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GLUTAMINE-PRPP AMIDOTRANSFERASE (PURF)-INDEPENDENT PHOSPHORIBOSYL AMINE SYNTHESIS FROM RIBOSE-5-PHOSPHATE AND GLUTAMINE OR ASPARAGINE*

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Running title: alternative phosphoribosyl amine synthesis

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Phosphoribosylamine (PRA) is the first intermediate in the common pathway to purines and thiamine, and is generated in bacteria by glutamine phosphoribosylpyrophosphate (PRPP) amidotransferase (PurF, EC 2.4.2.14) from PRPP and glutamine. Genetic data have indicated that multiple, non-PurF mechanisms exist to generate PRA sufficient for thiamine, but not purine synthesis. Here we describe the purification and identification of an activity (present in both *E. coli* and *S. enterica*) that synthesizes PRA from ribose-5-phosphate (R5P) and glutamine/asparagine. A purification resulting in greater than a 625-fold increase in specific activity identified eight candidate proteins. Of the candidates, overexpression of AphA (EC 3.1.3.2), a periplasmic class B non-specific acid phosphatase, significantly increased activity in partially purified extracts. Native purification of AphA to >95% homogeneity determined that the periplasmic L-asparaginase II, AnsB (EC 3.5.1.1), co-purified with AphA and was also necessary for PRA formation. The potential physiological relevance of AphA and AnsB in contributing to thiamine biosynthesis *in vivo* is discussed.

INTRODUCTION

In bacteria, phosphoribosylamine (PRA) is the first intermediate in the biosynthetic pathways for purines and the pyrimidine moiety of thiamine (1-4) (Figure 1). Glutamine-PRPP amidotransferase (E.C. 2.4.2.14), the product of the *purF* gene in *Salmonella enterica* (and *Escherichia coli*), catalyzes the synthesis of PRA from PRPP and glutamine. Strains lacking PurF have the expected nutritional requirement for purines, however under some growth conditions *S. enterica* strains lacking PurF are able to grow in the absence of thiamine (5,6). Genetic and biochemical studies have demonstrated this growth reflects the existence of a PurF-independent mechanism(s) to generate PRA that is utilized by subsequent purine and thiamine enzymes to generate the hydroxymethyl pyrimidine (HMP) moiety of thiamine (7,8). However, PRA generated in the absence of PurF is not sufficient to satisfy the cellular requirement for purines.

An inability to obtain mutants devoid of PRA formation suggests multiple activities that can generate this metabolite. PRA could be generated by side reaction(s) of enzyme(s) involved in other metabolic pathways, redistribution of metabolic flux, or a combination of the two. Results implicating the anthranilate synthase-phosphoribosyltransferase complex (TrpDE, EC 4.1.3.27, EC 2.4.2.18), required in

tryptophan biosynthesis, in synthesis of PRA (9,10) are consistent with this scenario. Genetic studies have shown that a functional oxidative pentose phosphate (OPP) pathway is required for at least one route of PRA biosynthesis independent of PurF and distinct from the Trp enzymes (6,9,11). Nutritional studies were consistent with a role of the OPP pathway in contributing ribose-5-phosphate (R5P) for PRA synthesis (Figure 1) (6,11,12).

The study described here was initiated to test the hypothesis that a PRA-forming activity utilizing R5P as a substrate is present in bacterial cells. Here we describe the identification and purification of a PRA-forming activity present in *E. coli* and *S. enterica* which converts R5P and asparagine or glutamine to PRA. Purification of this activity from crude extract determined it required two proteins encoded by *aphA* and *ansB*.

