A Plasmid-Based Approach for the Synthesis of a Histidine Biosynthetic Intermediate

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A novel plasmid DNA construct has been developed for use in the synthesis of a histidine biosynthesis intermediate, N^1-[(5″-phospho-β-D-ribosyl)formimino]-5-aminoimidazole-4-carboxamide ribonucleotide (5′-ProFAR, 1). The approach allows for a convenient, millimole-scale preparation of the bis-ribosyl nucleotide and provides material of suitable purity for spectral analyses and biochemical studies. A highly enriched source of the first three enzymes in the histidine biosynthetic pathway has been developed by constructing a recombinant DNA plasmid which directs the overexpression of these proteins. When used in a strain of *Escherichia coli* that is devoid of other histidine biosynthetic enzymes, cell-free extracts can be coupled with 5-phospho-D-ribosyl α-1-pyrophosphate synthetase and an ATP recycling system to prepare 1 directly from D-ribose 5-phosphate. This paper also presents the first spectral analysis of 5′-ProFAR and its major decomposition products.

Introduction

Histidine maintains a central role in nature since it is essential for the biosynthesis of proteins and of the immunoregulator histamine. The *de novo* biosynthesis of histidine occurs only in plants and microorganisms and requires ATP and 5-phospho-D-ribosyl α-1-pyrophosphate (PRPP) as metabolic precursors. There are eight intermediates in the pathway, and seven of these materials are ionic phosphate esters.[1] The first four intermediates of the pathway feature unusual bis-ribosyl nucleotides, the structures of which have been determined only by indirect methods.[2,3] Preparations of these materials are essential for detailed biochemical studies of the pathway enzymes. Furthermore, the intermediates have potential utility as biosynthetic precursors for natural products such as the antitumor agent coformycin.[4,5] *In vitro* studies of the enzymes involved in this pathway, not unlike many other multistep biosynthetic pathways, have been limited by this situation. These ionic intermediates pose significant synthetic challenges, and there have been no reported economical syntheses of these compounds to date. Biological sources of histidine biosynthetic precursors have been inefficient because these intermediates are not secreted to any great extent, and they are too unstable to allow for a buildup of significant concentrations *in vivo*.[6] A general strategy that could supply many of the substrates is the use of *in vitro* enzyme-mediated reactions.[3,7] Moreover, the development of enzymatic methods offers a useful approach to the synthesis or semisynthesis of analogs that could be important as biochemical probes or commercial products.[8-13] In this report we establish the feasibility of using a novel recombinant DNA approach to reconstitute *in vitro* a portion of the histidine biosynthetic pathway for the purpose of benchtop preparations of metabolic intermediates. As part of this endeavor, the first detailed spectral characterization of a member of a unique class of bis-ribosyl nucleotide intermediates is reported.

Results and Discussion

The initial target material was the key intermediate N^1-[(5′-phospho-β-D-ribosyl)formimino]-5-aminoimidazole-4-carboxamide ribonucleotide (5′-ProFAR, 1). Compound 1 occupies a central position in the pathway because it is the first intermediate in which a carbon and nitrogen of the adenine ring from ATP are committed to the production of histidine. *In vivo* production of this intermediate is not feasible because of the inefficient secretion of ionic materials, a common limitation to the use of simple eubacteria.[6] The original preparation of 1 used cell-free extracts of a mutant strain of *Salmonella typhimurium* as a source of enzymes for a portion of the histidine biosynthetic pathway.[3] However, in our experience this approach was unsuitable for the preparation of millimole quantities of purified products. Specifically, the necessary enzyme activities are elevated in the mutant strains grown under the proper conditions; the tedium involved in the multistep purification of each enzyme further discouraged the use of these hosts.

Instead, we chose to develop a biological source that contains the desired enzymes with a high degree of

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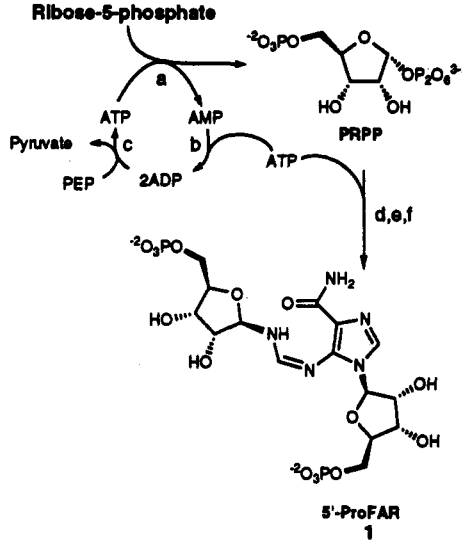
Scheme 1. (a) PRPP Synthetase, (b) Adenylate Kinase, (c) Pyruvate Kinase, (d) ATP Phosphoribosyltransferase, (e) N^1-5-(Phosphoribosyl)-ATP Pyrophosphohydrolase, and (f) N^1-5-(Phosphoribosyl)-AMP Cyclohydrolase



enrichment. The first three enzymes of the histidine biosynthetic pathway were the focus as they are required for the preparation of 1 from PRPP and ATP. A general ATP recycling system had been demonstrated previously for the preparation of PRPP from D-ribose-5-phosphate (R-5-P), and our aim was to couple this series of transformations in a single process, as outlined in Scheme 1.[14] As a general approach, a single DNA plasmid construct was designed to direct the overexpression of the *hisG*, *hisE*, and *hisI* genes in the host *Escherichia coli*. These genes respectively encode the enzymes ATP phosphoribosyl transferase, N^1-(phosphoribosyl)-ATP pyrophosphohydrolase, and N^1-(phosphoribosyl)-AMP cyclohydrolase.[3]

