Copyright © 1996 by the Genetics Society of America

Genetic Analysis of Metabolic Crosstalk and Its Impact on Thiamine Synthesis in *Salmonella typhimurium*

Leslie Petersen, Jodi Enos-Berlage and Diana M. Downs

Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

Manuscript received November 22, 1995
Accepted for publication February 1, 1996

ABSTRACT

The first five steps in *de novo* purine biosynthesis are involved in the formation of the 4-amino-5-hydroxymethyl-2-methyl pyrimidine (HMP) moiety of thiamine. We show here that the first enzyme in *de novo* purine biosynthesis, PurF, is required for thiamine synthesis during aerobic growth on some but not other carbon sources. We show that PurF-independent thiamine synthesis depends on the recently described alternative pyrimidine biosynthetic (APB) pathway. Null mutations in *zwf* (encoding glucose-6-P dehydogenase), *gnd* (encoding gluconate-6-P dehydrogenase), *purE* (encoding aminoimidazole ribonucleotide carboxylase), and *purR* (encoding a regulator of gene expression) were found to affect the function of the APB pathway. A model is presented to account for the involvement of these gene products in thiamine biosynthesis via the APB pathway. Results presented herein demonstrate that function of the APB pathway can be prevented either by blocking intermediate formation or by diverting intermediate(s) from the pathway. Strong genetic evidence supports the conclusion that aminoimidazole ribotide (AIR) is an intermediate in the APB pathway.

OUR research seeks to understand metabolic crosstalk in the cell at a molecular level. The goal is to understand how the cell coordinates its extensive array of biochemical pathways to produce an efficient metabolism. Our approach to understanding metabolic crosstalk in the cell has been driven by two assumptions. First, biochemical blocks in a pathway not only prevent end product formation but alter levels of metabolites before and after the block. Such alterations might be expected to produce regulatory effects elsewhere in metabolism (Turnbough and Bochner 1985; LaRossa *et al.* 1987). Second, particularly in pathways requiring low carbon flux, there may be alternative metabolic entries to the pathway. The cell could derive metabolites necessary for these entries by influx from other pathways that share common intermediates, or precursors to a given intermediate. In addition, the cell may have dedicated alternative pathways to provide intermediates for biosynthesis.

Thiamine biosynthesis in *Salmonella typhimurium* serves as a model system for these studies. As the biosynthetic pathway for an essential vitamin, thiamine biosynthesis must be intact for the cells to grow without exogenous thiamine. Therefore by assessing a cell's ability to grow in the absence of exogenous thiamine, we have a nutritional assay for endogenous thiamine synthesis that necessarily requires a small carbon flux. Thus, unlike work with a major biosynthetic pathway, the difference between growth and no growth in our system can reflect very small changes in carbon flux. In addition, this pathway has biochemical connections with the well-characterized, high carbon flux purine biosynthetic pathway (Newell and Tucker 1968a,b; Brown and Williamson 1987). In *S. typhimurium*, purines and the pyrimidine moiety of thiamine [4-amino-5-hydroxymethyl-2-methyl-pyrimidine, (HMP)] are reportedly derived from a common pathway that branches at aminoimidazole ribotide (AIR) (Newell and Tucker 1967, 1968a,b; Estramareix and Therisod 1984; Estramareix and David 1990). A pathway with one branch requiring high carbon flux to satisfy cellular needs (*e.g.*, purine) and one branch needing only a low carbon flow (*e.g.*, thiamine) would be expected to raise interesting regulatory challenges for the cell.

The *purF* gene encodes the enzyme phosphoribosylpyrophosphate amidotransferase (E.C. 2.4.2.14), whose product is phosphoribosylamine (PRA). PurF is the first of five enzymes shared by the purine and HMP biosynthetic pathway. As such, PurF is expected to be required for both purine and HMP biosynthesis (Newell and Tucker 1967).

We have previously shown thiamine biosynthesis can be independent of the *purF* gene product under two growth conditions. First, under anaerobic growth conditions, we isolated mutants that defined *apbA*, the first gene of the *a*lternative *p*yrimidine *b*iosynthetic (APB) pathway to be defined, and provided evidence that this pathway could bypass the requirement for all *pur* genes in thiamine synthesis (Downs 1992; Downs and Petersen 1994). Second, we have shown genetically that thiamine synthesis can occur independent of both *apbA* and *purF* if exogenous pantothenate is provided in the

Corresponding author: Diana M. Downs, Department of Bacteriology, 1550 Linden Dr., Madison, WI 53706.
E-mail: downs@vms2.macc.wisc.edu