EXPERIMENTAL PROCEDURES

Medium and chemicals. Culture media supplies were obtained from Difco (Sparks, MD). Glutamine, asparagine, glycine, ribose-5-phosphate, PRPP, magnesium acetate, ammonium chloride, ATP, EDTA, β-mercaptoethanol, phenylmethanesulfonyl fluoride (PMSF), protamine sulfate, DNase I (EC 3.1.21.1), and lysozyme (EC 3.2.1.17) were obtained from Sigma-Aldrich (St, Louis, MO). KCl, sucrose, $MgCl_2$, Tris base, methanol, and pyridine were from Fisher Scientific (Pittsburgh, PA). K_2HPO_4, KH_2PO_4, and ammonium sulfate were obtained from Mallinckrodt Baker (Phillipsburg, NJ). [1-^{14}C]-glycine was from New England Nuclear (Boston, MA). DEAE Sepharose Fast Flow and all chromatography resin were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Cellulose PEI plates were from Selecto Scientific (Suwanee, GA). Ultrafiltration YM membranes were from Amicon (Beverly, MA). All restriction enzymes used were from Promega (Madison, WI). AnsB (EC 3.5.1.1) from *E. coli* (catalog number A4887) was obtained from Sigma-Aldrich (St, Louis, MO).

Strains, cell growth, extract preparation. Strain DM1293 (*E. coli, purF::*Tn*10*) was grown in Luria-Bertani (LB) broth in a 16 L batch fermentor. Strain DM1936 (*S. enterica, purF2085*) was grown in Nutrient Broth (NB) in a 16 L batch fermentor. The medium was inoculated with one liter of an overnight culture and incubated at 37°C with aeration during 12 h. Cells (~88 g) were harvested by centrifugation at 4°C, washed twice with buffer A (0.05 M potassium phosphate buffer pH 7.5 containing 5 mM β-mercaptoethanol and 1 mM PMSF), and resuspended in 200 mL of the same buffer. The cells were disrupted using a French pressure cell at 10^4 kPa. Clarified cell-free extract was obtained by centrifugation at 23,700 X g during 45 min at 4°C. When grown on a smaller scale, media was inoculated with a NB overnight culture and incubated at 37°C with agitation until full cell density was achieved. Cell-free extracts were then generated as described above.

Assay for PRA-formation. A PRA-forming activity assay described for glutamine-PRPP amidotransferase (4,13) was modified to optimize sensitivity. PRA synthesis was assayed in 50 mM potassium phosphate buffer pH 8.0 in the presence of 6 mM magnesium acetate, 2.5 mM ATP, 2 mM ^{14}C-glycine (26 nCi), a pool of amino acids containing equal concentrations (6 mM) of glutamine, asparagine, and 2 μg of GAR synthetase. Reactions were started by the addition of R5P (13 mM final concentration), and incubated at 37°C for 4 h. Radio-labeled GAR and glycine were separated by thin-layer chromatography (TLC) on PEI-cellulose using a methanol:pyridine:water system (20:1:5). The

position of radioactive spots was detected using a Cyclone Storage Phosphor System (Packard Instrument Company), and their identity was confirmed with standards.

Protein purification. *Glycinamide ribonucleotide (GAR) synthetase (PurD)*. GAR synthetase was purified to 95% homogeneity from *E. coli* strain, DM1295 (*purF*::Tn*10*d/pJS187). Plasmid pJS187 provides for the over-expression of GAR synthetase (14). PurD protein was purified as described using ammonium sulfate fractionation, ion exchange chromatography (DEAE resin), and size exclusion chromatography (14). The activity of the purified protein was assayed by monitoring ^{14}C-GAR production from ^{14}C-glycine and chemically synthesized PRA (4,13,15). Alternatively, PurD was purified by affinity chromatography using the IMPACT CN Kit (New England Biolabs, Ipswich, MA). A C-terminal intein tag was created by amplifying the open reading frame of *purD* from *S. enterica* LT2 chromosomal DNA by PCR using Platinum Pfx DNA polymerase (Invitrogen, Carlsbad, CA). This construct was cloned into the *NdeI* and *SmaI* site of the plasmid pTYB2. Over-expression and purification of the protein were performed as recommended by the manufacturer and resulted in a native protein containing a single additional glycine at the N-terminal end. Preparations of PurD purified by either method were used interchangeably with no affect on ability to detect PRA forming activity.

