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prsB Is an Allele of the *Salmonella typhimurium prsA* Gene: Characterization of a Mutant Phosphoribosylpyrophosphate Synthetase

DAVID A. POST AND ROBERT L. SWITZER*

Department of Biochemistry, University of Illinois, 1209 West California, Urbana, Illinois 61801

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The *Salmonella typhimurium prsB* mutation was previously mapped at 45 min on the chromosome, and a *prsB* strain was reported to produce undetectable levels of phosphoribosylpyrophosphate (PRPP) synthetase activity and very low levels of immunologically cross-reactive protein in vitro (N. K. Pandey and R. L. Switzer, J. Gen. Microbiol. 128:1863–1871, 1982). We have shown by P22-mediated transduction that the *prsB* gene is actually an allele of *prsA*, the structural gene for PRPP synthetase, which maps at 35 min. The *prsB* (renamed *prs-100*) mutant produces about 20% of the activity and 100% of the cross-reactive material of wild-type strains. *prs-100* mutant strains are temperature sensitive, as is the mutant PRPP synthetase in vitro. The *prs-100* mutation is a C-to-T transition which results in replacement of Arg-78 in the mature wild-type enzyme by Cys. The mutant PRPP synthetase was purified to greater than 98% purity. It possessed elevated Michaelis constants for both ATP and ribose-5-phosphate, a reduced maximal velocity, and reduced sensitivity to the allosteric inhibitor ADP. The mutant enzyme had altered physical properties and was susceptible to specific cleavage at the Arg-101-to-Ser-102 bond in vivo. It appears that the mutation alters the enzyme's kinetic properties through substantial structural alterations rather than by specific perturbation of substrate binding or catalysis.

Phosphoribosylpyrophosphate (PRPP) synthetase catalyzes pyrophosphoryl transfer from ATP to ribose-5-phosphate (Rib-5-P) and is the first step of a highly branched pathway leading to purine, pyrimidine, and pyridine nucleotides and to histidine and tryptophan. PRPP synthetase from *Salmonella typhimurium* has been the subject of extensive kinetic and mechanistic studies (7, 28–30). Relatively little is known about the role of specific amino acid residues of the protein in catalysis. However, mutants with altered kinetic properties have been isolated in *S. typhimurium* (14, 20) and *Escherichia coli* (13). One of these mutant enzymes has been characterized, which has enabled structure-function relationships to be studied (3).

Pandey and Switzer (20) isolated an *S. typhimurium* mutant strain, PS-1, which was reported to have no assayable PRPP synthetase activity and only 2% of the immunologically cross-reactive material present in wild-type strains. This strain was also temperature sensitive; the temperature sensitivity was 88% cotransducible with the PRPP synthetase mutation. The temperature sensitivity and thus the linked PRPP synthetase mutation were mapped at 45 min on the *S. typhimurium* chromosome (20, 23). Subsequently, the structural gene of PRPP synthetase (*prsA*) was shown to be located at 35 min on the *S. typhimurium* chromosome (14). Thus, the mutation mapping at 45 min was thought to be involved in the regulation of *prsA* expression and was named *prsB*.

In this study, we sought to determine the biochemical function of *prsB* by cloning the gene. This led to the discovery that the *prsB* mutation had been mismapped. Instead, the *prsB* mutation is an allele of the *prsA* gene and encodes a mutant PRPP synthetase (*prs-100*) possessing altered kinetic and physical properties.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and plasmids. All *S. typhimurium* LT2 and *E. coli* K-12 strains used are listed in Table 1. Phage P22 (HT, Int) was used for transductions (5, 18). The *zdf-6601*::Tn*10* transposon closely linked to *hemA* was isolated from a P22 (Tn*10* pool) lysate provided by S. Maloy by transducing strain MS1116 to *hemA*$^+$ Tet^r. One candidate (DPM1) was analyzed and was used for further transductions. Plasmids used in this study are listed in Table 1.

Media and growth conditions. Minimal medium was M9 (18) or E medium (32) supplemented with 0.1% (wt/vol) glucose. Luria broth (18) was used as a rich medium. Amino acids (Sigma) were added at 50 μg/ml when required. δ-Aminolevulinic acid hydrochloride (Sigma) was added at 75 μg/ml to rich medium. Antibiotics were added to final concentrations in rich (minimal) media as follows: sodium ampicillin, 50 (25) μg/ml; and tetracycline hydrochloride, 20 (10) μg/ml. Liquid cultures were grown aerobically in a New Brunswick gyratory shaker at 300 or 350 rpm. Growth for overproduction of PRPP synthetase was done in 6-liter flasks in a New Brunswick gyratory shaker. Growth was monitored on a Klett Summerson colorimeter containing a red (no. 66) filter.

Cloning of the *prs-100* gene. The *prs-100* gene was cloned by a plasmid rescue technique using plasmid pPS2, which carries a partial *prsA* gene and is temperature sensitive for replication. This plasmid was constructed as shown in Fig. 1. This plasmid cannot replicate at nonpermissive temperatures; for the strain to maintain ampicillin resistance, the plasmid must integrate into the chromosome. Since the plasmid carries a partial *prsA* gene, it integrates by homologous recombination at the *prsA* locus. During growth at the permissive temperature, the plasmid can spontaneously excise from the chromosome to yield plasmids that contain either the chromosomal copy of the *prsA* gene from the *prs-100* host or the partial *prsA* gene originally cloned on the plasmid. These could then be distinguished by transforma-

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype or marker[a]	Origin or reference
E. coli		
JM83	*ara* Δ(*lac-proAB*) *rpsL thi* φ80D *lacZ*ΔM15	34
HO700	*metB udp deoD gsk-3 pncA supF relA spoT rspL lamB prs-3*::Kan^r	Hove-Jensen (11)
HO773	F$^-$ *ara*(Am) *araD* Δ(*lac*)U169 *trp*(Am) *mal*(Am) *rpsL relA deoD gsk-3 udp thi supF prs-4*::Kan^r	Hove-Jensen (11)
S. typhimurium		
MS1116	*hemA6*	S. Maloy
MS1115	*dadA2 dhuA13 his-3501 met-268*	S. Maloy
PS-1	*met prs-100*	20
DPM1	*zdf-6601*::Tn*10*	This study
DPM17	*hemA6 zdf-6601*::Tn*10*	This study
DPM27	*prs-100*	This study
Plasmids		
pEL3	Ap^r Ts	1
pPS2	Ap^r Ts	This study
pPS5	Ap^r Ts *prs-100*	This study
pPS5.1	Ap^r *prs-100*	This study
pBS111R	Ap^r *prsA*	S. Bower (4)
pBS201	Ap^r *prsA*	S. Bower (4)

[a] Abbreviations: Ap^r, ampicillin resistance; Ts, temperature sensitivity.