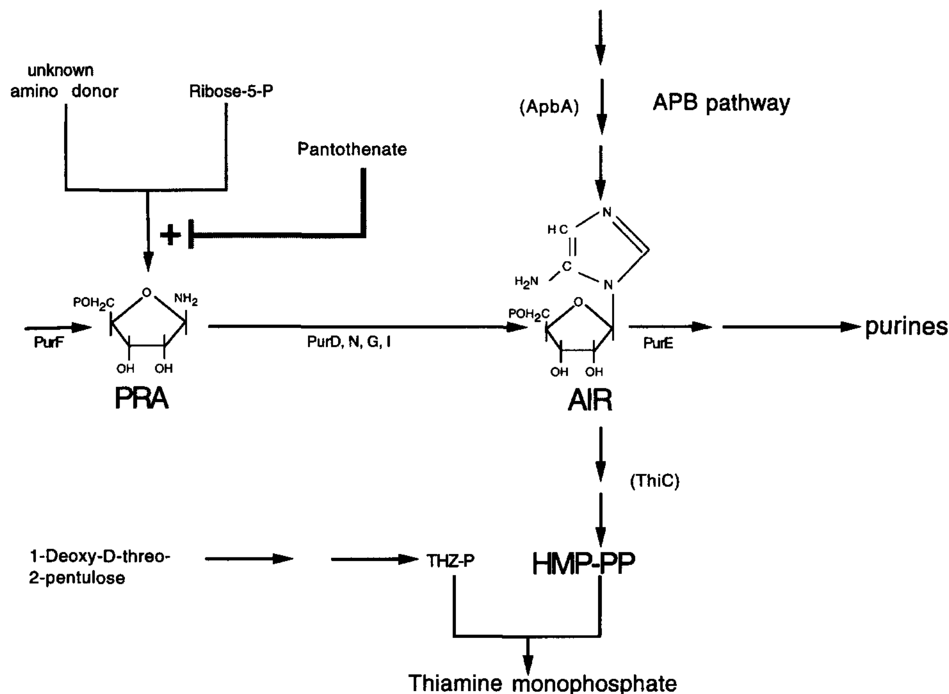

FIGURE 1.—Metabolic inputs to synthesis of 4-amino-5-hydroxy-methyl-2-methyl pyrimidine (HMP). Schematic of biosynthetic pathways involved in HMP synthesis. (1) The purine biosynthetic pathway with a known branch point to HMP at aminoimidazole ribotide (AIR). (2) The alternative pyrimidine biosynthetic (APB) pathway shown entering through AIR as per our model. Uncertainty about the particular biochemical steps performed by ApbA and ThiC (T. BEGLEY, personal communication) are indicated by parentheses. (3) Pantothenate-dependent pathway, shown with an involvement of ribose-5-P and a requirement for pantothenate (ENOS-BERLAGE and DOWNS 1996). HMP is subsequently phosphorylated to HMP pyrophosphate (HMP-PP) and joined with the independently formed 4-methyl-5(β-hydroxyethyl) thiazole phosphate moiety (THZ-P) to form thiamine monophosphate. Thiamine monophosphate is then further phosphorylated to yield the biologically active coenzyme thiamine pyrophosphate.

culture medium (DOWNS and ROTH 1991; ENOS-BERLAGE and DOWNS 1996). Recent work has shown that pantothenate-dependent thiamine synthesis requires a functional pentose phosphate pathway and that the specific contribution of this pathway is to supply ribose-5-phosphate (R-5-P) (ENOS-BERLAGE and DOWNS 1996). Our current view of the metabolic inputs to HMP biosynthesis is illustrated in Figure 1.

Here we report the results of genetic studies that identify metabolic interactions between the pentose phosphate pathway, purine biosynthesis and the thiamine biosynthetic pathway in *S. typhimurium*. We have identified aerobic growth conditions where PurF-independent thiamine synthesis occurs and is dependent on the APB pathway. We demonstrate by genetic means that mutations in a variety of genetically distinct loci, including *zwf* (encoding glucose-6-P dehydogenase), *gnd* (encoding gluconate-6-P dehydrogenase), *purE* (encoding aminoimidazole ribonucleotide carboxylase), and *purR* (encoding a regulator of gene expression) affect function of the APB pathway for thiamine synthesis. We present a model to explain the involvement of these gene products in thiamine synthesis.

MATERIALS AND METHODS

Bacterial strains: All strains used in this study are derivatives of *S. typhimurium* LT2 and are listed with their genotypes in Table 1. Tn*10*d(Tc) refers to the transposition-defective mini-Tn*10* described by WAY *et al.* (1984). MudJ refers to the defective transposon Mu dI*1734*, described elsewhere (CASTILHO *et al.* 1984). The *purF2085* mutation has been shown to be a deletion (DOWNS and ROTH 1991).

Culture media and biochemicals: No-carbon E medium supplemented with $MgSO_4$ (1 mM) (VOGEL and BONNER 1956; DAVIS *et al.* 1980), with addition of a carbon source (20 mM), was used as a minimal medium. Difco nutrient broth (8 g/liter) with NaCl (5 g/liter) was used as rich medium. Difco BiTek agar was added (15 g/liter) for solid medium. The following additives were included in media as needed (final concentrations given): adenine (0.4 mM), thiamine (25 μM), calcium pantothenate (50 μM), glucose-6-P (400 μM). Antibiotics were added as needed to the following concentrations in micrograms per milliliter (rich/minimal): tetracycline (20/10), kanamycin (50/125), ampicillin (30/15). When needed trace minerals were added at described concentrations (BALCH and WOLFE 1976). All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Transductional methods: All transductional crosses were performed with the high-frequency, generalized transducing bacteriophage P22 mutant HT*105/1*, *int-201* (SCHMIEGER 1972) as has been described previously (DOWNS and PETERSEN 1994). Transductants were purified by streaking on nonselective green indicator plates and putative phage-free clones were identified by their light green color (CHAN *et al.* 1972). Phage-free colonies were verified to be phage sensitive by cross-streaking with phage P22.

Mutant isolation: For the work described here, an Apb^- phenotype is defined as the inability of a *purF*-containing mutant to synthesize thiamine on medium-containing gluconate,

TABLE 1

Strain list

Strain	Genotype
LT2	Wild-type
DM400	*purF2085 zwf-21*::Tn*10*d[a]
DM574	*purF2085 gnd-175*::Tn*10*d
DM587	*purF2085 apbA7*::Tn*10*d
DM589	*purF2085 gnd-175*::Tn*10*d *apbA1*::MudJ[b]
DM929	*purF2085 purE884*::Tn*10*
DM932	*purF2085 purR2319*::Tn*10*
DM996	*purF2085 purE884*::Tn*10 apbA1*::MudJ
DM1224	*purF2085 purR2319*::Tn*10 purE2154*::MudJ
DM1761	*purF2085 purG2324*::MudJ
DM1763	*purF2085 purE2154*::MudJ
DM1764	*purF2085 purC2156*::MudJ
DM1782	*purF2085 purG2324*::MudJ *purR2319*::Tn*10*
DM1784	*purF2085 purE2154*::MudJ *purR2319*::Tn*10*
DM1785	*purF2085 purC2156*::MudJ *purR2319*::Tn*10*
DM1936	*purF2085*
DM1937	*purF2085*/pJS355[c] pPL1-1[d]

[a] Tn*10*d refers to the transposition-defective mini-Tn*10* (Tn*10*del-16 del-17).