As a starting point, a plasmid clone that encodes the entire histidine biosynthetic operon, pHC9800, was used to subclone these genes in a four-stage process summarized in Scheme 2.[15] The *hisG* gene is the first open reading frame for an enzyme in the histidine operon and resides in a Bgl II-Hpa I DNA fragment, which was cloned into the pUC18 vector at the BamH I-Hinc II restriction sites, to arrive at pLM-1. The insert fragment of pLM-1 contains an upstream transcriptional attenuation signal, a translational start signal, and an appropriate ribosome binding site for *hisG*. Downstream of *hisG* is 121 bp of the *hisD* gene, which shares natural, overlapping translational stop and start codons. In the distal region of the histidine operon reside the coding sequences for the HisI and HisE proteins. These enzymes are translationally fused in *Escherichia coli*, and so a single DNA fragment containing the *hisIE* gene was generated by means of the polymerase chain reaction (PCR).[16] Primers for this PCR reaction were chosen judiciously so that unique DNA restriction sites would flank the coding region. The sense strand primer retained the natural ribosome binding site AGGA required for translation initiation at a position −10 bp from the start codon.[17] The antisense primer also incorporated a translational stop codon contiguous with the unique Hind III recognition sequence. After digestion with the restriction enzymes Pst I and Hind III, the PCR product was cloned into the same sites in pLM-1 to arrive at pLM-2. Between the target coding regions in pLM-2 is an intervening sequence derived from the *E. coli his* operon which is efficiently transcribed but creates a shift of the translational reading frame. Therefore, all of the required coding DNA sequences for the target enzymes (HisG, HisI, and HisE) are positioned for expression in a single plasmid vector.

In order to ensure efficient and high-level expression of the desired genes, a modification of pLM-2 was required. A part of the natural transcription attenuation sequence remains in this clone and includes 94 bp upstream of the *hisG* translational start codon. To remove this region of DNA, pLM-2 was linearized by digestion with Sma I and then subjected to limited digestion with the enzyme Dra I. The large DNA fragment that had the intervening sequence removed was purified, religated, and amplified by transformation and plasmid purification to arrive at construct pLM-3. Digestion of pLM-3 with EcoR I and Hind III afforded a DNA cassette that contained all the desired coding sequences and allowed for strategic cloning into an efficient expression vector.

The final plasmid construct p*hisGIE-tac* is derived from the general expression vector pJF119EH, which contains a strong synthetic *tac* transcriptional promoter.[18] A second useful feature of this vector is that it carries the coding sequence for the LacI protein, which in the absence of a suitable chemical inducer (e.g., (isopropylthio)-β-D-galactoside, IPTG), serves to repress transcription of the cloned DNA and provides for careful regulation regardless of the *E. coli* host strain. A suitable strain for expression of the target proteins encoded in p*hisGIE-tac* is a histidine auxotroph (*E. coli* strain FB1) that is devoid of all the related biosynthetic genes.[19] Thus, the expression of the enzymes and synthesis of 1 could be conducted in a single cellular extract without the chance of further metabolism. As shown in Figure 1, *E. coli* strain FB1 transformed with p*hisGIE-tac* overexpresses the target proteins when induced with IPTG. The level of expression for the three enzymes approximates 30% of the total soluble protein extract which proved suitable for direct use after partial removal of nucleic acids.

Since the host strain contained no other enzymes that process histidine biosynthetic intermediates, cellular extracts enriched with HisG, HisI, and HisE did not require further protein fractionation before their use in the synthesis of 1. A basic catalytic unit was defined by rate analyses of the UV change at 300 nm in order to establish the stability of the extracts after fractionation from nucleic acids. In fact, these extracts could be stored at 4 °C without substantial losses in the desired catalytic properties. Typical experimental preparations of 1 on a 1 mmol scale required only 18 mg of protein extract to ensure completion of the reactions within 3–4 h. This amount of protein extract was possible from 45 mL of bacterial culture. It is