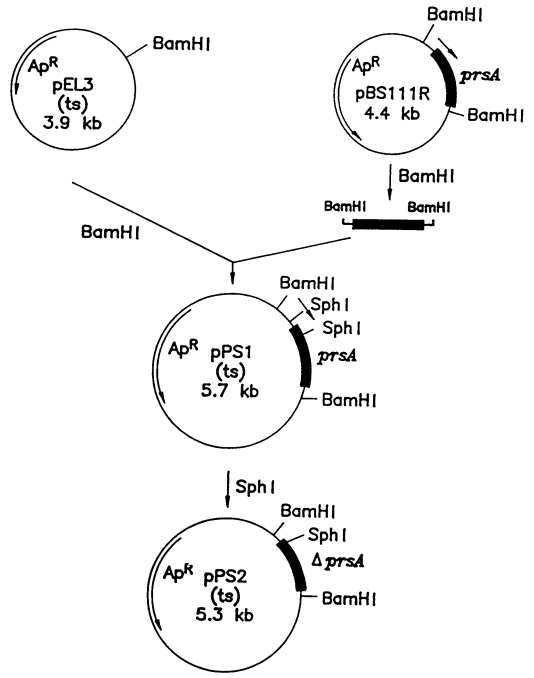

FIG. 1. Construction of plasmid pPS2. A 1.75-kb *Bam*HI fragment of pBS111R encoding the entire wild-type *prsA* gene was ligated into the single *Bam*HI site of pEL3, a plasmid with a temperature-sensitive replicon (1), to yield pPS1. A 409-bp *Sph*I fragment was deleted from pPS1 by digestion with endonuclease *Sph*I and religated to obtain plasmid pPS2. pPS2 contains a partial *prsA* gene and can undergo homologous recombination into the *prsA* gene on the *S. typhimurium* chromosome but cannot confer *prs*$^+$ in a *prs* mutant strain. Arrows indicate direction of transcription of relevant genes.

tion into strain HO700 (*prs-3*::Kan^r) on minimal medium, since this strain requires a plasmid-borne *prs* gene for growth.

The PS-1 strain used in these experiments was a revertant that is only slightly temperature sensitive with respect to wild-type strains. Thus, at nonpermissive temperatures for plasmid replication, strain PS-1 was not inhibited for growth and reversion of the *prs-100* mutation did not occur. However, when the *prs-100* mutation was transduced into a wild-type background, these strains became very temperature sensitive for growth. Secondary mutations in the original PS-1 strain, such as those described by Hove-Jensen (10, 11) in *E. coli* and Jochimsen et al. (14), in characterization of another *prsA* mutant, may account for the difference in the temperature sensitivity between PS-1 and other *prs-100* strains. That is, the loss of activity of the temperature-sensitive PRPP synthetase at elevated temperatures can be masked by the secondary mutations.

For cloning the *prs-100* gene, strain PS-1 was grown in a 500-ml sidearm flask, transformed with plasmid pPS2, and grown on rich medium at 30°C, using ampicillin for selection. At a turbidity of 200 Klett units, 5 ml of culture was inoculated into 200 ml of rich medium at the nonpermissive temperature (42°C) in a shaking water bath. When this culture reached 100 Klett units, a 5-ml aliquot was inoculated into another 200 ml of rich medium and grown at 42°C to 200 Klett units. A 5-ml sample of this culture was inoculated into 200 ml of rich medium at the permissive temperature (30°C) and grown into stationary phase. Plasmid DNA was isolated from 10 ml of this culture and used to transform strain JM83, selecting for ampicillin resistance. A second rapid plasmid isolation of the pooled initial transformation was performed to obtain the plasmid DNA used for selection of the *prs-100* gene in strain HO700.

Molecular biological methods. Standard techniques for manipulation of DNA were as described previously (5, 16, 25, 27). DNA nucleotide sequence was determined by the dideoxy-chain termination method for double-stranded plasmids (6) and for M13 bacteriophage (24), using the Sequenase kit (U.S. Biochemicals, Inc.). Sequence data were analyzed with software from DNAstar, Inc.

Enzymological procedures. Protein concentration was determined by using the bicinchoninic acid protein reagent (Pierce) according to the manufacturer's directions, with bovine serum albumin used as a standard.

