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Genetic Modification of Substrate Specificity of Hypoxanthine Phosphoribosyltransferase in *Salmonella typhimurium*

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Salmonella typhimurium strain GP660 (*proAB-gpt* deletion, *purE*) lacks guanine phosphoribosyltransferase and hence cannot utilize guanine as a purine source and is resistant to inhibition by 8-azaguanine. Strain GP660 was mutagenized and a derivative strain (GP36) was isolated for utilization of guanine and hypoxanthine, but not xanthine, as purine sources. This alteration was designated *sug*. The strain was then sensitive to inhibition by 8-azaguanine. Column chromatographic analysis revealed the altered phosphoribosyltransferase peaks for both hypoxanthine and guanine to be located together, in the same position as hypoxanthine phosphoribosyltransferase (*hpt* gene product) of the wild-type strain. Genetic analysis showed the *sug* mutation to be allelic with *hpt*. Therefore *sug* represented a modification of the substrate specificity of the *hpt* gene product.

Enteric bacteria have two separate and genetically distinct purine phosphoribosyltransferases (PRT) that are involved in the conversion of hypoxanthine, guanine, and xanthine to their respective ribonucleotides (3-5, 7). Mutants lacking these enzymes have been isolated, and genetic and biochemical analysis of the mutants has allowed characterization in terms of percent of substrate cross-specificity of the two enzymes (4). The hypoxanthine PRT (HPRT), the product of the *hpt* gene, actively converts about 65 to 75% of the available hypoxanthine to inosine 5'-monophosphate (IMP) and 10% of the guanine to guanosine 5'-monophosphate (GMP). The guanine PRT (GPRT), product of the *gpt* gene (formerly designated *gxu* [5]) is responsible for forming 25 to 35% of the IMP, 90% of the GMP, and is the sole vehicle for entrance of xanthine into the purine nucleotide interconversion pathway via xanthine 5'-monophosphate (XMP). Strains lacking the *gpt* gene product are resistant to inhibition by 8-azaguanine because the GPRT is the enzyme responsible for the conversion of this analogue to its active inhibitory component (5).

The HPRT and GPRT enzymes of *Salmonella typhimurium* have been separated on ecteola-cellulose columns, confirming the observations of Krenitsky et al. (7) in *Escherichia coli*. Two peaks of enzyme activity specific for hypoxanthine and guanine were detected in cell-free extracts of *S. typhimurium* strain LT-2. These peaks were identified as the *hpt* and *gpt* gene products when the appropriate mutants were similarly examined. The *gpt* gene is located in the *proAB* region of the *S. typhimurium* chromosome and is simultaneously lost in a *proAB* deletion mutant (5). Such a mutant carrying a *purE* mutation cannot use guanine or xanthine as efficient purine sources. An additional mutational event in this mutant has led to the isolation of a derivative which is able to grow on guanine.

In this report we describe the isolation and identification of this secondary mutant, designated *sug* (suppressor of guanine utilization). It was able to grow with guanine, but not xanthine, as the purine supplement, was sensitive to 8-azaguanine, had restored PRT activity for guanine, and appeared to be allelic with the *hpt* gene. We propose that this *sug* mutation created a modification of the *hpt* gene product with respect to its substrate specificity.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. It was shown previously (5) that the *proAB47* deletion also includes the *gpt* gene so that in a *pur*$^-$ derivative, such as strain GP660, the purine requirement can no longer be satisfied by guanine or xanthine. Strain GP36 is a derivative of GP660 obtained by selecting for the ability to utilize guanine as a purine source. This secondary mutation, designated *sug* (suppressor of guanine utilization), was obtained by inoculating GP660 into nutrient broth tubes containing twofold serial dilutions of nitrosoguanidine (NG). All tubes were incubated for