PRA-forming activity. Nucleic acids were precipitated by addition of protamine sulfate to the clarified cell-free extract at a final concentration of 0.1% with constant stirring over 10 min. The solution was then stirred for 30 additional min and centrifuged at 23,700 X *g* during 20 min. A two-step salt precipitation was performed. The clarified cell-free extract was brought to 35% saturation by the addition of solid ammonium sulfate with constant stirring during 30 min. The solution was then stirred for 30 additional min and centrifuged at 23,700 X *g* for 30 min. The same procedure was used in a second precipitation step performed at 70% saturation. The supernatant at 70% saturation was discarded and the precipitate was resuspended in buffer A (0.05 M potassium phosphate buffer pH 7.5 containing 5 mM β-mercaptoethanol and 1 mM PMSF) and dialyzed against the same buffer. The dialyzed fraction was applied at a flow rate of 2 mL/min to a DEAE ion exchange column (200 mL column volume) equilibrated with buffer A. The column was washed with two column volumes (CV) of buffer A and all PRA-forming activity was found to be in the flow-through and wash fractions. These fractions were pooled and concentrated using a 30 kDa cut-off Amicon ultrafiltration membrane. The concentrated fraction was applied at a flow rate of 0.5 mL/min to a HiLoad Superdex 75 size exclusion column (24 m CV) equilibrated with buffer A containing 100 mM KCl; 1-mL fractions were collected and assayed for PRA-forming activity. The active fractions were applied at a flow rate of 2 mL/min to a MonoQ (strong ion exchange) column (8 mL CV) equilibrated with buffer A. After a two CV wash with buffer A, a linear gradient from 0 to 1 M KCl was used to elute the proteins bound to the column; 1mL fractions were collected throughout the wash and elution. After dialyzing against buffer A, the fractions were tested for PRA-forming activity and stored at 4^{o}C.

An alternative step was used interchangeably with the DEAE column chromatographic step. In this alternative, 100 cubic centimeters of unpacked DEAE resin equilibrated with buffer A was mixed with the dialyzed fractions and the suspension was

placed on ice for 10 min. Trial experiments determined that this batch elution protocol resulted in purification of the PRA-forming activity with the same efficiency as collecting the flow-through from standard column chromatography. The supernatant was removed by filtration. The resin was washed with an additional 200 mL of buffer A, which was then pooled with the 200 mL supernatant and concentrated using a using a 30 kDa cut-off Amicon ultrafiltration membrane and applied to a Superdex 75 size exclusion column (see above).

AphA. Strain DM10089 (*purF2085 ansB1*::Kan pSU-*aphA*) was grown in NB with chloramphenicol (20 μg/mL) in a 16 L batch fermentor and the cells were collected by centrifugation. Proteins from the periplasmic space were isolated using a modified spheroplasting technique (16). The resulting protein preparation was subjected to the purification scheme described for the PRA-forming activity. SDS-PAGE protein bands were visualized by staining with a Silver Stain Plus kit from Bio-Rad (Hercules, CA) and the protein prep was determined to be >95% pure after passage through a Superdex 75 size exclusion column (data not shown). The protein prep was then stored at 4°C for two months or frozen and stored at -80°C for future use.

Protein quantification and manipulation. Protein was quantified by the method of Bradford (17) or by using a BCA Protein Assay Kit (Pierce). Ultrapure bovine serum albumin was used to generate a standard curve. After SDS-polyacrylamide gel electrophoresis (SDS-PAGE) protein bands were visualized by staining with either (a) 40:55:5:0.05 ethanol:water:acetic acid:Coomassie G-250 and distained in 40:55:5 ethanol:water:acetic acid; or (b) silver using a Silver Stain Plus kit from Bio-Rad (Hercules, CA).