[b] MudJ is used throughout the text to refer to the Mu *dl1734* transposon (CASTILHO *et al.* 1984).

[c] Plasmid pJS355 was provided by J. STUBBE (MEYER *et al.* 1992).

[d] Plasmid pPL1-1 was provided by M. KAINZ (KAINZ 1994).

fructose or glycerol as a sole carbon source. Mutants with an Apb$^-$ phenotype were isolated using insertion mutagenesis with one of two defective transposons, Tn*10*d(Tc) or MudJ. In either case, a P22 lysate was grown on a pool of cells containing >80,000 clones, each of which was assumed to carry one element randomly inserted in the chromosome. Recipient cells of DM1936 (*purF2085*) were transduced to the appropriate drug resistance on nutrient agar plates using the indicated phage lysate. The transductants were then replica printed sequentially to: (1) a minimal plate containing the desired carbon source and adenine, (2) a plate as in (1) with added thiamine. Putative mutants were identified by their inability to grow in the absence of exogenous thiamine. After being streaked out and verified as phage sensitive, these mutants were reconstructed by transducing the insertion into both a *purF2085* (strain DM1936) and wild-type (LT2) background. Only mutations allowing growth on minimal glucose medium in a wild-type (Pur$^+$) genetic background were pursued. This eliminated mutations in *thi* loci. The mutants characterized here were the result of independent mutant searches using fructose, glycerol and gluconate as carbon sources. A typical search involved screening 20,000 antibiotic resistant colonies and yielded two to three mutants that displayed an Apb$^-$ phenotype in a *purF* background (see RESULTS).

Phenotypic characterization: Phenotypes were assessed both in liquid and on solid growth media.

Growth curves: Strains to be analyzed were grown to full density in nutrient medium at 37°. After overnight incubation, cells were pelleted and resuspended in an equal volume of sterile saline (85 mM), and 0.2 ml of this suspension was used to inoculate 5 ml of the appropriate medium (2% v/v inoculum). Culture tubes were placed in a shaking water bath at 37°, and growth was monitored as optical density at 650 nm on a Bausch and Lomb Spectronic 20. Specific growth rate was determined as $\mu = \ln(X/X_o)/T$, where $X = OD_{650}$ during the linear portion of the growth curve and T = time. Routinely, the OD_{650} at time 0 was between 0.02 and 0.04. Overnight growth was scored as negative if the $OD_{650} < 0.2$; control cultures (with thiamine) reached $OD_{650} > 1.0$.

Plate tests: Nutritional requirements were measured in soft agar overlays as follows: a 0.2-ml aliquot of a saline cell suspension (prepared as above) was added to 4 ml of molten 0.7% agar and spread on an appropriate plate. Nutrients to be tested were spotted at the indicated volume and concentration after the agar had solidified.

β-galactosidase assays: β-galactosidase activity was assayed by the method of MILLER (1972) as reported previously (ESCALANTE-SEMERENA and ROTH 1987).

Plasmid manipulation: Plasmid pJS355 carries an *Eco*RI-*Pst*I fragment containing the *purEK* genes from *Escherichia coli* cloned into a λpL expression vector (MEYER *et al.* 1992). Plasmid pPL1-1 contains a source of λc*I857*, a temperature-sensitive λcI repressor (SUSSMAN and JACOB 1962; KAINZ 1994). Strain DM1937 was constructed by sequentially introducing these two plasmids into DM1936 by electroporation using an *E. coli* Pulser (BioRad Laboratories, Richmond, CA).

RESULTS

It was shown almost 40 yr ago that *purF* mutants require a source of both purines and the pyrimidine moiety of thiamine (HMP) when growing aerobically with glucose as sole carbon source (YURA 1956; NEWELL and TUCKER 1967). This is the expected phenotype for a mutant blocked in the common part of a branched pathway, *i.e.*, a requirement for both end products. However, we found the requirement for PurF in thiamine synthesis to be dependent on the growth condition of the cells, which raised questions about the role of this enzyme in thiamine synthesis in *S. typhimurium*. This observation has provided us with an ideal system to identify and characterize additional metabolic inputs into thiamine synthesis and to explore their role in the physiology of *S. typhimurium*. In the studies described here, we satisfied the purine requirement of the *purF* deletion strains by supplying adenine in the culture medium. By providing a source of purine, we were able to monitor the cell's ability to synthesis thiamine (a low carbon requirement) independent of the high carbon flux purine biosynthetic pathway. Throughout this work we equate a defect in thiamine synthesis with a defect in HMP synthesis since we are not altering the thiazole specific branch of the pathway (see Figure 1).