TABLE 1. *Bacterial strains*

Strain	Genotype	Source
LT-2	Wild type	M. Demerec
AB47	(*proAB-gpt*)Δ	M. Demerec
GP660	(*proAB-gpt*)Δ, *purE66*	M. Demerec
GP36	(*proAB-gpt*)Δ, *purE66*, *sug*	This study
JK1285	*leu D798*, *ara*, (*proAB-supQ285*)Δ	J. Kemper
GP39	*leu D798*, *ara*, *pro*$^+$ *gpt3*	Localized mutagenesis (6) of JK1285
TM445	*purC7*, *purI509*, *proA45*, *ilvA405*, *aroP505*, *rha461*, *str*, *hpt*, *guaC*	R. Martin (3)
GP57	as TM445, but *guaC*$^+$	*guaC*$^+$ transductant of TM445
GP58	as GP57, but *pro*$^+$, *gpt3*	*pro*$^+$ *gpt*$^-$ transductant of GP57 from GP39
GP59	as GP58, but *sug*	*sug*$^-$ transductant of GP58 from GP36

18 h at 37 C with aeration. The tube containing the highest dilution of NG that had a significant increase in turbidity (2 μg of NG per ml) was collected by centrifugation, and the cells were washed, resuspended in 5.0 ml of sterile saline, and plated on medium E (see below) containing casein hydrolysate and guanine. Colonies were checked for growth on hypoxanthine, xanthine, and casein hydrolysate with no purine supplement and for sensitivity to 8-azaguanine.

A *gpt* point mutation was obtained by localized mutagenesis (6) of a *pro*$^-$ mutant, strain JK1285. P22 phage grown on wild-type strain LT-2 was mutagenized with hydroxylamine by the method of Hong and Ames (6). This mutagenized phage was then used to transduce JK1285 to *pro*$^+$ with simultaneous selection for *gpt*$^-$ on the basis of 8-azaguanine resistance. The *pro*$^+$*gpt*$^-$ cotransductant so obtained, strain GP39, was confirmed to be *gpt*$^-$ by enzyme analysis. P22 phage was grown on GP39 and this was used to construct the *hpt*$^-$*gpt*$^-$ double mutant, by using strain GP57 as recipient and selecting for *pro*$^+$ in the presence of adenosine as purine source. Strain GP58 was one of the *pro*$^+$ recombinants which had also received *gpt3* by cotransduction. It grows poorly on adenine (or adenosine) as purine source and not at all on hypoxanthine, guanine, or xanthine. Growth on adenosine is stimulated by 2,6-diaminopurine or GMP. GP58 was confirmed to be a *hpt*$^-$*gpt*$^-$ double mutant by enzyme analysis.

sug transductants of GP58 were selected after exposure to P22 transducing phage derived from GP36. All transductants were surveyed for ability to grow on adenine, hypoxanthine, or guanine but not xanthine, indicative of the *sug* gentoype. These transductants were assigned the strain number GP59. Production of donor-transducing phages lysates and their assay followed standard procedures (1).

Media and culture techniques. The minimal salts medium used was medium E described by Vogel and Bonner (9) with glucose (0.2%) as the carbon source and casein hydrolysate (0.1%) for amino acid requirements. Purine supplements were always at the concentration of 20 μg of purine per ml of medium. Quantitative measurements were made in liquid media with a Klett-Summerson photocolorimeter using a green filter (no. 54). All cultures were incubated at 37 C. Tube cultures (5.0 ml) were aerated on a roller drum apparatus, and large broth cultures (100 to 400 ml) were incubated in flasks on a rotary-shaker water bath (New Brunswick). Nutrient broth (Difco) served as medium for preparation of bacteriophage lysates. Production of transducing phage lysates and their assay followed standard procedures (1).