Analysis of candidate genes. Candidate genes were amplified from *S. enterica* LT2 chromosomal DNA by PCR using Platinum *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA) with the primers shown in Table I. The resulting PCR products were purified and cloned into the multi cloning site of pSU19 (18). Plasmids were electroporated into *E. coli* strain DH5α and screened for vectors containing inserts. Constructs containing the cloned genes under the control of the *lac* promoter were confirmed by sequence analysis and introduced into *S. enterica* strain DM1936

Candidate proteins were partially purified from cell-free extracts of strains over-expressing them. Cells (~3 g) were resuspended in buffer A (6 mL) with 2mg/mL lysozyme and 1mg/mL DNase prior to disruption with a French pressure cell at 10^4 kPa. Clarified cell-free extract was obtained by centrifugation at 39,000 X *g* during 30 min at 4°C. The clarified cell-free extract was at 65°C for 5 min, centrifuged at 39,000 X *g* for 45 min at 4°C, and run by gravity feed over two DEAE columns (6 mL CV) equilibrated with buffer A running in tandem at 4°C. A 6 mL wash was combined with the flow-through fraction and concentrated with a 30 kDa cut-off Amicon ultrafiltration membrane.

RESULTS

Optimization of in vitro assay for PRA formation. Past protocols for PRA-formation have utilized a coupled reaction with glycinamide ribotide synthetase (PurD) in which the substrate (^{14}C-glycine) is separated from the product (^{14}C-GAR) by ion exchange chromatography, based on the charge conferred by the phosphate group in GAR (4). Use of this assay in crude cell-free extracts

was hampered by non-specific phosphatases that partially converted GAR to GARs and thus decreased the sensitivity of the assay. Because the activity of interest was expected to be weak, a TLC system using a mobile phase of methanol/pyridine/water (20:1:5) on PEI- cellulose plates was used to separate unincorporated ^{14}C-glycine from the ^{14}C-GAR/^{14}C-GARs.

Initial screens for PRA-forming activity were preformed in *S. enterica* and *E. coli* cell-free extracts lacking glutamine-PRPP amidotransferase. Either PRPP or R5P was provided as the carbon backbone donor and a pool of amino acids containing glutamine, asparagine, alanine, and valine (each at 6 mM) as the potential source of the amino group. Subsequent experiments determined that glutamine and asparagine were equally proficient as amino donors, and no other amino acid allowed PRA formation. Control experiments determined PRA was formed in the absence of extract (data not shown) if R5P was provided to the Tris-HCl buffer used in previous work (pH 8.0, 100 mM) (4). Since PRA can be chemically synthesized from R5P and ammonia this non-enzymatic formation was attributed to small amounts of ammonia in the buffer (15,19). Additional buffers (HEPES-NaOH, Clark and Lubs KH_2PO_4-NaOH; pH. 8, 50 mM) were discarded for a similar reason, or because the (PurD) activity was negatively affected. Potassium phosphate buffer (pH 8.0) was found to produce negligible background PRA formation and was used in subsequent experiments.

Initial characterization of a PRA-forming activity in cell-free extracts lacking PurF. Dialyzed cell-free extracts from *S. enterica* and *E. coli.* strains lacking glutamine-PRPP amidotransferase failed to support synthesis of PRA from either R5P or PRPP, glutamine and asparagine. It was subsequently found that synthesis of PRA from R5P, glutamine and asparagine was detectable after the cell-free extract had interacted with an anion exchange resin (DEAE, pH 7.5) and/or subjected to 65°C for 5 min. Detection of this activity is shown in Figure 2. Data in Figure 2A show that in the presence of fractionated extract, formation of GAR was detectable when R5P (lane 2), but not PRPP (data not shown) was provided with amino acids. The PRA-forming activity was decreased when heated at 98°C for 5 min (lane 3), and retained after dialysis (lane 4). Further, the activity was unaffected by treatment with either RNase or DNase (data not shown). Accumulation of GAR was proportional to the time of assay incubation up to 4 hr, as shown by data from a representative experiment in Figure 2B. Data in Figure 2C show that GAR synthesis was proportional to protein up to 15 μg, after which a plateau was reached. The data in Figures 2B and 2C are from a representative experiment and GAR concentration was determined from a linear curve of radio-labeled glycine generated under visualization conditions.

