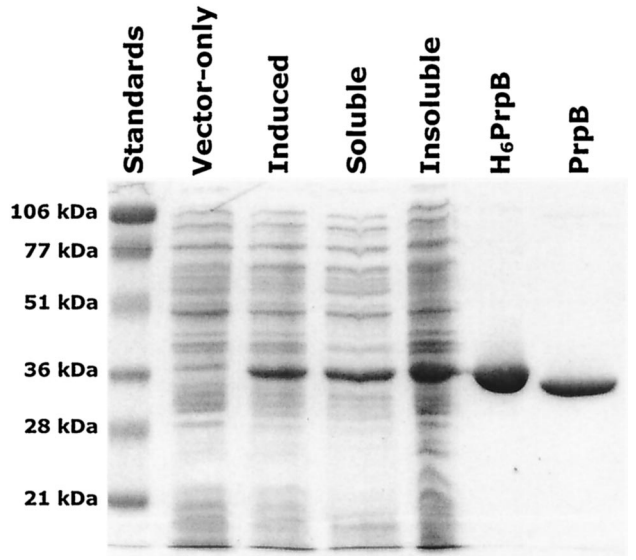

FIG. 3. Overproduction and purification of hexahistidine-tagged and detagged PrpB proteins. Lanes: Standards, Novagen Perfect Protein SDS-PAGE markers; Vector-only, pET15b vector-only control; Induced, H_6PrpB overproduction; Soluble and Insoluble, soluble and insoluble protein of H_6PrpB overproduction cell extracts, respectively; H_6PrpB, purification of H_6PrpB after nickel chelation-affinity chromatography; PrpB, purified detagged PrpB after cleavage with thrombin.

ICL assay. Due to the low detection limit of glyoxylate by the 2-MICL assay utilizing LDH as a coupling enzyme, a phenylhydrazine assay was used to test for isocitrate cleavage by PrpB and PrpB^{R122K} enzymes. The reaction mixture contained 2.5 mM $MgCl_2$, 4 mM phenylhydrazine-HCl, and 5 mM DTT. Progress of the reaction was monitored at 324 nm. Commercially available ICL (Sigma) was used as positive control. Reactions were also allowed to proceed for 24 h to determine if any remaining activity was associated with mutant proteins. The extinction coefficients of glyoxylate-phenylhydrazone (16.8 mM^{-1} cm^{-1}) and pyruvate phenylhydrazone (10.2 mM^{-1} cm^{-1}) have been reported (38).

Immunoblot analysis. Serovar Typhimurium strains JE3946 and JE3961 carry mutant *prpB* alleles (16). These strains along with *prpB*$^+$ strain TR6583 were grown overnight in 5 ml of an NCE medium (3) supplemented with methionine (0.5 mM), $MgSO_4$ (1 mM), glycerol (22 mM), and propionate (5 mM) to induce expression of the *prpBCDE* operon (42). One milliliter of each culture was harvested, cells were broken open with BugBuster (Novagen) according to the manufacturer's specifications, and 15 μg of protein from each cell extract was resolved by SDS-PAGE (22). Protein was transferred to nitrocellulose membrane and blocked with powdered milk (45). Blots were probed using polyclonal anti-PrpB antibodies generated against purified H_6PrpB protein at the University of Wisconsin—Madison Animal Care Unit. Blots were probed with donkey anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Pierce) and developed with ECLPlus Western blotting detection system (Amersham Pharmacia, Little Chalfont, Buckinghamshire, England).

RESULTS

Overproduction and purification of H_6PrpB protein. Serovar Typhimurium strain JE5256 [*metE205 ara-9 prpB121*::Tn*10*d(Tc)/pGP1-2 *rpo*$^+$ *kan*$^+$] was used to overexpress *prpB* from plasmid pPRP65. This strain and another containing pET15b (vector-only control) were grown under conditions that overexpressed *prpB*. H_6PrpB protein was purified using Ni-affinity chromatography. Samples were analyzed by SDS-PAGE (Fig. 3). H_6PrpB was identified as the prominent 34-

kDa band from crude whole-cell extracts. This band was absent in cell extracts of the control strain. Soluble H_6PrpB protein was separated by centrifugation from insoluble protein. Homogenous H_6PrpB protein was treated with thrombin protease to remove the hexahistidine tag. The resulting protein (32 kDa) was greater than 95% pure.