Chemicals. The tetrasodium salt of 5-phosphoribosyl-1-pyrophosphate was purchased from Sigma Chemical Co. (St. Louis, Mo.) and 8-azaguanine (2-amino, 6-oxy, 8-azapurine) was from Calbiochem (Los Angeles, Calif.). [8-^{14}C]hypoxanthine (3.07 mCi/mmol) and [8-^{14}C]guanine (5.41 mCi/mmol) were obtained from New England Nuclear (Boston, Mass.). [2-^{14}C]xanthine (48 mCi/mmol) was purchased from Schwartz/Mann (Orangeburg, N. Y.). The scintillation fluid used for counting in a Packard Tri-Carb scintillation counter was Omnifluor (New England Nuclear, Boston, Mass.) dissolved in toluene (Baker, analytical grade).

Enzyme assay. The growth, harvesting, disruption, and assay for purine phosphoribosyltransferase activites were described previously (5). The product of the radiochemical assay was quantitated by thin-layer chromatography as described (5) or by a modification of the DE-81 filter technique described by Atkinson and Murray (2). A sample (0.025 or 0.1 ml, depending on whether a crude extract or an ecteola-cellulose column fraction was being assayed) was spotted on a Whatman filter disk DE-81 (2.5 cm diameter, Reeve Angel, Clifton, N.J.), immersed into 1 liter of 4.0 mM ammonium bicarbonate solution for 45 min, rinsed twice with equal volumes of distilled water (30 min per wash), dried, and counted. Up to 100 disks could be handled together. There is nonspecific adsorption of guanine to the DE-81 disks, and this was significantly reduced by saturating each disk with cold guanine and drying before use. Xanthine PRT could not be measured by this disk procedure; however, the advantage of handling multiple samples permitted more rapid determination of enzyme activ-

ity in the column fractions.

Protein concentrations were determined by the Lowry method (8) with bovine serum albumin as the standard.

Ecteola-cellulose column chromatography. Cell-free extracts of 200- to 400-ml cultures were washed three times with 0.03 M sodium phosphate buffer, pH 7.4, suspended in the same buffer containing 2 mM mercaptoethanol, sonically treated (four 20-s bursts), and centrifuged for 1 h at 16,000 rpm in a refrigerated Sorvall (RC-2). A 3- to 5-ml volume (100 mg of protein total) of the cleared supernatant was applied to an ecteola-cellulose column (1 by 22 cm) and chromatographed by the procedure of Krenitsky et al. (7). The technique was modified only by the addition of mercaptoethanol (2 mM) to the eluting buffers. This alteration appeared to prevent deterioration of the enzyme for several days at 4 C. Fractions (3 ml) were collected, and 0.1 ml of every fraction after the gradient started was assayed for HPRT and GPRT activity as described above. When the tube containing peak activity was found, a xanthine PRT assay was performed and analyzed by thin-layer chromatography.

Incorporation of radioactive purines. The procedure for estimation of incorporation (5) was altered in that 0.1-ml samples were spotted on 3MM Whatman disks which had been saturated with the appropriate unlabeled purine base. These filters were washed three times with 5% cold trichloroacetic acid (45 min per wash) followed by a 30-min acetone wash. The disks were dried, immersed in 5.0 ml of scintillation fluid, and counted.

RESULTS

Isolation and phenotype of sug mutant. The initial isolation procedure utilized a purine-requiring mutant, strain GP660, which has a deletion rendering the strain deficient for *proAB* and *gpt*. Selection was made for the ability to grow on guanine as described in Materials and Methods. The mutant so obtained was designated *sug* because of an apparent suppression of guanine utilization. Colonies growing on a guanine medium were found to also utilize hypoxanthine and adenine, but not xanthine, as a purine source. In addition, the mutant was sensitive to inhibition by 8-azaguanine. Figure 1 illustrates the altered growth characteristics of GP36 and the parent strain GP660 on hypoxanthine and guanine. Whereas the growth responses of both strains were identical with hypoxanthine, there was a striking difference in the growth rate on guanine.