PurF-independent thiamine synthesis is carbon source specific: A *purF* deletion strain (DM1936) was assessed for growth on minimal medium supplemented with adenine (0.4 mM) and a particular carbon source (20 mM). Twenty carbon sources that can be utilized by *S. typhimurium* (GUTNICK *et al.* 1969) were tested, including: L-arabinose, Na-citrate, fructose, fucose, Na-fumarate, galactose, Na-gluconate, glucose, glycerol, Na-malate, maltose, D-mannitol, mannose, melibiose, rhamnose, ribose, sorbitol, Na-succinate, trehalose, and xylose. PurF-independent thiamine synthesis (equated with growth in the absence of exogenous thiamine) was monitored by replica printing on solid medium or by overnight growth in liquid (see MATERIALS AND METH-

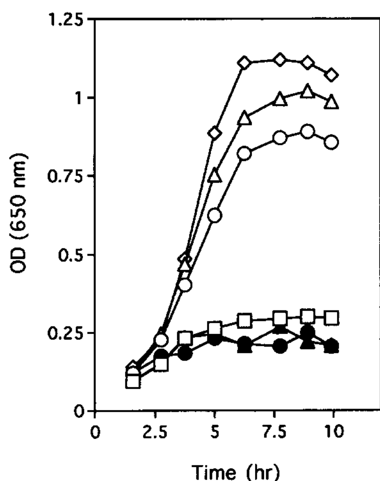

FIGURE 2.—APB-dependent thiamine synthesis occurs aerobically. Cultures were prepared and grown at 37° in shaking water baths as described in MATERIALS AND METHODS. Open figures represent strain DM1936 (*purF2085*). Solid figures represent DM587 (*purF apbA*). ○, minimal glucose adenine medium with 1 mM iron; △, minimal gluconate adenine medium; □, minimal glucose adenine medium; ◇, minimal glucose adenine thiamine medium.

ODS). Of the carbon sources tested, PurF-independent thiamine synthesis occurred on all but glucose, citrate, fumarate, malate and succinate containing medium. On all carbon sources, growth in the absence of exogenous thiamine was compared with that seen in the same medium containing thiamine (see MATERIALS AND METHODS). This allowed us to eliminate any effects, unrelated to thiamine synthesis, each carbon source had on the growth rate or final yield of the cultures. With the exception of glucose, all carbon sources unable to support PurF-independent thiamine synthesis were TCA cycle intermediates; the effects of these carbon sources were not pursued further.

We found that the exogenous addition of several compounds allowed PurF-independent thiamine synthesis to occur on glucose medium. Several possible nitrogen sources were tested and both glutamine and ammonium chloride were found to stimulate thiamine synthesis. We also noticed that trace minerals stimulated thiamine synthesis of this strain in liquid medium. We determined the stimulatory component of the trace mineral mix was iron (see Figure 2). Because of other work in the laboratory, we tested the ability of ribose-5-phosphate (R-5-P) to stimulate thiamine synthesis. We found that R-5-P allowed PurF-independent thiamine synthesis when glucose-6-P was simultaneously added to stimulate its uptake (SHATTUCK-EIDENS and KADNER 1981).

Each of these apparently unrelated compounds was efficient at stimulating thiamine synthesis when present at a final concentration of 1 mM; further titration to determine the minimal concentration required for significant stimulation was not performed. Pantothenate had been shown previously to allow PurF-independent thiamine synthesis at 50 μM (DOWNS and ROTH 1991; ENOS-BERLAGE and DOWNS 1996). A wide range of related vitamins and all amino acids were tested and no other compounds that allowed *purF* cells to grow without thiamine on glucose were found.

The *apbA* gene product is required for PurF-independent thiamine synthesis: We had previously described the existence of the *a*lternative *p*yrimidine *b*iosynthetic (APB) pathway. Synthesis of thiamine by this pathway does not require any of the purine genes under anaerobic conditions (DOWNS 1992). Although we originally reported that mutations in *apbA* block thiamine synthesis in *pur* backgrounds under anaerobic growth conditions (DOWNS and PETERSEN 1994), recent observations had suggested that this pathway functions at some capacity under aerobic growth conditions. To address the role of this pathway in the PurF-independent thiamine synthesis described above, we analyzed the growth behavior of *purF apbA* double mutants.

The following lines of evidence suggest that the APB pathway is responsible for the high reversion frequency to thiamine-independent growth previously reported for *purF* mutants (DOWNS and ROTH 1991), the PurF-independent thiamine synthesis stimulated on glucose by the range of compounds described above and the PurF-independent thiamine synthesis seen on alternative carbon sources.

When grown on glucose, strains carrying deletions of *purF* acquired secondary mutations (at a frequency of 10^{-5}–10^{-6}) that allowed them to synthesize thiamine (DOWNS and ROTH 1991). We found that when an *apbA* insertion mutation was introduced into *purF* deletion strains, this reversion rate was markedly reduced to 10^{-8}. This result suggested that the frequent secondary mutations that restored thiamine synthesis were either allowing function or increasing function of the APB pathway on glucose media. Consistent with this is our finding that mutations in *purE* are among these secondary mutations (see section on *purE* below).

Similar to the secondary mutations above, compounds that allowed PurF-independent thiamine synthesis on glucose medium required the APB pathway for their stimulation of thiamine synthesis. This conclusion was based on the fact that a mutation in *apbA* prevented thiamine synthesis in *purF* mutants inoculated in glucose media containing adenine and either glutamine, ammonium chloride, iron, or ribose-5-phosphate (Figure 2 and data not shown).

In addition, *apbA purF* strains were unable to synthesize thiamine on any nonglucose carbon sources (Figure 2).

Taken together, these data showed the APB pathway was responsible for thiamine synthesis occurring in a *purF* strain under all the conditions we had documented. Exogenous pantothenate is an exception to this generalization. We have shown that pantothenate allows thiamine synthesis in a *purF apbA* double mutant strain by affecting a different metabolic input point