Inability to detect PRA-forming activity in crude cell-free extracts prior to fractionation suggested the presence of an inhibitory compound. Subsequent experiments determined the target of inhibition was the PurD reaction, and it was not further characterized.

Purification of PRA-forming activity. PRA-forming activity was followed during fractionation of an *E. coli* strain lacking glutamine-PRPP amidotransferase (DM1293 (*E. coli purF77*::Tn*10*d)). A purification protocol was implemented and is summarized in Table II. A typical purification used 250 mL of cell-free extract (8 mg/mL) fractionated by precipitation with ammonium sulfate as described in Experimental Procedures. The proteins precipitated at 70% saturation were resuspended in buffer A and dialyzed against the same buffer. Ion exchange

chromatography was performed on the dialyzed fractions (200 mL). PRA-forming activity was detectable at this stage in the flow-through fraction and this activity was set to be 100% for subsequent yield determination.

After interaction with the DEAE resin, the PRA-forming activity was further purified and concentrated by passage through a 30 kDa cut-off Amicon ultrafiltration membrane, followed by size exclusion chromatography and ion exchange chromatography as described in Experimental Procedures. Data in Table II indicates a 625-fold final purification was achieved.

Proteins in the active fractions from the purification were visualized after separation on SDS-polyacrylamide gel. The fraction from the Mono Q column contained multiple protein bands (data not shown). Final active fractions from three independent purifications, and an inactive fraction from the final column were sent to Midwest Bio Services (Overland Park, KS) for in-gel extraction and mass spectrometry analysis. Table III summarizes the proteins identified in all active fractions, and not present in the inactive fraction.

Partially purified AphA has PRA forming activity. Genes encoding each of the eight proteins described in Table III were amplified from *S. enterica* and cloned into a pSU19 multi-copy plasmid. The resulting constructs were introduced into the *S. enterica* strain DM1936 (*purF2085*). Cell free extracts from the resulting strains were subjected to partial purification and assayed for PRA-forming activity. Of the eight strains, two had more PRA synthesis than the parental strain (Figure 3). The strain over-expressing AphA (lane 1), and the strain over-expressing AnsB (lane 2) were found to have increased PRA formation relative to the control strain DM1936 (lane 4). Purified AnsB was purchased (Sigma Chem. Co.) and tested for its ability to make PRA. While AnsB allowed PRA formation (lane3) two points were noted. First, 10μg of AnsB allowed less PRA formation than crude extract of the AphA overproducing strain. Second, as an asparaginase, AnsB produces NH_3 and thus PRA formation is expected to be at least partially due to the non-enzymatic combination of ammonia and R5P (15).

AphA and AnsB are required for PRA forming activity. AphA was purified from strain DM9518 (*purF2085* pSU-*aphA*) to >95% homogenicity and tested for PRA-forming activity. Ten micrograms of this protein preparation generated more PRA than the partially purified extract from DM9518 (data not shown), although there was a noticeable contaminating protein band of approximately the size of the AnsB protein (~35KD). To address the possible contribution of AnsB to the detected PRA-forming activity, a strain lacking AnsB and PurF activities was generated. Plasmid pSU-*aphA* was moved into this strain to create DM10089 (*purF2085 ansB1*::Kan pSU-*aphA*). AphA was then purified after over-expression in the *ansB* mutant background, and found to have no PRA-forming activity. Additional protein preparations confirmed that AphA is not sufficient to catalyze PRA formation.

Data in Figure 4 showed that while neither 10μg of AphA (lane 1) or AnsB (lane 2) allowed significant PRA formation, a mixture of the two proteins did. In the presence of less than molar amounts of AnsB, AphA was able to generate significant amounts of PRA. From these data it was concluded that the PRA-forming activity purified from crude extracts was due to a complex involving the periplasmic proteins AphA and AnsB.