Characterization of the 2-MICL activity. Kinetic parameters for the 2-MICL activity were determined using the continuous spectrophotometric assay described above to detect pyruvate. The pH optimum for 2-MICL activity was measured between 7.5 and 8.0. The PrpB enzyme showed almost maximal activity at pH 8.5 (96%) but retained only 16% of its specific activity at pH 6.0. pH values above 8.5 were not tested due to the low activity of the coupling enzyme LDH at higher pH. 2-MICL activity increased as a function of temperature up to 50°C; at 60°C, the enzyme lost all detectable activity, most likely due to the instability of the protein fold at high temperature. Although maximal activity was measured at 50°C, the more physiological temperature of 37°C was used for assays. For temperature stability analysis, the PrpB enzyme was incubated for 10 min at different temperatures and then assayed for activity at 37°C. The PrpB enzyme showed no decrease in specific activity when preincubated at 30 and 37°C, but only retained 14% of its activity after preincubation at 50°C. PrpB retained no detectable activity after preincubation at 60°C.

Requirement for a reducing agent. The PrpB enzyme from serovar Typhimurium required a reducing agent (DTT or reduced glutathione) for maximal activity. PrpB protein dialyzed for 4 h at 4°C in buffer without DTT lost all detectable activity. Partial activity (approximately 50%) was restored when the enzyme was incubated in the presence of 5 mM DTT for 15 min. However, greater than 95% of the original activity was retained if dialysis was performed for 4 h in the presence of DTT, indicating that the enzyme was stable under the dialysis conditions used as long as DTT was present in the buffer. To determine whether the reducing agent was needed to prevent formation of disulfide bond(s) or to avoid oxidation of a critical residue, the disulfide bond-specific reductant TCEP (9) was added to protein that had been dialyzed in the absence of reducing agent. TCEP failed to restore any detectable activity to PrpB suggesting that an oxidation-sensitive residue may play a crucial role in catalysis.

Metal requirement. The PrpB protein from serovar Typhimurium required a divalent metal cation for maximal activity. Mg^{2+} ions (2.5 mM) supported the highest specific activity of the enzyme. The apparent K_m for Mg^{2+} under these assay conditions was 38 μM. Equimolar concentrations of Mn^{2+} ions supported activity, but only at 28% of the specific activity of PrpB with Mg^{2+}. Ca^{2+} ions failed to support lyase activity and were in fact slightly inhibitory (data not shown).

Ionic strength. Increases in the ionic strength in the assay resulted in decreased specific activity of the PrpB enzyme. PrpB retained 81% of its specific activity at 50 mM NaCl, 63% at 100 mM NaCl, and only retained 41% of its activity at 250 mM NaCl. These data were consistent with reports of ICL inhibition by inorganic anions (43).

Substrate specificity. The PrpB enzyme of serovar Typhimurium appears to be specifically designed to cleave 2-MIC; PrpB did not cleave isocitrate at any detectable levels, even after incubation with isocitrate for 24 h.

TABLE 2. Kinetic parameters for PrpB and mutant PrpB proteins[a]

Protein	2-MIC activity		
	Mean K_m ± SD (μM)	Mean k_{cat} ± SD (tetramer s^{-1})	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
H_6PrpB	19 ± 1.8	105 ± 10	5.5×10^6
Detagged PrpB	18 ± 1.5	74 ± 6.8	4.2×10^6
H_6PrpB^{R122K}	22 ± 1.2	55 ± 0.3	2.5×10^6
H_6PrpB^{H125A}	10 ± 1.6	0.14 ± 0.003	1.4×10^4
H_6PrpB^{K121A}	57 ± 5.6	0.10 ± 0.006	1.8×10^3

[a] Mutant proteins H_6PrpB^{D58A} and H_6PrpB^{C123A} showed no detectable activity.

Kinetic parameters. Based on four independent trials, the K_m of H_6PrpB for 2-MIC was 19 μM, with a k_{cat} of 105 s^{-1} for the tetrameric enzyme. The K_m of the detagged PrpB protein for 2-MIC was 18 μM, with a k_{cat} of 74 s^{-1} (Table 2). The observed reduction in k_{cat} of detagged PrpB enzyme could be due to inactivation of the enzyme during removal of the hexahistidine tag, which was performed in the absence of reductant.