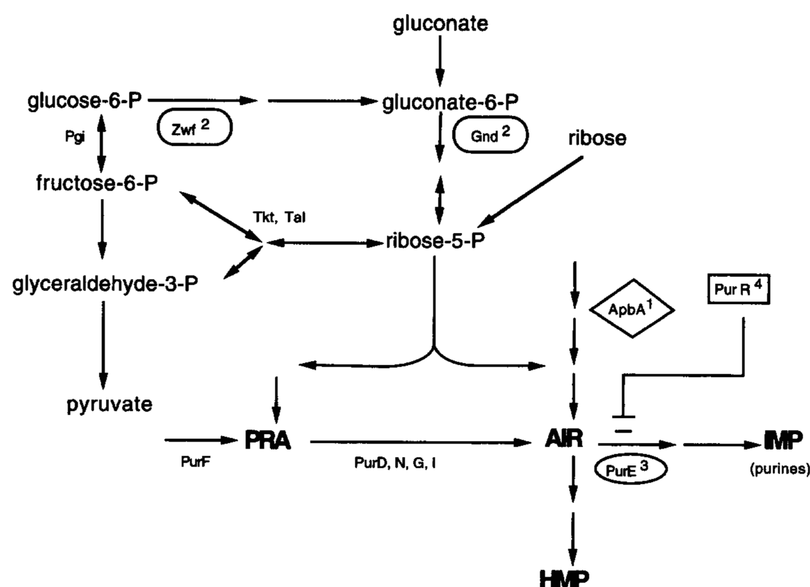

FIGURE 3.—Metabolic crosstalk that affects function of the APB pathway. A schematic representation of glycolysis, the pentose phosphate pathway, *de novo* synthesis of purines and HMP is shown. Numbered symbols represent gene products that can affect function of the APB pathway for HMP biosynthesis as follows: 1, mutations in ApbA are defined as preventing function of a biosynthetic enzyme in the APB pathway (M. FRODYMA, unpublished data). 2, mutations blocking the pentose phosphate pathway, proposed to eliminate the necessary intermediate ribose-5-phosphate. 3, mutations in *purE* increase HMP production via the APB pathway, proposed to be caused by an accumulation of the intermediate aminoimidazole ribotide (AIR). 4, mutations in *purR* divert AIR from HMP synthesis by constitutively expressing the *pur* genes, specifically *purE*.

into the thiamine pathway (ENOS-BERLAGE and DOWNS 1996).

Mutations that eliminate APB-dependent thiamine synthesis: To further define the metabolic components of this pathway, we pursued the identification of other genetic loci involved in APB-dependent thiamine synthesis. The approach was to isolate mutants unable to synthesize thiamine on carbon sources other than glucose in a *purF* genetic background. Mutant searches were performed and resulted in the isolation of 20 insertion mutants with an Apb$^-$ phenotype, four carried MudJ insertions and 16 carried Tn*10*d insertions. At present we have identified mutations in at least eight distinct genetic loci that prevent APB function (data not shown). Thus far we have been able to identify one of these loci as *gnd.* Additional strain constructions have determined that mutations in *zwf* or *purR* also prevent function of the APB pathway.

Oxidative pentose phosphate pathway is required for APB function: Four of the mutants isolated were defective in *gnd. gnd* encodes gluconate-6-phosphate dehydrogenase and is required for the oxidative pentose phosphate pathway (NASOFF *et al.* 1984) (see Figure 3). Additional strain constructions showed that mutations in *zwf* (ENOS-BERLAGE and DOWNS 1996), which encodes glucose-6-phosphate dehydrogenase, also caused an Apb$^-$ phenotype when transduced into a *purF* background.

Recent work in our laboratory had demonstrated a role for these two gene products in APB-independent, pantothenate-dependent, thiamine synthesis due to their contribution to R-5-P synthesis (ENOS-BERLAGE and DOWNS 1996). The following data supported the hypothesis that R-5-P from the pentose phosphate pathway was required for function of the APB pathway.

Strain DM574 (*gnd purF*) was unable to grow in minimal medium containing adenine in the absence of thiamine (an Apb$^-$ phenotype). However, addition of R-5-P to the medium allowed growth of this strain in the absence of exogenous thiamine. The inability of DM589 (*apbA purF gnd*) to grow under these conditions indicated the APB pathway was required for the R-5-P correction of the Apb$^-$ phenotype caused by a *gnd* mutation (data not shown).

In addition to the results above, other observations supported the hypothesis that the Apb$^-$ phenotype of the *gnd* and *zwf* mutants was due to their inability to produce sufficient R-5-P via the pentose phosphate pathway. These strains exhibit an Apb$^-$ phenotype only when grown on carbon sources that require the function of the respective enzyme for R-5-P synthesis (see Figure 3). For instance, strain DM400 (*zwf purF*) was not defective for thiamine synthesis on either gluconate or ribose. Strain DM574 (*gnd purF*) was not defective for thiamine synthesis when grown with ribose as a carbon source. Each of these results is predicted if the role of these gene products in the APB pathway is based on their ability to contribute to R-5-P pools and is consistent with R-5-P serving as a precursor to the pyrimidine moiety of thiamine (HMP) via the APB pathway.

Mutants in *purR* eliminate APB function: In the course of related work, it was found that insertion mutations in *purR* caused an Apb$^-$ phenotype in a *purF* deletion background. In *E. coli,* mutations in *purR* are reported to cause constitutive expression of all genes normally repressed by the wild-type PurR protein (ROLFES and ZALKIN 1988, 1990a,b), including all *de novo* purine biosynthetic genes. Each target gene is reported to respond to the lack of PurR with a characteristic level of derepression. The Apb$^-$ phenotype of a *purR purF* mutant was apparent on the carbon sources tested (glucose, gluconate, glycerol) and representative results (from glucose medium) are shown in Figure 4. As shown in Figure 4, in contrast to the *purR*$^+$ strain (DM1936), strain DM932 (*purF purR*) did not synthesize thiamine (and thus did not grow) when inoculated into

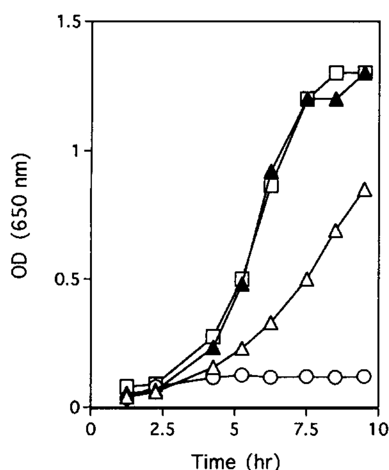

FIGURE 4.—Mutations in PurR prevent APB-dependent HMP synthesis. Cultures were prepared and grown at 37° as described in MATERIALS AND METHODS. Open symbols represent minimal glucose adenine medium with trace minerals. Closed symbols represent glucose adenine medium with trace minerals and thiamine. △, DM1936 (*purF*); ○, DM932 (*purF purR*); □, DM1224 (*purF purR purE*).

glucose medium containing adenine and trace minerals (to supply iron). All strains grew equally well when both adenine and thiamine were supplied in the medium (data not shown).