PRPP synthetase was assayed by two methods. For crude extracts, the assay of Bower et al. (4) was used. For the purified enzymes, the procedure published by Switzer and Gibson (29) was used, with the following modifications. For determination of kinetic constants, the assay was carried out at 25°C, because at low substrate concentrations at 37°C the reaction utilizing the *prs-100* mutant enzyme was not linear with enzyme concentration. The optimal substrate and divalent cation concentrations were 10 mM Rib-5-P, 8 mM ATP, and 12 mM $MgCl_2$ for the mutant enzyme and 5 mM Rib-5-P, 1 mM ATP, and 4 mM $MgCl_2$ for the wild-type enzyme. To stabilize the mutant enzyme at high dilutions, both substrates and divalent cations had to be added to the dilution buffer. The dilution buffer for the kinetic determinations contained 1 mg of bovine serum albumin per ml in 50 mM KP_i (pH 7.5), with the following modifications: for determination of the K_m for ATP, 20 mM Rib-5-P, 1 mM ATP, and 20 mM $MgCl_2$; for the K_m for Rib-5-P, 1 mM Rib-5-P, 20 mM ATP, and 25 mM $MgCl_2$; for the Mg^{2+} and Mn^{2+} saturation curves, 20 mM Rib-5-P, 5 mM ATP, and 6 mM $MgCl_2$ or

$MnCl_2$. For inhibition studies, the dilution buffer included 10 mM Rib-5-P, 8 mM ATP, and 12 mM $MgCl_2$. In these dilution buffers, the mutant enzyme retained maximal activity when assayed at 25°C and was stable up to 30 min on ice. The same dilution buffers were used for the wild-type enzyme, but the activity of the diluted wild-type enzyme was the same with or without the added substrates. Because both divalent cations and substrates were present in the dilution buffers, concentrations in the assay solution were corrected for carryover in the calculations of the kinetic constants. Furthermore, it was determined that the reaction proceeded by less than 0.2% on ice before the enzyme was assayed, so there was no significant inhibition by end products. All assays were done with two different enzyme concentrations for fixed times of 5 and 10 min. One unit of activity is defined as the amount of enzyme catalyzing formation of 1 μmol of product per min under the stated assay conditions.

For thermal inactivation experiments, the purified proteins were incubated in 50 mM KP_i (pH 7.5)–1 mM $MgCl_2$–10% glycerol at a concentration of 0.8 mg/ml. At each time point, 3 μl of the sample was diluted into 42 μl of dilution buffer containing 12 mM $MgCl_2$, 10 mM Rib-5-P, and 8 mM ATP on ice. Assays were then carried out at 25°C under the respective saturating conditions.

Purification of PRPP synthetase. Wild-type PRPP synthetase from strain SB139 containing plasmid pBS201 was purified as described by Bower et al. (4).

The *prs-100* mutant enzyme was purified as follows. All procedures were carried out at 4°C unless otherwise noted. Thawed cell paste (50 g) was suspended in 650 ml of KP_i (pH 7.5) on ice. Phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM. The cells were disrupted in a Heat Systems W-375 sonicator by pulsing at 50% power for 5 min and cooling for 2.5 min. Total sonication time was 30 min. The extract was centrifuged for 20 min at 10,000 × *g*.

A 1/10 volume of 10% (wt/vol) solution of streptomycin sulfate in 50 mM KP_i (pH 7.5) was added to the resultant supernatant. After being stirred on ice for 5 min, the milky solution was centrifuged at 23,000 × *g* for 30 min.

The supernatant from the streptomycin sulfate step was brought to 35% saturation with $(NH_4)_2SO_4$ by the slow addition (20 min) of a saturated solution of $(NH_4)_2SO_4$ in 50 mM KP_i (pH 7.5) and then gently stirred on ice for 30 min. The suspension was then centrifuged for 30 min at 23,000 × *g*. The resulting precipitate was resuspended in 200 ml of 50 mM KP_i (pH 7.5) and quickly frozen in liquid nitrogen; 100 ml of this fluid was thawed and centrifuged for 20 min at 23,000 × *g*.

The clarified $(NH_4)_2SO_4$ fraction was loaded onto a column (2.6 by 10 cm) of DEAE-Sepharose CL-6B (Pharmacia) that had been equilibrated with 50 mM KP_i (pH 7.5). The column was washed with 150 ml of 150 mM KCl in 50 mM KP_i (pH 7.5) and developed with a 250-ml linear gradient from 150 to 300 mM KCl in 50 mM KP_i (pH 7.5). Fractions containing the highest activity were pooled (100-ml total volume) and concentrated to a volume of 40 ml with an Amicon model 52 apparatus with a PM10 membrane. The solution was desalted by repeated cycles of concentration and dilution with 50 mM KP_i (pH 7.5) on the Amicon unit to a final volume of 40 ml. Concentrating the mutant enzyme on the Amicon unit caused a loss of 50% of the enzyme as a precipitate. Further concentration of the mutant enzyme was done on a Centricon 10 microconcentrator (Amicon). In contrast to the Amicon model 52 concentrator, little protein or activity loss was seen with the microconcentrator. The mutant enzyme was concentrated to 8 mg/ml (1.5-ml total volume) and remained a clear solution while retaining 100% of its original activity. The mutant enzyme was also unstable to freezing and thawing, as judged from the appearance of a precipitate and the loss of activity, but addition of $MgCl_2$ to a final concentration of 1 mM and glycerol to a final concentration of 10% stabilized the enzyme.

The concentrated eluate (1.5 ml) was loaded onto a Sepharose S-300 gel filtration column (2.2 by 90 cm; Pharmacia), which had been equilibrated with 50 mM KP_i (pH 7.5), containing 100 mM KCl, 1 mM $MgCl_2$, and 10% glycerol. The peak of protein, as determined by the Bradford assay, was divided into three fractions, consisting of the leading edge of the peak (fraction 1, 10 ml), the center of the peak (fraction 2, 14 ml), and the trailing edge of the peak (fraction 3, 17 ml). These fractions were then concentrated on microconcentrators to final volumes of 1 ml for fraction 1, 1.5 ml for fraction 2, and 2 ml for fraction 3. These fractions were then desalted by repeated cycles of concentration and dilution with 50 mM KP_i (pH 7.5), containing 1 mM $MgCl_2$ and 10% glycerol, on the Centricon units. The samples were divided into 50-μl aliquots, quickly frozen in liquid nitrogen, and stored at −80°C. Further analysis of these fractions is presented in Fig. 4.

Protein sequencing. Protein sequencing was performed by electroblotting 5 μg of the purified mutant enzyme onto a polyvinylidene difluoride membrane as described by Matsudaira (17). The band corresponding to the 23-kDa fragment was excised, and the blotted sample was sequenced by the University of Illinois Biotechnology Center in an Applied Biosystems 470A gas phase sequenator.