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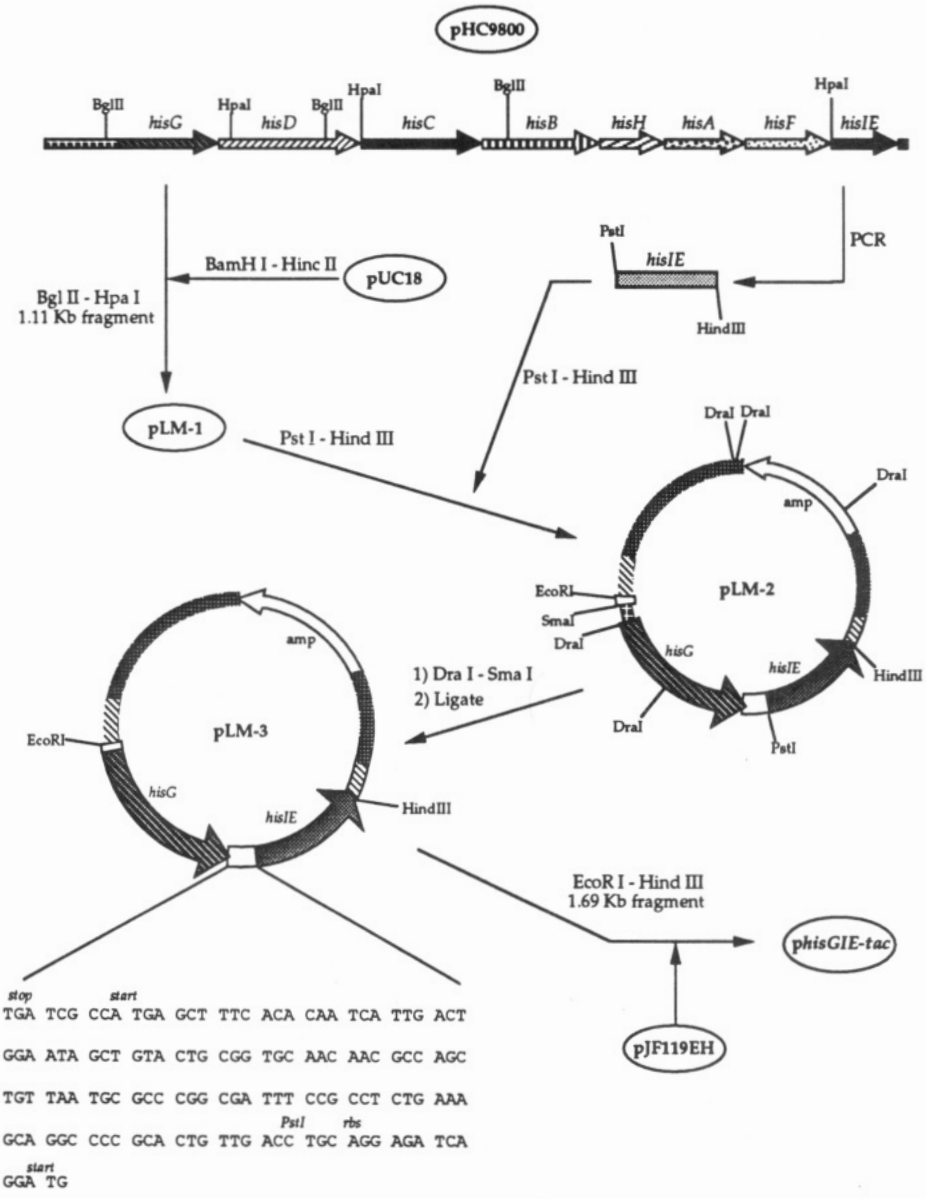
Scheme 2. Plasmid DNA Construction of p*hisGIE-tac*. Plasmid Clone pHC9800 Contains the *E. coli* Histidine Operon Cloned in the Vector pBR325 at the Hind III Sites[15]



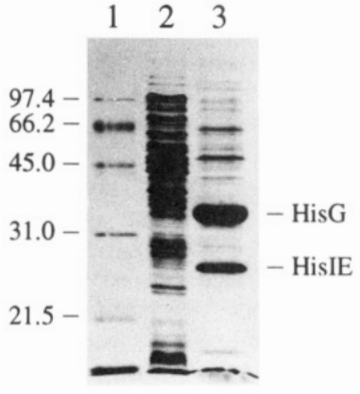


Figure 1. SDS–PAGE of protein extracts from *E. coli* FB-1/p*hisGIE-tac* cultures after treatment with streptomycin sulfate: lane 1, molecular weight markers; lane 2, extract from noninduced cultures; lane 3, extract from induced cultures used in the synthesis of 1.

clear then that this process is amenable to direct scale up without the need for excessive amounts of biological materials.

The utility of commercially-available PRPP is limited by its modest purity, high expense, and short shelf life. Commercial preparations of PRPP synthetase are of lower specific activity, and so we adopted a method for efficient expression and purification of the *S. typhimurium* enzyme.[20,21] Using a recombinant vector for the overexpression of the PRPP synthetase, we were able to optimize an *in situ* generation of PRPP. Critical to the efficiency of the process were the nucleotide substrate concentrations and the ratio of phosphoenol pyruvate (PEP) to R-5-P. Synthesis of 1 from R-5-P requires 2 mol equiv of ATP, one for synthesis of PRPP and one for synthesis of N^1-phosphoribosyl-ATP. One equiv of ATP is provided in the reaction mixture, and the second equivalent is generated from recycling of a 0.3 mol excess. To obtain a desirable rate of PRPP production, the concentration of AMP was minimized because of its inhibitory properties toward PRPP synthetase and ATP phosphoribosyltransferase.[1,21] Recycling of AMP to ATP using soluble forms

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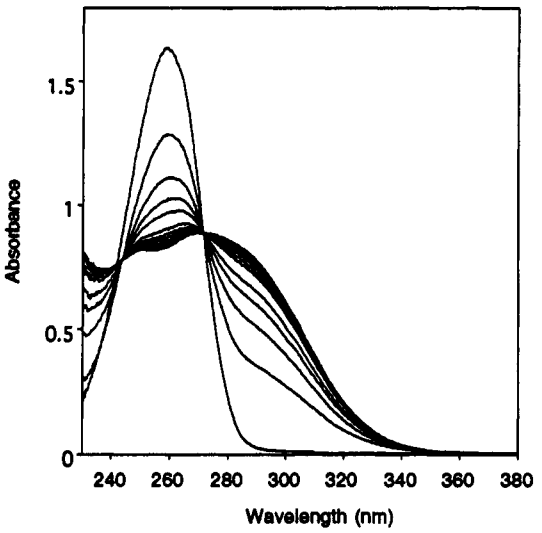