To explain the negative effect *purR* mutations had on the APB pathway, we first confirmed the regulatory phenotype of the *S. typhimurium purR* mutants (T. GUO MING, unpublished data) by assaying the expression of β-galactosidase from MudJ transcriptional fusions in various *pur* genes. We assayed mutants in a *purF* deletion background carrying a MudJ fusion in either of three *pur* genes, *purG, C, I,* under repressing growth conditions (*e.g.*, in the presence of both adenine and thiamine) in *purR* and *purR+* backgrounds. In each case, the *purR* strain contained greater than sixfold higher levels of *pur* transcription as measured by β-galactosidase expression (*purG,* 42/371; *purE,* 38/300; *purC,* 27/185). In addition to being consistent with previous results, these data eliminated the possibility that the presence of a *purF* mutation in the strain was altering the constitutive expression of *pur* genes caused by a defect in PurR.

To address the Apb$^-$ phenotype of *purR* mutants, we focused on the constitutive expression of the *pur* genes. Based on our current view of metabolic inputs into HMP synthesis (see Figure 1), we hypothesized that an elevated level of the PurE enzyme caused aminoimidazole ribotide to be diverted from HMP synthesis toward purine (IMP) synthesis. This would decrease flux through the synthetic pathway for the pyrimidine moiety of thiamine and would result in a thiamine auxotrophy. This hypothesis predicted that a mutation in *purE* would reverse the Apb$^-$ phenotype caused by a *purR* mutation. As shown in Figure 4, our results were consistent with this hypothesis. Strain DM1224 (*purF purR purE*) regained the ability to synthesize thiamine compared with strain DM932 (*purF purR*). Mutations in *purC, G,* or *I* failed to produce a similar effect (data not shown).

Overexpression of PurE reduces synthesis of thiamine through the APB pathway: Because mutations in *purR* affect the regulation of a number of genes in addition to *pur* genes (MENG *et al.* 1990; STEIERT *et al.* 1990; WILSON *et al.* 1993), we sought to more rigorously show that the Apb$^-$ phenotype on *purR* mutations was due solely to overexpression of *purE*. We constructed a *purF* strain carrying plasmid pJS355, which contains a clone of *purEK* from *E. coli* under the control of the λpL promoter (MEYER *et al.* 1992). In addition to pJS355, we introduced pPL-1 as a source of lambda *cI857* (SUSSMAN and JACOB 1962; KAINZ 1994). Thus the final strain, DM1937, was a *purF* mutant with a temperature-inducible source of PurE. Strain DM1937 could grow on glucose medium containing adenine and trace minerals at 30°, as expected. However, if monitored at 37°, growth in the absence of thiamine was markedly reduced when compared with the growth with exogenous thiamine (final OD_{650} of 0.3 *vs.* 1.1). This result is consistent with the hypothesis that increased levels of PurE can divert AIR to IMP synthesis, thus generating a nutritional requirement for thiamine.

First evidence that AIR is an intermediate in the APB pathway: It is important to emphasize here that use of glucose medium provided a growth condition where we could alter (with the presence or absence of iron) the activity of the APB pathway. This provided a growth medium where we could assess both positive and negative effects on APB pathway function without altering the wild-type growth rate as changing carbon sources would.

From the results shown in Figure 4 (compare DM1936 *vs.* DM1224), it appeared that a *purE* mutation not only eliminated the deleterious effect of the *purR* mutation but actually enhanced APB-dependent thiamine synthesis. We pursued this observation directly by determining whether a *purE* mutation had any effect on the thiamine synthesis of a *purF* mutant on glucose medium (in the absence of iron). We found that a *purE* mutation allowed thiamine synthesis in a *purF* mutant on glucose medium, necessarily overriding the glucose specific inhibition described in the first section of RESULTS. A comparison of growth rates in glucose adenine medium showed that while DM1936 (*purF*) had a specific growth rate (μ) of <0.1, the *purE* containing strain (DM929) showed a specific growth rate of 0.37. Thiamine synthesis was dependent on ApbA, as judged by the fact that in the above experiment strain DM996 (*purF purE apbA*) had a specific growth rate of <0.1. This result supported the hypothesis that the APB pathway was functioning to a limited extent on minimal glucose medium and also provided strong genetic evidence that AIR was an intermediate of the APB pathway, as previously suggested (DOWNS 1992).

DISCUSSION

The results presented herein further our understanding of the recently discovered APB pathway (DOWNS 1992) for thiamine synthesis and delineate interactions with other central metabolic pathways that regulate APB activity as a function of carbon source quality and carbon flux through *de novo* purine biosynthesis. Our genetic data show that the activity of the APB pathway can be regulated both by blocking metabolic input and diverting metabolic output.