Phosphoribosyltransferase activities. A study of the enzymatic capacities of GP36 was performed to detect a difference in the levels of PRT activities. Cell-free extracts of GP36 were assayed for PRT activity. These results are listed in Table 2 in comparison with data on

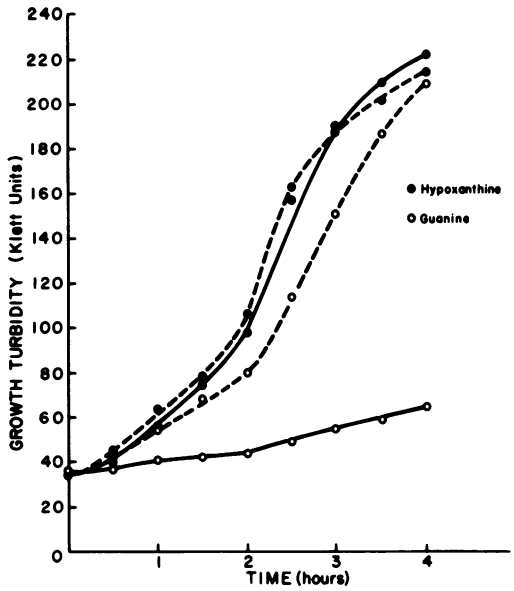

FIG. 1. *Growth response of GP660 (continuous lines) and GP36 (dashed lines) on hypoxanthine (closed circles) or guanine (open circles). Growth turbidity was measured every 30 min after 1/20 dilution of an overnight culture into medium plus purine supplement.*

TABLE 2. *Hypoxanthine and guanine phosphoribosyltransferase activities*

Strain	Relevant genotype	Phosphoribosyltransferase activities[a]		
		IMP	GMP	XMP
LT-2	Wild type	28.0	18.2	24.1
GP660	*gpt*⁻	21.2	0.16	0.36
GP36	*gpt*⁻*sug*⁻	15.3	13.2	2.1

[a] Activity measured as nanomoles of purine bases converted to ribonucleotide per minute per milligram of protein in cell-free extracts.

wild type and GP660. IMP synthesis in GP660 was lower than LT-2 by the amount contributed by the *gpt* activity and this was consistent in all the *gpt* mutants we examined. The measured levels of GMP synthesis in GP660 and XMP synthesis in both GP660 and GP36 ranged from 2 to less than 1. A portion of this activity was determined to be an artifact of the assay system (see Materials and Methods for explanation). The ability of GP36 to convert guanine to GMP increased markedly over that of its parent strain, and approached the level observed with wild type. There was, however, almost a 50% decrease in the amount of IMP synthesized

during the assay period. The XMP value was regarded as insignificant. Thus the ability to enzymatically convert guanine to GMP had been partially restored in GP36 with a concomitant decrease in IMP activity.

Chromatographic separation of HPRT and GPRT. The resolution of HPRT and GPRT activities in *E. coli* cell-free extracts was described by Krenitsky et al. (7). We repeated his observations using *S. typhimurium* as the enzyme source and obtained the activity patterns illustrated in Fig. 2. The IMP activity was found predominantly in the first peak with a smaller level present in the second. The GMP activity was considerably less than the IMP level and was approximately equal in both fractions. Examination of *gpt* mutants established clearly that the first peak contained only the *hpt* gene product (Fig. 3b). In addition, there was no detectable GMP activity in this fraction or any other sample taken during column characterization of this mutant, collecting over 300 ml of eluant. Figure 3a presents the data on the pattern of strain GP36. It can be noted that (i) there was a single peak of PRT activity, (ii) nearly identical levels of IMP and GMP activities were present in the same enzyme fraction, and (iii) the protein eluted slightly earlier, but in the same region as the *hpt* peak of AB47 and LT-2. For completion, Fig. 3 also shows the pattern (Fig. 3c) obtained with strain TM445, an *hpt*$^-$*gpt*$^+$ mutant. As expected this shows only one peak corresponding in position to the *gpt* product and possessing normal activities for IMP, GMP, and XMP (not shown).