Figure 2. UV–vis spectra used to monitor the conversion of ribose 5-phosphate and 1.1 equiv of ATP to 5′-ProFAR by the enzyme coupled system outlined in Scheme 1. The incubation was conducted at 30 °C, and the spectra were taken at 5-min intervals for a total of 1.5 h. The progress of large-scale incubations was monitored in a similar manner after dilution of aliquots into the reaction buffer.

of adenylate kinase and pyruvate kinase required a 5-fold molar excess of PEP to maintain efficiency as the product pyruvate accumulated. In the absence of an appropriate buffer, production of pyruvate altered the pH of the reaction mixture and resulted in significant rate retardation. A Tris–phosphate buffer mixture at pH 7.6 was selected to maintain maximal activity of the PRPP synthetase and to control the pH throughout the reaction course. Finally, the addition of inorganic pyrophosphatase was important to ensure the irreversibility of the phosphoribosyltransferase and to circumvent enzyme product inhibition due to the buildup of inorganic pyrophosphate in steps d and e of the process.

Characteristic shifts in the UV–vis spectra were used to monitor the progress of these reactions as shown in Figure 2. A diminution in absorbance at 260 nm and a corresponding increase at 290 nm occur as a result of hydrolysis of the adenine ring of ATP. Purification of the desired nucleotide was achieved by application of the incubation mixture to an anion-exchange column followed by a gradient elution with NH_4HCO_3 buffer. The material purified in this manner is suitable for standard chemical and biochemical studies.

As part of our mechanistic studies of histidine biosynthesis, we required a more detailed spectral characterization of 1. The first structural characterization of 1 was indirect and involved biological processing to 5-amino-1-(5′-phospho-β-D-ribosyl)imidazole-4-carboxamide (AICAR, 2), chemical derivatization, and chemical or enzymatic degradation for analyses.[2] This information, combined with the unique UV–vis spectrum of the intermediate, led to the proposed structure. After additional purification by ion pair reversed-phase HPLC of the material prepared by our procedure, a ^1H NMR spectrum revealed two distinct aromatic resonances at 7.99 and 7.96 ppm along with a unique NH resonance at 5.07 ppm. These signals allowed for an initial assignment of the remainder of the ^1H resonances from ^1H COSY data. Despite the overlapping resonances for several of the ribosyl protons, the correlation of a single anomeric proton signal at 5.74 ppm with the NH resonance at 5.07 ppm allowed for the initial assignment of each ring system. Final verification of these proton assignments was made from ^1H–^{13}C HMQC and HMBC spectra, which enabled the complete assignment of the ^{13}C resonances. Two distinct ^{31}P resonances were observed at the expected chemical shifts, confirming the two phosphate monoesters. High-resolution FABMS data were also consistent with the predicted molecular formula for the free acid form of the nucleotide.

Three dephosphorylated forms of 1 (compounds 3–5) were also prepared using alkaline phosphatase to cleave one or both phosphate ester groups (Scheme 3). After purification and characterization of 3–5, they were tested as alternate substrates or inhibitors in our enzyme systems. These materials were also used for corroborative information regarding the structural assignments in 1. As was the case with 1, the ^1H NMR spectra of 3–5 were assigned from ^1H COSY data. The positions of the monophosphate ester groups were confirmed by chemical degradation and HPLC chromatographic analysis of the resulting 6 or 2 arising from cleavage of the C7–N6 bond (Scheme 3). All these data are consistent with the structure for 1 and support the NMR spectral assignments.

The unusual nucleotide 1 has several bonds that are potentially hydrolyzable and result in a limited lifetime under biological conditions. The decomposition process(es) and the related products could have secondary effects on cellular metabolism. In addition, the practical aspects of handling the material prompted a study of the hydrolytic stability of 1. Under conditions relevant to our studies, the decomposition was followed by HPLC analysis. The loss of starting material always exhibited pseudo-first-order kinetics, and the half-life values are shown in Table 1. The salt form or nucleotide concentration had minimal effects on the rates of decomposition; however, decomposition was accelerated substantially at lower pH. Reaction mixtures from decomposition of 1 consistently contained 2 as the major component. The latter material was characterized by coinjection with authentic material on HPLC and by comparisons of the ^1H NMR and FABMS spectra. Depending upon the reaction conditions, at least three additional products could be detected, but these have not been characterized at this time.

The development of reliable sources of biosynthetic intermediates like 1 is essential for *in vitro* biochemical studies of the constituent enzymes. In addition, a source of intermediates in histidine biosynthesis will also aid in the study of secondary metabolic pathways such as that suggested for the nucleoside antibiotic 2′-deoxycoformycin.[4,5] Use of R-5-P as a starting material and the irreversible property of the system enhances the potential for application of these enzymes in the synthesis of nucleotide analogs from simple carbohydrate precursors.