Important conclusions from this work include:

1. Thiamine synthesis occurs aerobically in the absence of the PurF protein. PurF-independent thiamine synthesis is due to the APB pathway since introduction of an *apbA* mutation eliminated growth in the absence of exogenous thiamine.
2. Under aerobic growth conditions, function of the APB pathway is dependent on the carbon source available. When growing on some but not other carbon sources, *S. typhimurium* can bypass the requirement for PurF in the synthesis of thiamine. This finding illustrates the effect that changing growth conditions can have on cellular metabolism and emphasizes the importance of monitoring mutant phenotypes under a variety of growth conditions if we are to understand the subtle complexities of metabolic regulation and crosstalk.
3. The ability of the APB pathway to produce thiamine aerobically in a *purF* mutant is dependent on the remaining *pur* genes involved in the synthesis of AIR. Under anaerobic conditions these genes are not required for APB-dependent synthesis of thiamine (DOWNS 1992). This conclusion was based on results with insertion mutations in the *pur* genes. Current work suggests that point mutations in these *pur* genes can result in different phenotypes, some of which allow APB-dependent thiamine synthesis aerobically (J. ZILLES, unpublished results). The genetic analysis of these point mutants is in progress to determine why these *pur* gene products are required for APB activity under aerobic but not anaerobic growth conditions.
4. Mutations blocking the pentose phosphate pathway (*e.g.*, *gnd*, *zwf*) prevent function of the APB pathway due to their inability to synthesize ribose-5-P. The role of ribose-5-P in thiamine synthesis remains to be established but it is postulated to be a precursor to the pyrimidine moiety synthesized via the APB pathway.
5. Mutations in *purR* eliminate activity of the APB pathway by diverting a metabolite from synthesis of the pyrimidine moiety of thiamine (HMP) to purine synthesis. We conclude that the Apb$^-$ phenotype caused by *purR* mutations is due to the overexpression of the *purE* gene, whose product uses aminoimidazole ribotide (AIR) as a substrate. We suggest that increased levels of PurE result in increased conversion of AIR to carboxyaminoimidazole ribonucleotide (CAIR) thereby eliminating carbon flux normally available for the synthesis of the pyrimidine moiety of thiamine. In contrast to the classical notion of an auxotrophy being caused by blocking metabolite formation, we show here an auxotrophy caused by draining a metabolite already formed.
6. Consistent with the above scenario, we showed that a block in *purE* allows APB-dependent thiamine synthesis on glucose medium. From this result we make two conclusions: first, the APB pathway is weakly functional on glucose medium and the lack of thiamine synthesis in a *purF* mutant is due to insufficient flux through the APB pathway to satisfy the HMP requirement of the cell once the AIR diverted to purine synthesis is taken into consideration. Second, the result with a *purE* mutant strongly supports the hypothesis that AIR is an intermediate in the APB pathway.

In addition, the data demonstrate that the branch point at AIR is an important regulatory point for directing metabolic flux to thiamine (HMP) synthesis. We present a model (described in Figure 3) for thiamine synthesis meant to emphasize that function of a biosynthetic pathway can be prevented either by blocking metabolic input or by diverting an intermediate. From other work in the lab we know that an *apbA* mutant is a thiamine auxotroph under some growth conditions where purines are being synthesized *de novo* (M. FRODYMA, unpublished results). This further supports the notion that the pathway branch point at AIR may be actively regulated to ensure proper distribution of carbon flow. This system is reminiscent of branch point regulation of the TCA cycle and the glyoxlyate shunt where the flux through two pathways is determined by properties of the enzymes competing for the same metabolite and the level of production of each (WALSH and KOSHLAND 1984). As the study of cellular metabolism progresses, more regulatory branch points are likely to emerge. Thiamine synthesis offers a model system where carbon flux can be monitored *in vivo* and the regulatory strategies used by the cell at branch points can be probed.

In summary, we have defined a genetic system that will allow us to dissect the metabolic crosstalk between biochemical pathways, with the focus of these interaction being the effect they have on thiamine synthesis. This work is the foundation for biochemical and genetic analyses of these interactions, including the elucidation of the metabolites and enzymes involved as well as determining the regulation of such interactions.

We thank J. STUBBE for providing plasmid pJS355, M. KAINZ for providing plasmid pPL1-1 and J. ROTH for providing *purR* mutants. We thank J. C. ESCALANTE-SEMERENA and G. ROBERTS for critical reading of the manuscript. This work was supported by National Institutes of Health grant GM-47296 to D.M.D. J.L.E. was supported by an National Science Foundation predoctoral fellowship.

LITERATURE CITED

Balch, W. E., and R. S. Wolfe, 1976 New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. Appl. Environ. Microbiol. **32:** 781–791.

Brown, G. M., and J. M. Williamson, 1987 Biosynthesis of folic acid, riboflavin, thiamine, and pantothenic acid, pp. 521–538 in *Eshcerichia coli and Salmonella typhimurium Cellular and Molecular Biology,* edited by F. C. Neidhardt. Am. Soc. Microbiol., Washington, DC.

Castilho, B. A., P. Olfson and M. J. Casadaban, 1984 Plasmid insertion mutagenesis and *lac* gene fusion with mini Mu bacteriophage transposons. J. Bacteriol. **158:** 488–495.

Chan, R. K., T. Botstein, T. Watanabe and Y. Ogata, 1972 Specialized transduction of tetracycline resistance by phage P22 in *Salmonella typhimurium.* II. Properties of a high tranducing lysate. Virology **50:** 883–898.

Davis, R. W., D. Botstein and J. R. Roth, 1980 *Advanced Bacterial Genetics.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Downs, D. M., 1992 Evidence for a new, oxygen-regulated biosynthetic pathway for the pyrimidine moiety of thiamine in *Salmonella typhimurium.* J. Bacteriol. **174:** 1515–1521.

Downs, D. M., and L. Petersen, 1994 *apbA,* a new genetic locus involved in thiamine biosynthesis in *Salmonella typhimurium.* J. Bacteriol. **176:** 4858–4864.

Downs, D. M., and J. R. Roth, 1991 Synthesis of thiamine in *Salmonella typhimurium* independent of the *purF* function. J. Bacteriol. **173:** 6597–6604.