Genetic analysis. For this portion of the work we assumed, for the sake of objectivity, that *sug*, *hpt*, and *gpt* were separate genes and that *sug*$^+$ is the natural state of the gene. Thus GP36 would contain a *sug*$^-$ mutation. With this

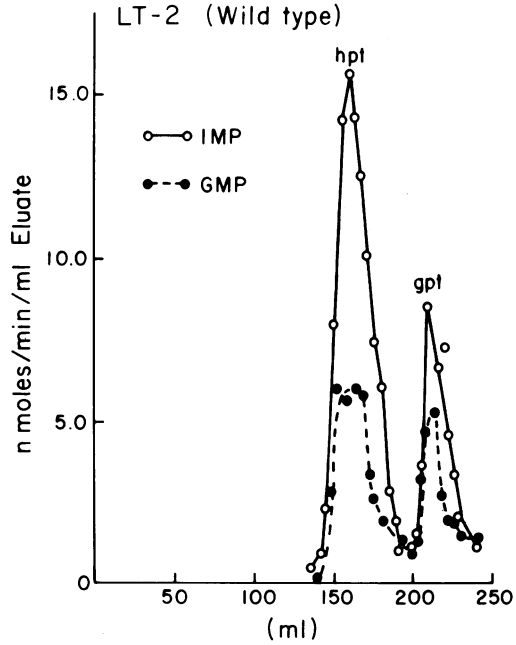

FIG. 2. *Pattern of purine phosphoribosyltransferase activities of wild type LT-2 from ecteola-cellulose column.*

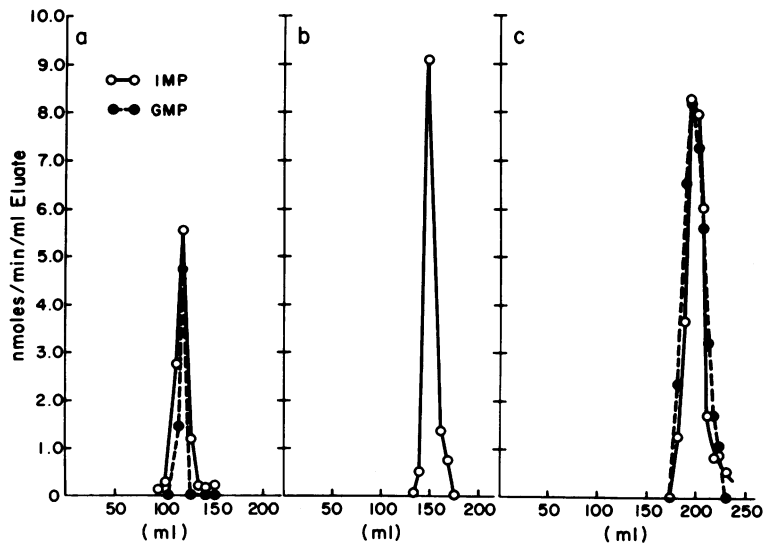

FIG. 3. *Pattern of elution of the purine phosphoribosyltransferase activity from an ecteola-cellulose column. (a) GP36 (sug*$^-$*gpt*$^-$*); (b) AB47 (gpt*$^-$*); (c) TM445 (hpt*$^-$*).*

as a guide, the following experiment was performed. A generalized transducing P22 phage was prepared from strain GP36, and GP54 (an *hpt-gpt* double mutant) was the recipient strain (Table 3). The *pur* markers in these strains were necessary to facilitate detection of the phenotypes of the PRT mutants. In this particular instance, transductants were selected for the ability to grow on hypoxanthine and tested for utilization of other purines. The ability to grow on hypoxanthine or guanine, but not xanthine, would correspond to the genotype, *gpt⁻sug⁻*. A total of 69% of the recombinants fit this category, with the remaining 31% appearing as *pur⁺* recombinants. If *hpt* and *sug* were not allelic, a certain percentage of the recombinants should have grown only with hypoxanthine but not guanine, indicative of the genotype *hpt⁺ gpt⁻*. Since none were found, this implies 100% linkage between *hpt* and *sug*, and hence allelic identity.