Residues essential for 2-MIC cleavage. Previous studies on ICL enzymes and elucidation of the crystal structure of the enzymes with bound inhibitors (5, 6, 21, 26, 34), and similar studies of PEP mutase from *Mytilus edulis* (19, 20), identified residues that affect the catalytic activities of these enzymes. Mutations in equivalent residues in the *prpB* gene were constructed, including residues C123 (the proposed active site base of ICL), D58, H125, K121, and R122 (Fig. 2). Mutant PrpB proteins from plasmids pPRP82, pPRP84-86, and pPRP132 were overproduced and purified using nickel chelation chromatography. Immunoblot analysis showed that all mutant proteins were stable (data not shown) and showed only minor differences in the electrophoretic behavior relative to that of the wild-type protein in nondenaturing polyacrylamide gels; these differences were likely due to subtle changes in the isoelectric points of the proteins (Fig. 4). The electrophoretic behavior of all mutant PrpB proteins was consistent with the reported tetrameric quaternary state of PrpB from *E. coli* (7, 11) and serovar Typhimurium (H. Holden and I. Rayment, unpublished results).

2-MICL activity of mutant PrpB proteins. Table 2 shows the kinetic values obtained for wild-type and mutant PrpB proteins for the cleavage of 2-MIC. Proteins PrpB^{R122K}, PrpB^{H125A}, and PrpB^{K121A} retained measurable activity. The subtle R122K change resulted in a 50% decrease in k_{cat}, while the other mutations of the proposed active site loop region of PrpB ($K^{121}RCGH^{125}$) resulted in more drastic decreases in activity of the enzyme. The C123A change (corresponding to a residue in the active site loop of ICLs) abolished all detectable activity, as did the D58A mutation. These results showed that both the C123 and D58 residues of PrpB were essential for catalysis. With the exception of protein PrpB^{R122K}, all mutant PrpB proteins were unable to compensate for the lack of PrpB in *prpB* mutants during growth on propionate. Even though the catalytic efficiency of the PrpB^{R122K} was only half of that of the wild-type enzyme, no measurable difference in the doubling time during growth on propionate was observed between strains carrying plasmid pPRP75 (encoding PrpB) or pPRP132 (encoding PrpB^{R122K}) (doubling time = 4.2 h each).

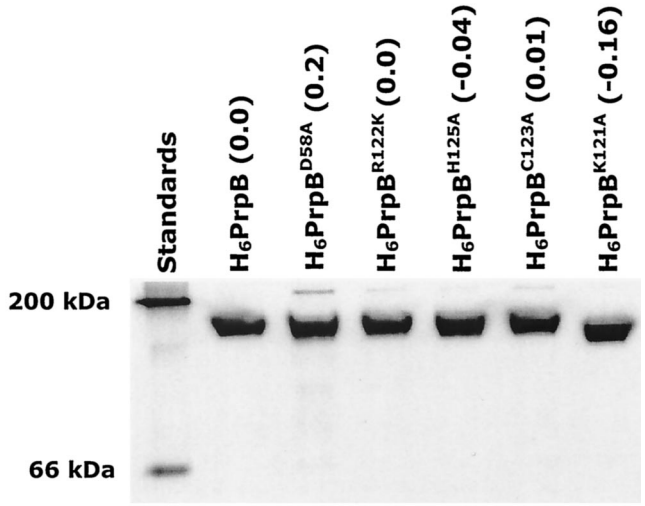

FIG. 4. Electrophoretic behavior of wild-type and mutant PrpB proteins under nondenaturing conditions. Conditions for nondenaturing PAGE were as described above. The protein used is indicated above each lane. Numbers in parentheses indicate the change in isoelectric point in comparison to H_6PrpB. Size markers used were bovine serum albumin (66 kDa) and β-amylase (200 kDa) (Sigma).

Can PrpB be converted into an ICL by mutation? Ways of distinguishing genes encoding ICLs from those encoding 2-MICLs have been suggested (28). Because all characterized ICLs contain a KKCGH motif while all known 2-MICLs contain a corresponding KRCGH motif, it has been proposed that 2-MICL enzymes might be recognized by a KRCGH motif while ICLs may be recognized by the KKCGH sequence (7). To determine whether residue R122 of PrpB was involved in substrate specificity, a R122K change was introduced by site-directed mutagenesis and the resulting protein was assayed for ICL and 2-MICL activities. The $PrpB^{R122K}$ protein retained about 50% of the 2-MICL activity, and still failed to cleave isocitrate at any detectable level. These results indicate that a single change in the KKCGH motif does not change the specificity of the 2-MICL enzyme for its substrate. A likely explanation for having R122 in 2-MICLs is that an arginine residue is required in this position of the protein to facilitate closure of the active site loop over the substrate, similarly to that proposed for K189 of *Mycobacterium tuberculosis* ICL (34).