There are distinct advantages of plasmid-based overexpression systems for the preparation of advanced biosynthetic intermediates. The use of plasmid-based multigene constructs or ("mini operons") can lead to highly enriched sources of biosynthetic enzymes that are suited for direct use without further purification. One of the most important applications of this approach is in the preparation of ionic materials such as 1 because of the low propensity of bacterial cells to secrete such compounds. Even in cells that overexpress the first three enzymes in histidine biosynthesis, the highest detectable concentration of 1 in the culture medium was only 10 μM (data not

Scheme 3. Decomposition and Dephosphorylation of 1: a, Alkaline Phosphatase; b, Sodium Acetate/55 °C/pH 5.0; c, See Table 1

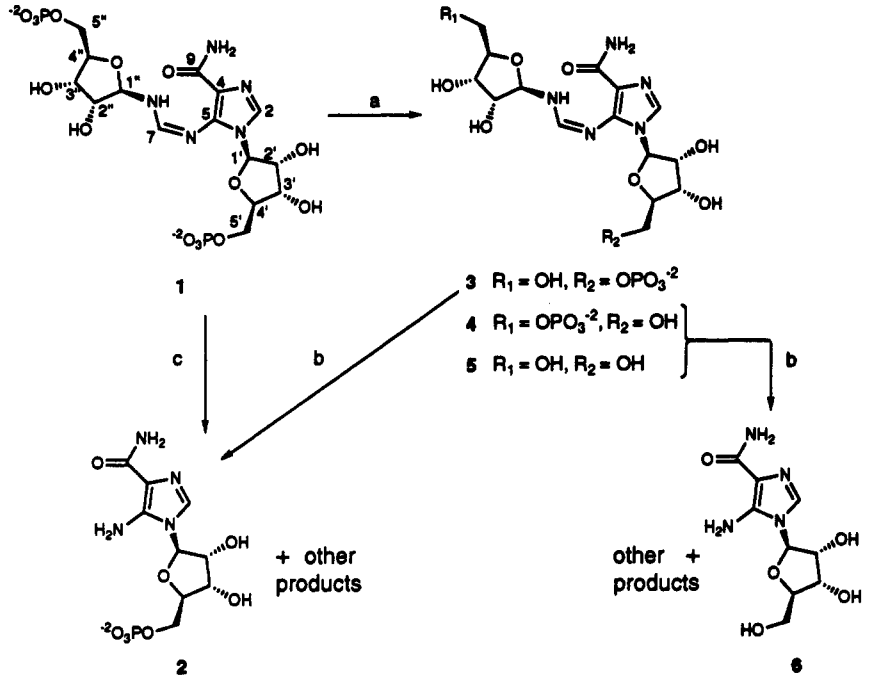

Table 1. Half-Life (min) of 5′-ProFAR in Aqueous Solution

concn (mM)	salt form	pH	55 °C	37 °C	22 °C
50	NH_4^+	6.0	76	208	409
0.5	NH_4^+	6.0	50	nd	nd
0.5	Na^+	6.0	57	305	533
0.5	Na^+	4.5	nd	13	38
0.5	Na^+	8.0	87	1307	nd

shown). Further extensions of this approach are currently in progress and are directed at the preparation of all of the intermediates in histidine biosynthesis.

Experimental Section

General. All preparations of protein extracts including centrifugations were conducted at 4 °C using a Sorvall SS-34 rotor. All enzyme assays were performed at 30 °C in a Varian Cary 3 spectrophotometer. The NMR spectra were obtained on a Varian VXR-500 MHz spectrometer using D_2O as the sample solvent. The ^1H and ^{13}C chemical shifts are reported relative to internal sodium (trimethylsilyl)propanesulfonate or dioxane, and the ^{31}P spectra were referenced to external H_3PO_4. The HMBC and HMQC experiments were implemented using the Varian VNMR 2.2 software. The samples for these experiments were prepared in 99% D_2O and analyzed without spinning at 22 °C using a presaturation pulse to null the water signal. Analytical HPLC analyses of 5′-ProFAR were conducted with a Hamilton PRP-1 column (4.1 mm × 25 mm) operating at 0.7 mL min^{-1} with 25 mM diisopropylethylamine acetate pH 7.0/2% acetonitrile as the eluent and detection by UV absorbance at 290 nm.

Materials. Biochemicals and enzymes were purchased from Sigma, with the exception of pyruvate kinase, which was purchased from Boehringer Mannheim. Ammonium bicarbonate and diisopropylethylamine were purchased from Aldrich. Synthesis of PEP-K^+ was according to a literature procedure.[22] All other chemicals were reagent grade and used without further purification. Q Sepharose FF was purchased from Pharmacia. Dowex AG 50W-X8 cation exchange resin (100–200 mesh) was purchased from BioRad. Cellulose TLC plates with fluorescent indicator were purchased from American Scientific Products and were visualized by short-wave ultraviolet light and sulfosalicylic acid–ferric chloride stain.[23] Diisopropylethylamine was purified before use by distillation from ninhydrin. All other solvents were of HPLC grade and used without purification. Oligonucleotides were prepared at the Purdue Center for Macromolecular Structure on an ABI synthesizer. The recombinant plasmids were amplified in *E. coli* XL1-Blue (Stratagene), and the strain used for the expression studies was *E. coli* FB1 Δ(*hisGDCBHAFIE*)-750 *gnd rhaA* which was obtained from C. Bruni. The following plasmids were obtained as gifts: pBS9800 (C. Bruni), pBS111R (R. Switzer), and pJF119EH (E. Lanka).