DISCUSSION

The current presentation raises as many questions as were answered. A mutation has been induced in a *gpt* purine auxotroph which allowed growth with guanine as the sole purine source. Measurement of enzyme levels in crude extracts of the new mutant strain GP36 indicated approximately equal levels of IMP and GMP synthesis. The parent of GP36 had very little GMP activity. This evidence was interpreted to mean an existing PRT had been modified, possibly in the active substrate site, to permit a fairly efficient synthesis of guanine ribonucleotides. The most likely candidate for modification was the *hpt* gene product since there is an apparent similarity in the molecular structure of the two purine bases—hypoxanthine and guanine. This notion would suggest that (i) guanine and hypoxanthine activities should be inseparable on chromatographic elution, (ii) both enzyme activities should be contained in the fraction corresponding to the *hpt* gene product, and (iii) *sug* and *hpt* should be allelic. We compared the ecteola-cellulose chromatography pattern of the two PRT mutants and wild type with GP36. These studies clearly confirmed the observations of Krenitsky et al. (7) with an *E. coli* PRT prototroph. In addition, as predicted above, the PRT activities of GP36 banded together in the region of the HPRT. The modification of the *hpt* substrate reactive site was at the loss of overall efficiency for the natural substrate of *hpt*. The proof of allelism was final evidence indicating that the *sug* mutation was an alteration in the substrate specificity of the *hpt* gene product. It is interesting that this alteration did not include activity for xanthine. We suggest that the use of these mutants might be valuable tools for the study of the molecular construction of the substrate site on HPRT.

Another interesting observation may be made regarding the column chromatography data. The LT-2 profile indicates an equal division of GMP activity in both fractions, and this contradicts the results of the mutant studies which suggested a 90–10 distribution of the guanine activity between GPRT and HPRT. The *gpt* mutants showed only one peak corresponding to the HPRT activity and no GPRT was found. We also profiled the *hpt* mutant and only a single peak corresponding to the *gpt* product was found with the expected activities for hypoxanthine, guanine, and xanthine. It appears possible that the *gpt* product is required for optimal guanine activity of the *hpt* product and possibly that the *gpt* product stabilizes the *hpt* product in vitro. The additional consideration is that in vivo these two products exist naturally as a complex of enzymes instead as separate entities. Finally, we noted that despite the application of identical amounts of protein during the column chromatography, the quantity of enzyme appeared to be considerably lower in the various PRT mutants, as seen in the lower activity peaks (compare Fig. 2 and 3).

The *sug* mutation was considered to be an apparent suppressor mutation since the phenotypic expression of the first mutation (*gpt*) was suppressed with respect to quanine utilization by the second mutation (*sug*). No specific mechanism was implied by the nomenclature, but it is now clear that this represents a type of physiological suppression whereby the second mutation altered the substrate specificity of another enzyme (HPRT) so that it could then supply part of the function of the missing enzyme.

TABLE 3. *Transduction results*[a]

Transductants (%)	Purine growth response				
	None	Hypoxanthine	Guanine	Xanthine	Phenotype
69	−	+	+	−	*gpt⁻ sug⁻*
0	−	+	−	−	*hpt⁺ gpt⁻*
31	+	+	+	+	*pur⁺*

[a] Transduction: the donor was GP36 (*gpt⁻ sug⁻* [*hpt⁺*] *purE⁻*) and the recipient was GP58 (*gpt⁻* [*sug⁺*] *hpt⁻ purC*). Selection was in hypoxanthine and transductants were scored for the ability to grow on other purine sources. The total number of transductants examined was 90.

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