Identification of lesions in *prpB* generated by random chemical mutagenesis and stability of the resulting proteins. The *prpB* allele of previously isolated *prpB* mutant strains (JE3946 and JE3961) was sequenced to determine whether the mutations were in the active site of PrpB. Both strains carried only one amino acid change. In strain JE3946 *prpB* had a change at position E188 to a lysine (E188K), while strain JE3961 contained a G55K change. Mutant protein $PrpB^{G55K}$ was stable as determined by immunoblot analysis. This mutation most likely caused a propionate phenotype due to a disruption in the positioning of the nearby crucial D58 residue. In contrast, protein $PrpB^{E188K}$ was unstable (data not shown), suggesting that this amino acid change likely disrupted protein folding making the protein sensitive to degradation.

DISCUSSION

This work was undertaken to gain insights into the reaction mechanism of the 2-MICL (PrpB) enzyme of serovar Typhimurium. The PrpB enzyme was purified and kinetically characterized, and the roles of conserved residues were investigated both in vitro and in vivo.

The wild-type PrpB enzyme of serovar Typhimurium. Kinetic parameters have been determined for the PrpB enzyme from *E. coli.* In the study by Brock et al. (7), PrpB from *E. coli* was determined to have a K_m of 19 μM for 2-MIC and a K_m of 35 μM for Mg^{2+}. The close agreement of the kinetic parameters determined for the PrpB enzyme of serovar Typhimurium (K_m of 19 μM for 2-MIC and a K_m of 38 μM for Mg^{2+}) to those reported for the *E. coli* PrpB protein was expected considering that the amino acid sequence of these two enzymes is 90% identical. Unlike the *E. coli* enzyme, however, the serovar Typhimurium enzyme was able to use Mn^{2+} instead of Mg^{2+} ions, albeit poorly. The activity of PrpB from serovar Typhimurium required a reducing agent for maximal activity, which has also been observed in ICL and in PrpB enzymes from *E. coli* and *A. nidulans* (7, 43). However, failure of the disulfide-specific reductant TCEP to reactivate oxidized, inactive PrpB enzyme, suggested the need to prevent a redox active residue (such as a cysteine) from oxidizing, rather than preventing a disulfide bond from forming.

Insights into the PrpB reaction mechanism. Hypotheses of the PrpB reaction mechanism were proposed based on mechanisms for ICL and PEP mutase proteins with which the PrpB protein shares homology (7, 20). On the basis of the work reported here, it is hypothesized that the mechanism of PrpB action is similar to that of ICL based on the similarities in structure of substrates, and in similarities between the active site loop region between ICL and 2-MICL, i.e., the K(R/K)CGH motif. Both ICL and *E. coli* PrpB can be inactivated by treatment with cysteine-modifying agents (7, 31). Our data indicate that residue C123 is critical to catalysis.

The three-dimensional crystal structure of serovar Typhimurium apo-PrpB has been solved to a 2.1-Å resolution (H. Holden and I. Rayment, unpublished results). The structure confirms the oligomeric state of PrpB to be a dimer of dimers; the structure of the monomer is an atypical α/β-barrel similar to the one of PEP mutase and ICL (5, 6, 19, 34). The region of the polypeptide spanning residue C123, however, is disordered, most likely due to the lack of substrate in the active site. Based on similarities of PrpB to ICL and data presented here, it is hypothesized that residue C123 serves as the active site base of PrpB and residue D58 is required for the binding of the Mg^{2+} ion. Elucidation of the structure of PrpB complexed with its substrate in the active site will provide further insights into the reaction mechanism of this enzyme.

During the review of this work, the three-dimensional crystal structure of *E. coli* PrpB protein was published (11). In this structure, the active-site loop was modeled in place based on the structure of the ligand-bound *M. tuberculosis* ICL. In this structure, residue D58 appears critical to the positioning of a Mg^{2+} ion in the active site. Our results would support the proposed role for residue D58. In the same structure, active-site residue R122 does not appear to play a role in substrate recognition because it points away from the active site in the

modeled loop. The authors postulate that the reaction mechanism proceeds identically to the ICL mechanism based on similarities in structure.

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