Radiochemicals. Radiochemicals were obtained from the following sources: iodo[^{14}C]acetamide (ICN), $^{32}P_i$ (ICN), [^{125}I]protein A (ICN), and [α-^{35}S]ATP (Amersham). [γ-^{32}P]ATP was synthesized by the procedure of Johnson and Walseth (15), with modifications described by Harlow (8a).

Immunological techniques. One milligram of purified wild-type PRPP synthetase was further purified by high-performance liquid chromatography, using a gel filtration column (Zorbax GF-250) in 100 mM KP_i (pH 7.5), to greater than 99% purity, as judged from silver staining of an overloaded sodium dodecyl sulfate (SDS)-polyacrylamide gel. Polyclonal antibodies against PRPP synthetase were raised by Colcalico. Serum was used at a 1/2,000 dilution, and quantitative determination of cross-reactive protein was performed by immunoblotting, labeling with [^{125}I]protein A, according to the general procedures of Grandoni et al. (8).

RESULTS

The *prsB* phenotype revisited. The *prsB* mutant strain described by Pandey and Switzer (20) possessed no assayable PRPP synthetase activity and only 2% of the cross-reactive material of wild-type strains. Our reanalysis of strain PS-1 consistently showed a maximum of 20% of PRPP synthetase activity of the wild type in crude extracts and 100% of the cross-reactive material present in wild-type strains. Furthermore, strain PS-1 had a temperature-sensitive PRPP synthetase activity in vitro, and immunoblots of crude extracts of *prsB* strains revealed an immunologically cross-reactive band with M_r 27,000, in addition to the expected band corresponding to PRPP synthetase (M_r 34,000).

Mapping of the *prsB* allele. The mutation in strain PS-1 originally described by Pandey and Switzer (20) was reported to be 88% linked to an unknown temperature sensitivity at 45 min on the *S. typhimurium* linkage map. A

TABLE 2. Transductional crosses used to map *prs-100*

Donor or marker	Recipient	Selected marker	Unselected marker	Unselected/ selected transductants
DPM1	PS-1	Tet^r	*prsA*$^+$	17/36
DPM17	DPM27	Tet^r	Ts$^+$[a]	223/499
			hemA[b]	102/499
PS-1	MS1115	*dadA*$^+$[c]	*prs-100*	6/498
zdf-6601::Tn*10*	MS1115	*dadA*$^+$[c]	Tet^r	78/497

[a] The temperature sensitivity (Ts) was assumed to be the *prs-100* mutation.
[b] Of 102 *hemA* transductants, 100 were Ts$^+$
[c] *dadA*$^+$ transductants were selected by requiring growth on D-methionine (33).

temperature sensitivity was identified that was 50% linked to *hisW* and 12% linked to *cdd*. Thus, the *prsB* locus was assumed to be linked to *hisW* and *cdd* because it was linked to a temperature sensitivity, but it was never linked to these alleles directly. Since the *prsB* allele had never been transduced into a wild-type background, we attempted to cure the *prsB* phenotype and to transduce the allele with the tetracycline resistance transposon (*zeh-754*::Tn*10*), which is 92% linked to *hisW* (22) and was shown by Pandey and Switzer (20) to be linked to a temperature sensitivity and the *prsB* allele. Of 495 transductants, none were found to have cotransduced the *prsB* allele with the Tn*10*. Since the *prsB* allele was not cotransducible with the Tn*10*, further genetic analysis with markers mapping near 45 min was done. Cotransduction frequencies between *hisW* (22), Nal^r (*gyrA*) (22), *glpT* (23), *cdd* (20, 23) and *zeh-754*::Tn*10* (22) were determined and agreed with those previously reported. However, the *prsB* allele was not cotransducible with any of these genes, and it was concluded that the reported location of the *prsB* mutation was incorrect. Thus, we investigated the possibility that *prsB* is actually an allele of the *prsA* gene.

It has been shown previously that the *hemA* gene is 51% linked to the *prsA* gene (14). A strain carrying a tetracycline resistance transposable element (*zdf*-6601::Tn*10*) inserted near *hemA* was isolated as described in Materials and Methods. We determined that the *prsB* phenotype could be cured by a *prsA*$^+$ donor strain with a cotransduction frequency to *hemA* of 46%. The area around the *prsA* gene was mapped by two- and three-factor crosses (Table 2 and Fig. 2). All of the phenotypes described in the previous section that are associated with the *prsB* gene could be simultaneously cured in *prsB* strains or transduced together into wild-type backgrounds. However, upon transduction of the *prsB* allele into a wild-type background, the strains become very temperature sensitive for growth at 42°C. This temperature sensitivity in vivo correlates with the temperature-sensitive PRPP synthetase activity in vitro. Therefore, we conclude that the original map position for *prsB* reported by Pandey and Switzer (20) is incorrect and that it is an allele of *prsA*. Thus, the *prsB* mutant was renamed *prs-100*.

Cloning and characterization of the *prs-100* gene. The *prs-100* mutant allele was cloned by plasmid marker rescue techniques as described in Materials and Methods. The plasmid DNA obtained was used to transform *E. coli* HO700 (*prs-3*::Kan^r) to *prs* prototrophy on minimal medium at 30°C. Several plasmids were isolated that supported growth of HO700 in minimal medium. Two plasmids (pPS3 and pPS5) were shown to produce a protein of M_r 34,000, which is the M_r of PRPP synthetase. Plasmid pPS5 produced much more of the protein than did plasmid pPS3. A 1.7-kb *Bam*HI fragment from plasmid pPS5 was cloned into the *Bam*HI site of pUC19 (plasmid pPS5.1; Fig. 3). Cells bearing plasmid pPS5.1 overexpressed a temperature-sensitive PRPP synthetase activity in vitro and produced both 34- and 27-kDa cross-reactive proteins detected by immunoblotting. Strain HO773/pPS5.1 was also temperature sensitive in vivo. Plasmid pPS5.1 complemented strain HO773 (*prs-4*::Kan^r) at 32°C but not at 39°C. Strain HO773 containing the wild-type *prsA* gene on plasmid pBS111R grew normally at both temperatures. Thus, the PRPP synthetase encoded by plasmid pPS5.1 showed all of the characteristics of the enzyme specified by the *prs-100* allele.