Physiological role of AphA and AnsB in thiamine biosynthesis. Results described here

showed that AphA and AnsB have the ability to produce PRA *in vitro*. Strain DM10259 (*purF2085 ansB1*::Kan *aphA15*::Cm) was constructed and analyzed for growth under various conditions known to allow PurF-independent PRA formation. In all growth conditions tested, the strain lacking *ansB* and *aphA* grew as well as the parental *purF* mutant, indicating this PRA-forming activity was not essential for growth. When *aphA* or *ansB* was placed in multi-copy, neither allowed a *purF* mutant strain to grow in the absence of thiamine under non-permissive conditions (data not shown).

DISCUSSION

This study describes the identification and purification of a cellular activity from both *E. coli* and *S. enterica* that synthesizes phosphoribosyl amine (PRA). Data herein showed that AphA, a periplasmic class B non-specific acid phosphatase/phosphotransferase (EC 3.1.3.2), in combination with submolar ratios of the periplasmic L-asparaginase II, AnsB (EC 3.5.1.1), can produce PRA from R5P and either asparagine or glutamine *in vitro*. Titration experiments determined that (at pH 7.5) optimal PRA-formation occurred when AphA and AnsB were provided at a 13:1 molar ratio. We suggest that AphA binds R5P and catalyzes the formation of PRA when presented with the ammonia released by hydrolysis of asparagine or glutamine by AnsB.

Based on the enzymes involved, a scenario in which either one could produce PRA is feasible. Although AphA has been characterized primarily for its ability to dephosphorylate mononucleotides and various other phosphomonoesters, the enzyme has been shown to catalyze dephosphorylation of R5P. This finding indicates that the enzyme can bind R5P, though it is acted on with a relatively low catalytic efficiency (K_{cat}/K_M of 5.0 x 10^3 M^{-1}x s^{-1} pH 6) (20). At physiological pH the protein exhibits sub-optimal activity, potentially decreasing this catalytic efficiency further, raising the possibility that R5P could bind in the active site without dephosphorylation occurring. In this scenario, AphA could serve to concentrate R5P, allowing PRA formation to occur when ammonia is present. Since AnsB hydrolyzes both asparagine and glutamine optimally at pH 7.5 (21), a simple scenario suggests that AnsB produces ammonia that allows non-enzymatic formation of PRA in the presence of R5P (15).

Although more complex scenarios for the generation of PRA by the individual enzymes could be imagined, several observations suggested AphA and AnsB together were responsible for the PRA production detected herein. First, AphA and AnsB co-purified through several chromatographic steps, consistent with at least a loose association of the two proteins. The fact that both enzymes are located in the periplasm is consistent with this interpretation. Secondly, the amount of PRA formed with the addition of both proteins was noticeably more than that generated by AphA, even with excess ammonia. We suggest a model in which PRA is formed in the active site of AphA. By this scenario R5P would occasionally bind the AphA enzyme, blocking the active site due to the poor catalytic activity of the enzyme with this substrate. This association would remain until the glutaminase/asparaginase releases ammonia in close proximity, perhaps allowing the interaction of R5P and ammonia resulting in PRA formation. The inability of PRPP to allow PRA formation could be due to the binding specificity of AphA. Alternatively, the inability of PRPP to participate in the reaction could support the non-enzymatic mechanism proposed. If PRA is easily released from the enzyme, this could benefit the organism by restoring the availability of

the active site for mononucleotide dephosphorylation. Testing this model will require additional kinetic analyses *in vitro*.

While AphA/AnsB could synthesize PRA *in vitro*, a condition where these proteins were essential for thiamine biosynthesis *in vivo* was not identified. Localization studies showed that AphA is present in the cytoplasm in addition to its predominant location in the periplasm (22), thus is it rational to suggest some PRA is generated by the mechanism described herein. Previous work in our lab has emphasized the multiple inputs to PRA formation (9,10,23). In all cases separate from that described here, these inputs have been identified using sensitive *in vivo* genetic analyses. The work described here emphasizes that the cell has additional capacity to generate metabolites that may not be apparent by only addressing activities essential for growth. Further, there is not a good way of determining that the appropriate condition to detect essentiality has been tested.