Plasmid Constructs. The restriction enzyme digests, DNA ligations, bacterial cultures, and plasmid isolations were modeled after standard protocols.[24] Analyses of DNA sequences were conducted using the dideoxy method and modified T7 DNA polymerase (Sequenase, USB). All oligonucleotides used in these studies were purified by elution from urea–polyacrylamide gels after separation by electrophoresis. The sequences of the primers used in the PCR reaction for the *hisIE* gene are, for the sense strand, 5′-GAA TTC TGC AGG AGA TCA GGA TAT GTT AAC-3′, and for the antisense strand, 5′-GGG AAG CTT ACT GAT GCC GTT TAC GCA GGT-3′. The remainder of the insert DNA in this vector is derived from the natural sequence of the *his* operon.[15] The plasmid DNA construct pLM-3 was derived from pLM-2 by complete digestion of 8 μg DNA with 10 U SmaI in a total volume of 40 μL at 25 °C for 14 h. The mixture was incubated briefly at 37 °C and 2.5 U DraI was added. Six equal aliquots were taken over 0–15 min and kept on ice. DNA fragments in each sample were separated immediately on a 0.7% agarose electrophoresis gel, stained with ethidium bromide, and visualized under long UV light. The fragment of appropriate length was excised from the gel and extracted using the Geneclean protocol (Bio 101). Ligation of the recovered vector with T4 DNA ligase under standard conditions was followed by amplification in *E. coli* XL-1Blue providing several recombinants that were analyzed by a series of restriction digests, partial DNA sequencing, and transformation of the appropriate *E. coli* strains bearing genetic deficiencies for these three enzymes.[15] This last complementation test served to verify the functional features of the plasmid-encoded DNA when expressed *in vivo*. The resulting

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5'-region between the EcoR I site of pUC 18 and the *hisG* gene is truncated in this plasmid and has the following sequence: GAATTCGAGCTCGGTACCCAAAGAGGAATAACAAAATG.

Isolation of PRPP Synthetase. This procedure is a modification of that described by Switzer.[21] Two hundred mL of LB medium containing 75 μg/mL of ampicillin in a 500-mL culture flask was inoculated with an overnight culture (3 mL) of *E. coli* HB101 transformed with plasmid pBS111R and shaken at 37 °C for 9 h. Constitutive expression of the *S. typhimurium* enzyme is directed by the plasmid in these cells, and the expression is enhanced by growth under slightly anaerobic conditions. The cells were harvested by centrifugation (15 min at 10000*g*), and the pellet was suspended in 50 mM potassium phosphate (20 mL, pH 7.5), ultrasonicated (6 × 20 s pulses with 15-s rests), and centrifuged (20 min at 23500*g*). The precipitate was discarded, and to the supernatant was added 10% w/v streptomycin sulfate (2 mL). The solution was heated to 54 °C in boiling water, immediately placed in a 55 °C water bath for 5 min, and cooled to 5 °C on ice. The cloudy suspension was centrifuged (20 min at 23500*g*), and the precipitate was discarded. To the supernatant was added $(NH_4)_2SO_4$ (4.18 g), and the resultant suspension was allowed to stand on ice for 30 min and then centrifuged (40 min at 23500*g*). After centrifugation, the pellet was suspended in a buffer solution (2 mL) containing 50 mM potassium phosphate (pH 7.5) and 1.09 M $(NH_4)_2SO_4$. The solution was diluted to 10 mL with water and acidified to pH 4.6 with 1 N acetic acid, and the precipitate was collected by centrifugation (20 min at 23500*g*). The protein was dissolved in 50 mM potassium phosphate (9 mL, pH 7.5) and stored at −80 °C for 2 h. After thawing, the mixture was centrifuged (10 min at 10800*g*), and to the supernatant was added 50 mM potassium phosphate saturated with $(NH_4)_2SO_4$ (4.5 mL). The mixture was allowed to stand on ice for 10 min and centrifuged (20 min at 10800*g*). The final pellet was suspended in 50 mM potassium phosphate (2 mL, pH 7.5) and stored at 4 °C. The specific activity of the enzyme was 30 U/mg.

Assay of PRPP Synthetase. The assay was adapted from a previously reported method that relies on enzymatic coupling of AMP production to the reduction of pyruvate.[25] The assay mixture (1 mL) contained 100 mM Tris–phosphate pH 7.6, 10 mM magnesium chloride, 15 mM KCl, 5 mM ribose 5-phosphate, 5 mM ATP, 1.5 mM PEP, 0.2 mM NADH, 20 U of pyruvate kinase, 5 U of myokinase, and 9 U of lactate dehydrogenase. The reaction was initiated by addition of PRPP synthetase (0.1 μg), and the progress of the reaction was monitored by the decrease in absorbance at 340 nm. One unit of activity refers to the formation of 1 μmol of product per min at 30 °C, using a molar extinction coefficient of 6220 M^{-1} for NADH.