To locate the putative mutation(s) in the *prs-100* gene, pPS5.1 was used as a source of restriction fragments analogous to fragments from the wild-type plasmid pBS111R (Fig. 3). Heterologous plasmids were constructed and transformed into JM83. Strains bearing these plasmids were analyzed for PRPP synthetase activity at 37 and 42°C and by immunoblots. The mutation giving rise to the phenotypes of the *prs-100* gene was found to be located 5′ to the *Bgl*II site (Fig. 3). Determination of the nucleotide sequence of this region revealed a single change at nucleotide 794 (4) from C to T, so that codon 79 encoded a cysteinyl residue instead of an arginyl residue. This change was verified by chemical modification of the cysteinyl residues in purified preparations of the mutant and wild-type proteins with iodo[^{14}C]acetamide. The mutant protein contained 5.2 ± 0.2 cysteinyl residues per mol of protein (expected: 5), and the

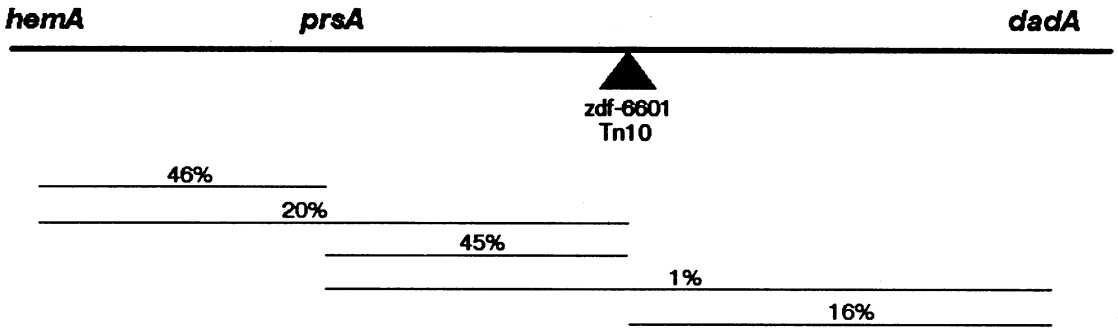

FIG. 2. Map of the *prsA* region of the *S. typhimurium* chromosome. Approximate locations of the *hemA*, *prsA*, and *dadA* genes and the Tn*10* insertion (*zdf-6601*) are shown. The thin lines indicate percent cotransduction between various markers, summarized from the data in Table 2.

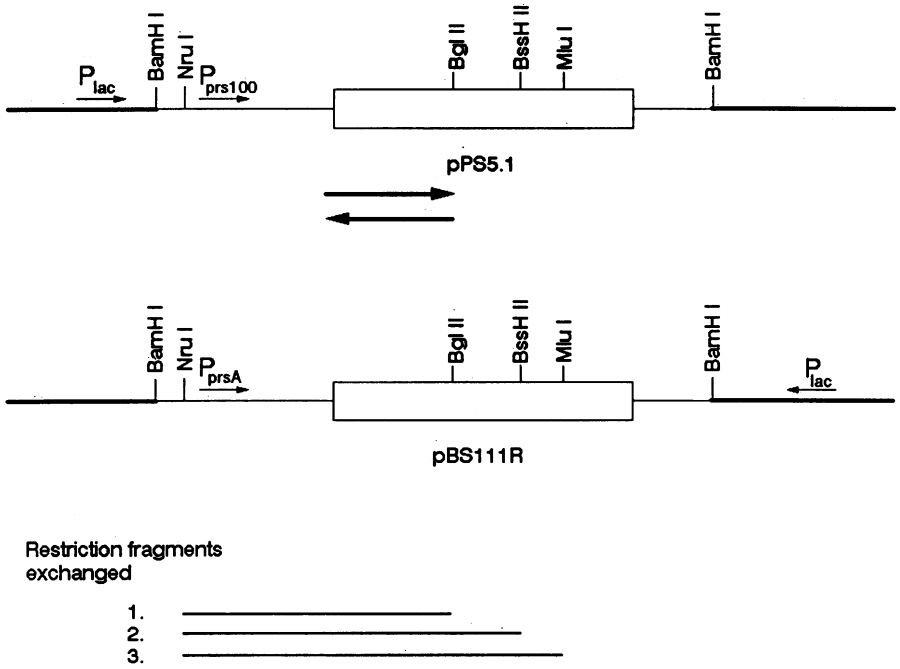

FIG. 3. Linear maps of pBS111R encoding wild-type *prsA* and pPS5.1 encoding *prs-100* to show localization of the mutation in *prs-100*. Restriction fragments 1 (*Nru*I to *Bgl*II), 2 (*Nru*I to *Bss*HII), 3 (*Nru*I to *Mlu*I) (represented by lines at the bottom) were excised from pPS5.1 and inserted in place of the equivalent fragments from pBS111R to locate the *prs-100* mutation. The region corresponding to the heavy arrows was then sequenced. The box represents the coding region for PRPP synthetase. Promoters are labeled, and arrows show the direction of transcription from each.

wild type enzyme contained 4.1 ± 0.2 cysteinyl residues (expected: 4).