The robustness of cellular metabolism predicts that multiple inputs to many (if not all) metabolites will exist. Such overlap can be considered to occur by evolutionary pressure, or simply be the natural result of biochemical reactions that involve compounds of similar structure. Regardless of the evolutionary questions, from the perspective of understanding the metabolic network in the cell it is important to define not only reactions that are easily recognized as essential for growth, but the potential for robustness provided by minor reactions. Based on the work herein, AphA is component of the cell that can be probed with *in vitro* biochemical approaches to generate a better understanding of metabolic robustness with respect to PRA formation.

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[1] The abbreviations used are: PRPP, phosphoribosylpyrophosphate; R5P, ribose-5-phosphate; PRA, phosphoribosylamine; OPP, oxidative pentose phosphate; PMSF, phenylmethanesulfonyl fluoride; PurF, Glutamine PRPP amidotransferase; GAR, 5'-phosphoribosylglycinamide; PurD, GAR Synthetase; AS-PRT, anthranilate synthase-phosphoribosyltransferase; AphA, class B non-specific acid phosphatase/phosphotransferase; AnsB, L-asparaginase II.

Table I
Primers used in this study

Plasmid [a]	Primers used to amplify insert
pIR-*mdh*	5'-GCCTGTGTCACGCCTCGCAAATAA-3' 5'-GCGACCTGCATGTGCCTGTTGG-3'
pIR-*mdoG*	5'-CACGTATTCTCAGATTTTTCACCT-3' 5'-CGCGAGTAAGTCCGATGCT-3'
pIR-*ydcG*	5'-CTGAAGAATGCTGCGTGAGG-3' 5'-GAGCAGCACTTAATAAACCCAGAG-3'
pSU-*aphA*	5'- GTCGACCCGTTACTGGCGTTATGGTC-3' 5'- GGATCCGTGCAGCAAGTCTGGAAAAG-3'
pSU-*cpdB*	5'-CTGCAGGGAACGATATCGGGTTTCAC-3' 5'-GGATCCCGGGAACGTTTATCAGATGG-3'
pSU-*ansB*	5'-CTGCAGTAAACAATGGCGCAGATCG-3' 5'-GGATCCGTGCGAGAGGTCTTCCAAAG-3'
pSU-*yghA*	5'-GAGAGTCGACGAATACGGGCGAAGCATAAG-3' 5'-CAGAGGATCCAAGTGGCGCCTTGCTTAAC-3'
pSU-*talA*	5'-GAGACTGCAGGCCTGTCTGCTATGCTTTTTG-3' 5'-GAGAGGATCCCATTGGCAAGGTCTTTACGG-3'

[a] For all plasmids, pSU19 (Cm^r) was used

Table II
Purification of PRA-forming activity [a]

Purification Step	**Specific activity U[c]/mg protein**	**Purification**	**% Yield**
Cell-free extract	ND[b]	-	-
$(NH_4)_2SO_4$ precipitation	ND	-	-
DEAE ion exchange chromatography (flow-through)	506	1	-
Superdex 75 size exclusion chromatography	10848	22	68%
MonoQ ion exchange chromatography	316492	625	89%

[a]PRA synthesis from R5P, glutamine and asparagine was determined a function of radio-labeled 14C-GAR production
[b]ND: none detected
[c]U: Arbitrary units detected by the Cyclone Storage Phosphor System

Table III
Identification of potential PRA-forming enzymes by mass spectrometry analysis