Enos-Berlage, J. E., and D. M. Downs, 1996 Involvement of the oxidative pentose phosphate pathway in thiamine synthesis in *Salmonella typhimurium.* J. Bacteriol. **178:** 1476–1479.

Escalante-Semerena, J. C., and J. R. Roth, 1987 Regulation of cobalamin biosynthetic operons in *Salmonella typhimurium.* J. Bacteriol. **169:** 2251–2258.

Estramareix, B., and S. David, 1990 Conversion of 5-aminoimidazole ribotide to the pyrimidine of thiamin in enterobacteria: study of the pathway with specifically labeled samples of riboside. Biochem. Biophys. ACTA **1035:** 154–160.

Estramareix, B., and M. Therisod, 1984 Biosynthesis of thiamine: 5-aminoimidazole ribotide as the precursor of all the carbon atoms of the pyrimidine moiety. J. Am. Chem. Soc. **106:** 3857–3860.

Gutnick, D., J. M. Clvo, T. Klopotowaski and B. N. Ames, 1969 Compounds which serve as the sole source of carbon or nitrogen for *Salmonella typhimurium* LT2. J. Bacteriol. **100:** 215–219.

Kainz, M., 1994 Transcription antitermination mediated by the phage lambda Q protein: defining the reaction *in vivo.* Thesis. Cornell University, Ithaca, NY.

Kupor, S. R., and D. G. Fraenkel, 1972 Glucose metabolism in 6-phosphogluconlactonase mutants of *Escherichia coli.* J. Biol. Chem. **247:** 1904–1910.

LaRossa, R. A., T. K. Van Dyk and D. R. Smulski, 1987 Toxic accumulation of α-ketobutyrate caused by inhibition of the branched chain amino acid biosynthetic enzyme acetolactate synthase in *Salmonella typhimurium.* J. Bacteriol. **169:** 1372–1378.

Meng, L. M., M. Kilstrup and P. Nygaard, 1990 Autoregulation of PurR repressor synthesis and involvement of *purR* in the regulation of *purB, purC, purL, purMN,* and *guaAB* expression in *Escherichia coli.* Eur. J. Biochem. **187:** 373–379.

Meyer, E., N. J. Leonard, B. Bhat, J. Stubbe and J. M. Smith, 1992 Purification and characterization of the *purE, purK,* and *purC* gene products: identification of a previously unrecognized energy requirement in the purine biosynthetic pathway. Biochemistry **31:** 5022–5032.

Miller, J. H., 1972 *Experiments in Molecular Genetics.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Nasoff, M. S., H. V. Baker, II and R. E. Wolf, Jr., 1984 DNA sequence of the *Escherichia coli* gene, *gnd,* phosphogluconate dehydrogenase. Gene **27:** 253–264.

Newell, P. C., and R. G. Tucker, 1967 New pyrimidine pathway involved in the biosynthesis of the pyrimidine of thiamine. Nature **215:** 1384–1385.

Newell, P. C., and R. G. Tucker, 1968a Biosynthesis of the pyrimidine moiety of thiamine. Biochem. J. **106:** 279–287.

Newell, P. C., and R. G. Tucker, 1968b Precursors of the pyrimidine moiety of thiamine. Biochem. J. **106:** 271–277.

Rolfes, R. J., and H. Zalkin, 1988 *Escherichia coli* gene *purR* encoding a repressor protein for purine nucleotide synthesis. J. Biol. Chem. **263:** 19653–19661.

Rolfes, R., and H. Zalkin, 1990a Autoregulation of *Escherichia coli purR* requires two control sites downstream of the promoter. J. Bacteriol. **172:** 5758–5766.

Rolfes, R., and H. Zalkin, 1990b Purification of the *Escherichia coli* purine regulon repressor and identification of corepressors. J. Bacteriol. **172:** 5637–5642.

Schmieger, H., 1972 Phage P22 mutants with increased or decreased transduction abilities. Mol. Gen. Genet. **119:** 75–88.

Shattuck-Eidens, D. M., and R. J. Kadner, 1981 Exogenous induction of the *Escherichia coli* hexose phosphate transport systems defined by *uhp-lac* operon fusion. J. Bacteriol. **148:** 203–209.

Steiert, J. G., R. J. Rolfes, H. Zalkin and G. V. Stauffer, 1990 Regulation of the *Escherichia coli glyA* gene by the *purR* gene product. J. Bacteriol. **172:** 3799–3803.

Sussman, R., and F. Jacob, 1962 Sur un systeme de repression thermosensible che2 le bacteriophage d' *Escherichia coli.* C. R. Acad. Sci. Paris **254:** 1517.

Turnbough, Jr., C. L., and B. R. Bochner, 1985 Toxicity of the pyrimidine biosynthetic pathway intermediate carbamyl aspartate in *Salmonella typhimurium.* J. Bacteriol. **163:** 500–505.

Vogel, H. J., and D. M. Bonner, 1956 Acetylornithase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218:** 97–106.

Walsh, K., and D. E. J. Koshland, 1984 Determination of flux through the branch point of two metabolic cycles. J. Biol. Chem. **259:** 9646–9654.

Way, J. C., M. A. Davis, D. Morisato, D. E. Roberts and N. Kleckner, 1984 New Tn*10* derivatives for transposon mutagenesis and for construction of *lacZ* operon fusions by transposition. Gene **32:** 369–379.

Wilson, R. L., L. T. Stauffer and G. V. Stauffer, 1993 Roles of the GvcA and PurR proteins in negative regulation of the *Escherchia coli* glycine cleavage enzyme system. J. Bacteriol. **175:** 5129–5134.

Yura, T., 1956 Evidence of nonidentical alleles in purine requiring mutants of *Salmonella typhimurium.* Publ. Carnegie Instn. **612:** 63–75.

Communicating editor: N. L. Craig