Purification and characterization of the *prs-100* mutant PRPP synthetase. The mutant enzyme was purified as described in Materials and Methods and Table 3. As seen from the low recovery of the mutant PRPP synthetase, the stability of the enzyme declined as the enzyme was more highly purified. The mutant PRPP synthetase was purified to 98% purity, as judged by densitometry of an SDS-polyacrylamide gel. During the purification of the mutant enzyme, a 27-kDa fragment copurified throughout every step. This fragment cross-reacted with the anti-PRPP synthetase antibodies (Fig. 4). There was no observable increase in the 27-kDa fragment relative to the native protein as determined by immunoblotting (data not shown) during the purification, so it is probably not an artifact of the purification. The peak from the gel filtration column, which was the final step of purification, was subdivided into three fractions. An SDS-polyacrylamide gel and immunoblot of these three fractions appear in Fig. 4. The 27-kDa fragment was partially sequenced as described in Materials and Methods, yielding the following sequence: Ser-Ala-X-Val-Pro-Ile-Thr-Ala-Lys-Val-Val-Ala-X-Phe-Leu-X-X-Val-Gly-Val. The 16 residues identified match exactly with a sequence that starts at amino acid 102 (codon 103) in the PRPP synthetase sequence (4), which would result in a 23-kDa fragment (visualized as a 27-kDa fragment on an SDS-polyacrylamide gel). Thus, the mutation in the *prs-100* enzyme renders it susceptible to a very specific proteolysis. Interestingly, much lower levels of this fragment have also been observed when the wild-type enzyme is overproduced.

Kinetic studies of purified *prs-100* mutant PRPP synthetase. Kinetic measurements were carried out with the mutant and wild-type PRPP synthetases at 25°C, at which both enzymes were stable. At 37°C, the activity of the mutant enzyme was linear only when both divalent cation and substrates were at saturating concentrations. However, the mutant enzyme was stable at 25°C for 10 min if divalent cation and the substrate whose K_m was not being determined were saturating and the variable substrate was at or above its K_m. If the variable substrate was below its K_m, the assay was linear for only 5 min.

TABLE 3. Purification of the mutant *prs-100* PRPP synthetase

Purification step	Vol (ml)	Protein (mg/ml)	Sp act (U/mg of protein)[a]	Total activity (U)	Recovery (%)	Fold purification
Crude extract	670	6.3	0.74	3,150	100	
Streptomycin sulfate precipitation	690	5.3	2.4	8,120	270	3.2
Ammonium sulfate fractionation	56	3.4	56	10,500	330	76
DEAE chromatography	35	1.4	63	3,080	98	85
Gel filtration chromatography	1.5	0.8	88	150	5	119

[a] Assayed at 37°C under saturating conditions as described in Material and Methods.

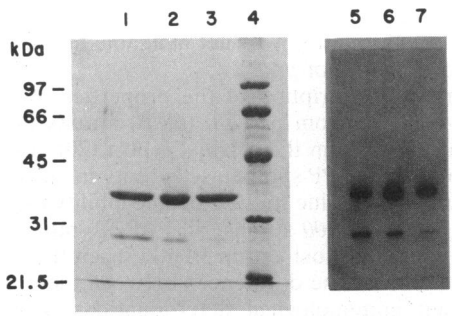

FIG. 4. SDS-polyacrylamide gel electrophoresis analysis of highly purified *prs-100* mutant PRPP synthetase. Electropherograms were analyzed by Coomassie blue staining (lanes 1 to 4) or immunoblotting (lanes 5 to 7). Lanes 1 to 3 contained 20 μg of protein, and lanes 5 to 7 contained 500 ng. Samples analyzed were fraction 1 (lanes 1 and 5), fraction 2 (lanes 2 and 6), and fraction 3 (lanes 3 and 7) from the Sepharose S-300 gel filtration column as described in Materials and Methods. Molecular weight standards (Bio-Rad) were analyzed in lane 4; sizes are shown on the left.

Both substrates and divalent cation had to be added to the dilution buffer in order to achieve maximal activity. If they were not included, the enzyme had only about a quarter of full activity. However, the loss of activity upon dilution in standard dilution buffer was not irreversible. Maximal activity was regained if both substrates and divalent cation were added to the previously diluted enzyme.

Michaelis constants for ATP and Rib-5-P were determined at 25°C for the purified mutant and wild-type *S. typhimurium* PRPP synthetases. The K_m values for ATP and Rib-5-P were 36 ± 7 and 165 ± 11 μM, respectively, for the wild-type enzyme. The corresponding values were 380 ± 10 and 410 ± 10 μM for the mutant enzyme. The maximal velocity for the mutant enzyme was 60% of the maximal velocity for the wild-type enzyme under saturating conditions for the mutant enzyme. Under normal assay conditions for the wild-type enzyme (2 mM ATP, 5 mM Rib-5-P, 5 mM $MgCl_2$) at 37°C, the specific activity of the purified mutant enzyme was 30% that of the wild-type enzyme. This agrees with the observed value for the activity of the *prs-100* mutant seen in crude extracts, which is 20% of wild-type activity.

Saturation curves for Mg^{2+} and Mn^{2+} were determined for the wild-type and *prs-100* enzymes. All curves were sigmoid except for Mg^{2+} activation of the wild-type enzyme, which was hyperbolic. The concentration of Mg^{2+} required to give 50% maximal activity was 0.7 mM for the wild-type enzyme and 4.75 mM for the mutant enzyme. Mn^{2+} concentrations of 2.25 and 2.75 mM were required for half-maximal activity of wild-type and mutant enzymes, respectively.

Inhibition of the *prs-100* mutant enzyme. ADP is both a competitive and an allosteric inhibitor of PRPP synthetase (7, 30). Allosteric inhibition of PRPP synthetase by ADP becomes much more pronounced when Rib-5-P is saturating (7). Thus, two levels of Rib-5-P were used to study inhibition by ADP; ATP was held constant at 1 mM for the wild-type enzyme and 5 mM for the *prs-100* enzyme (Fig. 5). The mutant enzyme was less sensitive to inhibition by ADP. At saturating Rib-5-P, the $I_{0.5}$ (concentration at 50% inhibition) value was 50 μM for the wild-type enzyme and 325 μM for the mutant. At subsaturating Rib-5-P, the $I_{0.5}$ values were 250 and 850 μM for the wild-type and mutant enzymes, respectively. Thus, the *prs-100* mutant enzyme was 3.4- to 6.5-fold less sensitive to ADP inhibition than the wild type but was not desensitized to allosteric inhibition altogether.