Preparation of HisGIE Extract. Cells transformed with plasmid p*hisGIE-tac* were selected by growth on LB medium in the presence of ampicillin (70 μg/mL), which was present in all cultures. An overnight culture (3 mL) of *E. coli* FB1/p*hisGIE-tac* was used to inoculate 20 mL of fresh medium. After being shaken at 37 °C for 16 h, this culture was used to inoculate 1 L of prewarmed medium and and then shaken at 37 °C. When this culture reached late logarithmic phase (indicated by $OD_{550} \approx 1.0$), IPTG was added to a final concentration of 1.5 mM, and the cells were harvested 9 h later by centrifugation (15 min at 8700*g*). The cell pellets were resuspended in LB medium (total volume 100 mL), pelleted in 1.5-mL aliquots, and stored at −80 °C. For routine preparations, a pellet was resuspended in 100 mM Tris–HCl (2 mL, pH 7.8), lyzed by ultrasonication, and centrifuged (20 min at 12000*g*) to remove insoluble cell debris. To the supernatant was added 10% w/v streptomycin sulfate (200 mL), and the mixture was allowed to stand on ice for 2 h. The cloudy suspension was centrifuged (20 min at 12000*g*), and the precipitate was discarded. Enzymatic activity in the supernatant was analyzed as described below. A typical value was 0.30 U/mg. Extracts at this stage of purity could be stored at 4 °C for at least 1 month without any detectable loss of enzyme activity.

Assay of p*hisGIE* Extract. The assay is based on continuous formation of 5'-ProFAR which can be detected by an increase in absorbance at 300 nm ($\Delta\epsilon_{300}$ = 6069 L M^{-1} cm^{-1}). This is a variation on a previously reported assay.[25] The assay mixture (1 mL) contained 50 mM potassium phosphate pH 7.5, 16 mM $MgCl_2$, 1 mM EDTA, 150 μM ATP, 3 mM PRPP, and 0.6 U of inorganic pyrophosphatase. The reaction was initiated by addition of HisGIE extract (1–6 μg), and a linear increase in absorbance was observed after a 2–3 min lag. A unit of activity is defined as the formation of 1 μmol of product per minute under the specified conditions.

N^1-[(5″-Phospho-β-D-ribosyl)formimino]-5-aminoimidazole-4-carboxamide Ribonucleotide (1). A 100-mL solution of 50 mM potassium phosphate/100 mM Tris–HCl/1 mM EDTA, pH 7.5, containing 0.31 g (1 mmol) of ribose 5-phosphate, 0.775 g (1.3 mmol) of ATP, and 1.03 g (5 mmol) of PEP was adjusted to pH 7.6 with 5 M sodium hydroxide. To this solution were added magnesium chloride (final concentration 16 mM), 200 U of pyruvate kinase, 180 U of myokinase, and 10 U of PRPP synthetase, and the solution was incubated at 30 °C for 1 h. Final additions of 130 U of inorganic pyrophosphatase and 5 U of HisGIE extract were made, and incubation was continued for an additional 4 h. The solution was filtered through a 0.45-μm syringe disk and divided into four portions. Each portion was applied to a column of Q Sepharose FF (2.5 cm × 11 cm) and eluted with a linear gradient composed of 180 mL of 60 mM NH_4HCO_3 and 180 mL of 250 mM NH_4HCO_3. After lyophilization, a white solid (559 mg, 86%) was obtained. An analytical sample was prepared by preparative HPLC on a Hamilton PRP-1 column (2.1 cm × 25 cm) operating at 7 mL min^{-1} using the same eluent as that described for the analytical system. The sample was dried by lyophilization and exchanged to the sodium form by cation-exchange chromatography on Dowex AG 50W-X8 (Na^+, 2.5 cm × 5 cm) and elution with water. The eluent was lyophilized to a white powder and stored dry at −20 °C. Alternatively, aliquots (750 μL) of 8 mM 5'-ProFAR were stored frozen at −80 °C: TLC (cellulose, R_f 0.18; 3:3:4 CH_3CN/2-propanol/0.1 M NH_4HCO_3); ^1H NMR (D_2O) δ 7.99 (1, s), 7.96 (1, s), 5.76 (1, d, J = 4.78 Hz), 5.74 (1, d, J = 5.29 Hz), 5.07 (1, br, N(8)), 4.55 (1, dd, J = 4.78, 4.86 Hz), 4.41 (1, dd, J = 4.28 Hz, 4.86 Hz), 4.33 (1, ψt, J = 4.35 Hz), 4.29 (1, dd, J = 4.35, 5.29 Hz), 4.25 (1, m), 4.17 (1, m), 3.95 (2, m), 3.87 (2, m); ^{31}P NMR (D_2O) δ 4.13 (s), 4.04 (s); ^{13}C NMR (D_2O) δ 167, 157, 145, 132, 118, 87, 85, 84, 83, 75, 74, 71, 70, 64, 63; HRMS (FAB, -Ve glycerol) calcd m/z 576.0744, found 576.0763.

Dephosphorylation of 1. In a total volume of 2 mL of 15 mM Tris–Cl pH 8.0 were combined 5'-ProFAR (21 μmol) and 4.2 U of alkaline phosphatase (*E. coli*, Sigma). The mixture was incubated at 25 °C for 5 h, and the process was monitored by HPLC, as described above. The reaction was stopped by the addition of 0.5 mM EDTA (20 μL), and the mixture was stored at −80 °C. Separation of the dephosphorylated materials was performed by preparative HPLC using 25 mM diisopropylethylammonium acetate (pH 7.0). Three materials were identified by UV, pooled, and dried by lyophilization. The monophosphate esters were exchanged to the sodium salt form by passage through Dowex AG 50W X-8 (Na^+).