Gene	Strain [a]	Gene product [b]	Size	Function
mdoG	DM9380	Putative periplasmic glycoprotein	57 kDa	Periplasmic glucans biosynthesis, addition of branches to linear glucan backbone
ydcG	DM9831	Putative periplasmic glycoprotein	61 kDa	Periplasmic glucans biosynthesis
mdh	DM9379	Malate dehydrogenase	32 kDa	Interconversion of L-malate and oxaloacetate
talA	DM9652	Transaldolase A	35 kDa	Reversible three-carbon ketol unit transfer from sedoheptulose 7-P to glyceraldehyde 3-P to form erythrose 4-P and fructose 6-P
yghA	DM9615	Hypothetical oxidoreductase NAD(P)-binding	31 kDa	Belongs to the short-chain dehydrogenases/reductases (SDR) family
cpdB	DM9520	2',3'-cyclic nucleotide 2'-phosphodiesterase	70 kDa	Salvage of nucleosides and nucleotides (interconversion)
ansB	DM9584	Periplasmic L-asparaginase II	35 kDa	Hydrolysis of asparagine to aspartic acid and ammonia
aphA	DM9518	Periplasmic class B phosphatase/ phosphotranserase	25 kDa	Non-specific acid phosphatase/phosphotranserase

[a] *purF2085* strain with gene cloned into pSU19

[b] At least two different peptides matching the identified protein were detected by mass spectrometry

Fig. 1. **Pathway schematics.** Biosynthetic pathway for purine and thiamine synthesis. Some purine gene products are indicated above the reactions they catalyze. The proposed substrates for the alternative PRA synthesis are R5P and an unknown amino donor. Abbreviations: R5P, ribose-5-phosphate; PRPP, phosphoribosylpyrophosphate; PRA, phosphoribosylamine; GAR, 5'-phosphoribosylglycinamide; AIR, aminoimidazole ribotide; HMP-PP, 4-amino-5-hydroxymethylpyrimidine pyrophosphate; THZ-P, 4-methyl-5-(β-hydroxyethyl)thiazole-phosphate.

Fig. 2. **PRA-forming activity in a partially purified extract from an *E. coli purF* mutant. *A*)** Synthesis of PRA as a function of ^{14}C-GAR production. Reactions were performed as described in Experimental Procedures adding an amino acid pool (glutamine and asparagine) and R5P as substrates, in the absence (lane 1) and the presence of a fresh (lane 2), boiled (lane 3) or dialyzed (lane 4) partially purified cell extract. After incubation at 37^{o}C during 4 h, radio-labeled ^{14}C-GAR and ^{14}C-glycine were separated on PEI-cellulose using a methanol/pyridine/water (20:1:5) solvent system. ***B*)** GAR synthesis vs. time. GAR synthesis from R5P and an amino acid pool in the presence (■) and the absence (□) of the partially purified extract (10 mg total protein). ***C*)** Synthesis of GAR in the presence of increasing amounts of protein after 4 h incubation at 37^{o}C.

Fig. 3. **PRA forming activity in partially purified protein extracts**. PRA formation as a function of radio-labeled ^{14}C-GAR from R5P, asparagine and glutamine using: 10µg of DM9518 (*purF* pSU-*aphA*) (lane 1), 10µg of DM9584 *(purF* pSU-*ansB*) (lane 2), 10µg pure AnsB from Sigma (lane3), 10µg DM1936 (*purF*) (lane 4), no protein control (lane

5). After incubation at 37°C during 4 h, radio-labeled ^{14}C-GAR and ^{14}C-glycine were separated by TLC, and the ^{14}C-GAR spots are shown.

Fig 4. **Formation of PRA from AphA and AnsB.** PRA formation using purified protein as a function of radio-labeled ^{14}C-GAR from R5P, asparagine and glutamine using: 10µg AphA (lane 1), 10µg AnsB (lane 2), 0.1µg AnsB (lane 3), 10µg AphA+1.0µg AnsB (lane 4), 10µg AphA+ 0.1µg AnsB (lane 5), 10µg AphA+10ng AnsB (lane 6), 10µg AphA+5.0ng AnsB (lane 7), 10µg AphA+1.0ng AnsB (lane 8), and no protein control (lane 9). After incubation at 37°C during 4 h, radio-labeled ^{14}C-GAR and ^{14}C-glycine were separated by TLC and the ^{14}C-GAR spots are shown.