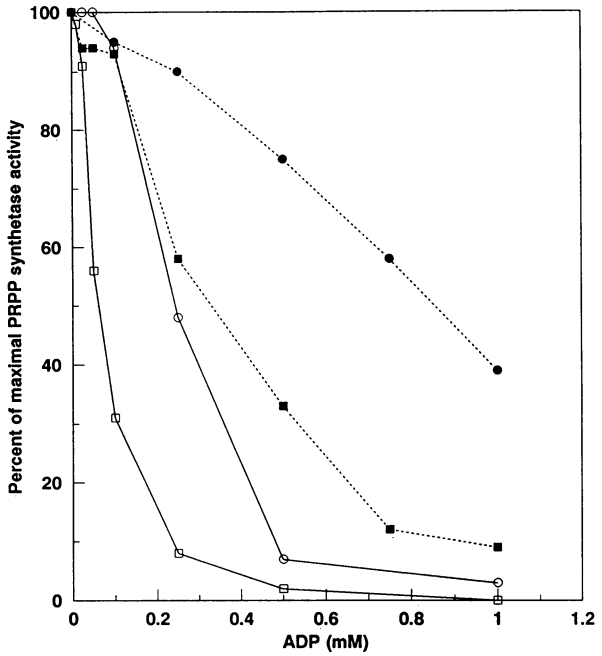

FIG. 5. Inhibition of wild-type and *prs-100* mutant PRPP synthetases by ADP. Saturating (5 mM) (□) and subsaturating (0.5 mM) (○) concentrations of Rib-5-P were used for the wild-type enzyme. For the mutant enzyme, the saturating level of Rib-5-P was 5 mM (■) and the subsaturating level used was 0.75 mM (●). The concentrations of Rib-5-P chosen for subsaturating Rib-5-P experiments were determined so that both enzymes were at 70% of their respective maximal velocities.

Nucleoside triphosphates (GTP, CTP, and UTP, all at 10 mM) were investigated as inhibitors of the PRPP synthetases; the mutant and wild-type enzymes were inhibited similarly by these nucleotides, which probably act only by competitive binding at the ATP site (30).

Thermal inactivation of *prs-100* mutant PRPP synthetase. Temperature affected the *prs-100* enzyme two different ways. Half of the activity of the mutant enzyme was lost upon heating the purified enzyme for 2 min at 49°C; in contrast, 7.5 min at 49°C was required to inactivate half of the wild-type enzyme. When assayed with saturating substrates and Mg^{2+} at 42°C, the *prs-100* mutant enzyme had only 25% of the activity seen when it was assayed at 25°C. However, if the mutant enzyme was incubated at 42°C for the same period of time (5 min) and then assayed at 25°C, it had 70% of the activity of a sample incubated at 25°C and assayed under the same conditions. This finding suggests that at 42°C the mutant enzyme undergoes both irreversible thermal denaturation and reversible conformational changes, leading to decreased specific activity. In contrast, the wild-type enzyme was completely stable under these conditions at 42°C and was three times as active at 42°C as at 25°C.

DISCUSSION

The *prsB* mutant (strain PS-1) was originally described (20) as having no detectable PRPP synthetase activity in vitro and only 2% of the immunochemically cross-reactive protein as a wild-type *S. typhimurium* strain. The lesion was mapped to 45 min on the chromosome. The results of the present study establish unequivocally that the mutation

affecting PRPP synthetase in strain PS-1 maps in the structural gene, *prsA*, and results from a substitution of a cysteinyl residue for Arg-78 in the mature enzyme. We also consistently observed about 20% of the activity and 100% of cross-reactive material of wild-type cells. How do we account for the discrepancies between our present results and the previous conclusions? First, we considered the possibility that the temperature-resistant partial revertant of PS-1 used in our studies (the original temperature-sensitive PS-1 strain has been lost) had reverted in the *prsB* locus, giving the phenotype and *prsA* mutation that we have characterized. A second derivative of the original PS-1 strain, which had the *prsB* mutant phenotype, was also available to us. This strain had been transduced to tetracycline resistance and temperature insensitivity with the transposon *zeh-754*::Tn*10*, which maps at 45 min, by Pandey and Switzer (20). We isolated a tetracycline-sensitive derivative of this strain, and like the PS-1 partial revertant used in our studies, it was always transduced to $prsB^+$ (wild-type *prsA*) by selecting for tetracycline resistance (*zdf-6601*::Tn*10*) and then for *hemA* at 35 min on the chromosome. Since the two *prsB* strains were independent isolates obtained from the original PS-1 strain and had not undergone further selection for *prsA* mutations, it seems highly unlikely that a reversion in the *prsB* locus occurred in both isolates. Second, a careful study of the original data describing the mapping of PS-1 (19a) reveals a number of observations that tend to undermine the reliability of the original mapping of the *prsB* locus. The *prsB* mutation was not originally transduced into a wild-type genetic background prior to characterization, and the mapping of the *prsB* locus relied on a very small number of transductants. Only the temperature sensitivity locus, not the *prsB* phenotype, was scored with the Hfr matings and the transductional analysis with *hisW* and *cdd*. The only direct linkage of the *prsB* locus to 45 min on the chromosome is the reported linkage to the tetracycline resistance transposon (*zeh-754*::Tn*10*) (20). However, only six transductants were obtained in a single experiment in which, for reasons that are not clear to us, the P22 lysate used had been treated with hydroxylamine. Pandey and Switzer (20) also reported that the F'32 episome cured the temperature sensitivity and the *prsB* phenotype of PS-1. However, the recipient strains were not shown directly to contain an F' episome. The surviving culture of the donor strain now available in our laboratory harbors an Hfr, not an F' episome. Third, it is clear that strain PS-1, which was derived from harsh mutagenesis with ethyl methanesulfonate, carried multiple mutations, including more than one temperature sensitivity mutation, which would have confused the original mapping. The derivative of PS-1 that we worked with is only slightly temperature sensitive for growth, whereas when *prs-100* is transduced into wild-type backgrounds, the transductants become very temperature sensitive for growth, even though both contain PRPP synthetase activities that are equally temperature sensitive in vitro. Hove-Jensen (10, 11) and Jochimsen et al. (14) also reported that mutations in the *gsk*, *deoD*, and *udp* alleles can permit *prsA* mutants to grow normally. There is also another temperature-sensitive mutation closely linked to *prsA* in PS-1, because a high-copy-number plasmid carrying the wild-type *prsA* gene cannot completely compensate for the temperature sensitivity of some *prs-100* transductants. Thus, PS-1 probably contained at least three mutations that confer temperature-sensitive growth. We suggest that Pandey and Switzer (20), who were unaware of this, accidentally mapped multiple temperature-sensitive mutations. Finally, our characterization of the *prsB* allele was aided by the knowledge that *prsA* is closely linked to *hemA*; that information was not available at the time of the original description of *prsB*.