As a final check of the spectral assignments of the two monophosphate esters, samples of each were hydrolyzed at 55 °C in 20 mM sodium acetate (pH 5.0) for 5 h. Commercial samples of AICAR and 5-amino-1-(β-D-ribosyl)imidazole-4-carboxamide were used for HPLC retention time standards.

N^1-[(β-D-Ribosyl)formimino]-5-aminoimidazole-4-carboxamide Ribonucleotide (3): ^1H NMR (D_2O) δ 7.94 (1, s), 7.75 (1, s), 5.75 (1, d, J = 5.07 Hz), 5.73 (1, d, J = 4.85 Hz), 5.09 (1, m), 4.51 (1, d, J = 5.07 Hz), 4.36–4.30 (3, m), 4.17–4.11 (2, m), 3.89–3.84 (2, m), 3.86 (1, dd, J = 2.38 Hz, 12.36 Hz), 3.77 (1, dd, J = 4.72 Hz, 12.36 Hz); ^{31}P NMR (D_2O) δ 4.14.

N^1-[(5″-Phospho-β-D-ribosyl)formimino]-5-aminoimidazole-4-carboxamide Ribonucleoside (4): ^1H NMR (D_2O) δ 7.96 (1, s), 7.95 (1, s), 5.76 (1, d, J = 5.31 Hz), 5.73–5.68 (1, m), 5.09–5.06 (1, m), 4.64–4.59 (1, m), 4.42 (1, dd, J = 3.80 Hz, 4.99 Hz), 4.24–4.17 (3, m), 4.07–4.02 (1, m), 3.98–3.91 (2, m), 3.82–3.79 (1, m), 3.72–3.65 (1, m); ^{31}P NMR (D_2O) δ 3.99.

N^1-[(β-D-Ribosyl)formimino]-5-aminoimidazole-4-carboxamide Ribonucleoside (5): ^1H NMR (D_2O) δ 7.95 (1, s), 7.75 (1, s), 5.76 (1, d, J = 5.29 Hz), 5.73–5.68 (1, m), 5.11–5.07 (1, m),

(25) Morton, D. P.; Parsons, S. *Arch. Biochem. Biophys.* **1976**, *175*, 677–686.

4.54 (1, ψt, J = 5.29 Hz), 4.34–4.30 (1, m), 4.23–4.18 (2, m), 4.13–4.09 (1, m), 4.07–4.02 (1, m), 3.85 (1, dd, J = 3.45 Hz, 12.75 Hz), 3.76 (1, dd, J = 4.92 Hz, 12.75 Hz), 3.71–3.66 (2, m).

Analysis of Nucleotides in Bacterial Cultures. An overnight culture (3 mL) of *E. coli* FB1/p*HisGIE-tac* was used to inoculate a flask with 20 mL of fresh LB medium with ampicillin (70 μg mL^{-1}). Similarily, an overnight culture of *E. coli* FB1 (2 mL) was used to inoculate 20 mL of LB medium. When the cell cultures had reached late logarthmic growth, IPTG was added to a final concentration of 1.5 mM in both flasks. After 3 h, the cells were removed by centrifugation, and the medium was retained for analysis. Filtered samples (10 μL) were analyzed by HPLC on a PRP-1 column (0.4 cm × 25 cm, Hamilton) using 25 mM diisopropylethylammonium acetate (pH 7.0)/2% CH_3CN as the eluent. Chromatograms were compared from induced and noninduced FB1 cultures, and 5′-ProFAR was quantified by coinjections of known amounts of authentic samples. Under the specified conditions, 5′-ProFAR eluted in a transparent region of the chromatogram detectable to a lower limit of 5 μM.

Kinetics of Decomposition. A solution (0.5 mL) of 50 mM 5′-ProFAR (NH_4^+ salt, pH 6.0) was placed into each of three Eppendorf tubes. One tube was allowed to stand at room temperature, and the second and third tubes were incubated in 37 °C and 55 °C water baths. Other samples of different concentration, salt form, and pH were prepared similarily using 20 mM sodium acetate pH 4.5 or 20 mM Tris pH 8.0 as buffers. Aliquots from each reaction were analyzed by HPLC at specified time points, and the final mixtures were analyzed by HPLC with UV detection at 240, 260, and 290 nm. The natural log of the peak area of 5′-ProFAR (elution time 12.3 min) was plotted against time to determine the half-life of 5′-ProFAR.

Decomposition Products from 5′-ProFAR. 5′-ProFAR (17.5 μmol) was decomposed as described above, and the mixture was separated by preparative HPLC on a Hamilton PRP-1 column (2.1 cm × 25 cm). The products were eluted with 25 mM diisopropylethylammonium acetate (pH 7.0)/2% acetonitrile at a flow rate of 6.5 mL min^{-1} and were detected by their absorbance at 260 nm. One compound eluted at 19.5 min and decomposed to multiple products during attempts at further purification. A second compound eluted at 25.5 min (67% yield) and was found to be identical to commercial AICAR by chromatographic properties on HPLC and TLC, as well as UV, ^{1}H NMR, and FABMS spectral characteristics.

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