Our present description of the properties of the mutant PRPP synthetase from *prs-100* (*prsB*) strains in vitro also differs markedly from the original report (20). The previous failure to detect PRPP synthetase activity in vitro in extracts of PS-1 is probably due to the extreme lability of the *prs-100* enzyme. In all *prs-100* strains, all PRPP synthetase activity in crude extracts is lost within 30 min when the extracts are incubated on ice. The differences between the previous and the present immunological determinations of PRPP synthetase may have resulted from the degradation of the mutant enzyme during the incubations required for the previous complement fixation assays but not for our immunoblotting experiments. We have also shown that our current preparation of antiserum is highly specific for PRPP synthetase on immunoblots, whereas the preparation used in the previous work is not monospecific, which would cause the complement fixation assays to be flawed.

A prominent characteristic of the *prs-100* mutant PRPP synthetase is its instability in comparison with the wild-type enzyme. The enzyme is thermolabile in vivo and in vitro. Thermal inactivation of the enzyme has both reversible and irreversible components. The enzyme was also unstable to a variety of other treatments, even at 0 to 4°C, such as freezing and thawing, concentration by ultrafiltration on an Amicon apparatus, and high dilution. It could be stabilized to dilution by the addition of Mg^{2+} ions, glycerol, and substrates. No particular substrate or divalent cation alone will stabilize the mutant enzyme totally, but rather all are needed to stabilize the mutant enzyme upon dilution. These observations point to a mutant enzyme whose native conformation is easily unfolded. These properties required us to develop a purification procedure that was quite different from that used to purify the wild-type enzyme (29).

The *prs-100* mutant enzyme has greatly increased susceptibility to specific cleavage at the Arg-101-to-Ser-102 bond, which gives rise to the 23-kDa fragment always found in cells producing the *prs-100* mutant enzyme. This cleavage probably occurs in vivo, since the 23-kDa fragment is found in whole *prs-100* cells and the abundance of the fragment does not increase markedly during purification. Increased amounts of the fragment have been observed upon incubation of crude extracts at 0°C, however. Interestingly, the same fragment can be observed in much lower amounts in cells that overproduce the wild-type PRPP synthetase. The 23-kDa carboxyl-terminal fragment copurifies with the uncleaved 34-kDa *prs-100* PRPP synthetase through many steps, although the two forms were partially resolved by gel filtration chromatography (Fig. 4). Native PRPP synthetase is known to exist in pentameric and decameric states (26). We speculate that the *prs-100* enzyme may exist in hybrid aggregates of 34- and 23-kDa polypeptides. We have failed to detect an 11-kDa amino-terminal fragment in the purified *prs-100* enzyme.

The kinetic alterations in the *prs-100* mutant enzyme are not limited to a single element of substrate binding or catalysis. The maximal velocity and the Michaelis constants for both substrates were substantially altered, as were divalent cation activation curves and sensitivity to allosteric inhibition by ADP. These changes, together with the increased lability of the enzyme and its increased susceptibility to cleavage at a site that is some distance from the amino acid substitution in the mutant, point to substantial and rather widespread structural changes in the mutant enzyme.

In the absence of a high-resolution three-dimensional structure for the enzyme, it is not possible to draw precise deductions about the nature of these folding changes or implications for structure-function relationships with this enzyme.

The PRPP synthetases of various organisms share extensive sequence similarities. When the sequences of the rat (31), human (21), *Bacillus subtilis* (19), *S. typhimurium* (4), and *E. coli* (12) PRPP synthetases are aligned, it is evident that the site of the mutation in *prs-100* (Arg-78) is not conserved. This residue is an Arg in the bacterial PRPP synthetases, but is replaced by Ile in the mammalian enzymes. We suggest that Arg-78 lies in a region which is important for correct folding of the enzyme but is not directly involved in substrate binding or catalysis. The site of facile proteolytic cleavage of the *prs-100* enzyme, Arg-101–Ser-102, lies in a sequence from Tyr-90 to Lys-110 (numbers refer to the mature enzyme, which has lost the initiator Met residue) that is highly conserved (15 identical residues or conservative replacements out of 18) and very basic (six Arg or Lys residues). This region appears to be exposed to the surface of the enzyme, because it is susceptible to proteolytic cleavage. A temperature-sensitive *E. coli* PRPP synthetase mutation, *prs-2* (9), also maps to this general region of the *prs-100* mutation (11a). A mutant human PRPP synthetase, which has properties reminiscent of *prs-100*, is also known (2). This mutant has an elevated maximal velocity, is reduced in sensitivity to allosteric inhibitors, and is thermally labile (2). It will be of interest to learn whether these *E. coli* and human mutants are altered in the same region of the PRPP synthetase as identified for the *S. typhimurium prs-100* mutant enzyme.

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