Imidazole Glycerol Phosphate Synthase: The Glutamine Amidotransferase in Histidine Biosynthesis†

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ABSTRACT: Two proteins essential for the biosynthesis of the amino acid histidine in *Escherichia coli* have been overexpressed and purified to apparent homogeneity. The protein encoded by the *hisF* gene has an ammonia-dependent activity that results in the conversion of the biosynthetic intermediate N^1-[(5′-phosphoribulosyl)formimino]-5-aminoimidazole-4-carboxamide ribonucleotide (PRFAR) to imidazole glycerol phosphate (IGP) and 5-aminoimidazole-4-carboxamido-1-β-D-ribofuranosyl 5′-monophosphate (AICAR). The second protein encoded by the *hisH* gene exhibits no detectable catalytic properties with biosynthetic intermediate PRFAR, glutamine, or ammonia. In combination, the proteins are capable of a stoichiometric conversion of glutamine and PRFAR to form AICAR, IGP, and glutamate. Neither protein alone is capable of mediating a conversion of the nucleotide substrate to a free metabolic intermediate. The HisH and HisF proteins form a stable 1:1 dimeric complex that constitutes the IGP synthase holoenzyme. Steady-state kinetic parameters for the holoenzyme indicate that glutamine is a more efficient substrate relative to ammonium ion by a factor of 10^3. The HisF subunit will support an ammonia-dependent reaction with a turnover number similar to that of the holoenzyme with glutamine. The glutaminase activity for the holoenzyme is 0.8% of that in the presence of the nucleotide substrate PRFAR. There are critical subunit interactions that mediate the catalytic properties for glutamine hydrolysis. The catalytic turnover of glutamine can be increased up to 37-fold by the addition of either the product IGP or the biosynthetic precursor N^1-[(5′-phosphoribosyl)formimino]-5-aminoimidazole-4-carboxamide ribonucleotide (5′-ProFAR). The mechanistic significance of this glutaminase activity compared to other *trpG* type glutamine amidotransferases is discussed.

For many years, the interdependence of the histidine biosynthetic pathway and the de novo synthesis of purines in microorganisms has been recognized as a significant feature of microbial metabolism (Shedlovsky & Magasanik, 1962). This is illustrated by the fact that mutations in the early steps of histidine biosynthesis have been shown to lead to purine auxotrophic phenotypes and underscores the high degree of metabolic expense represented in the pathway (Johnston & Roth, 1979; Stougaard & Kennedy, 1988; Shioi et al, 1982). Since the biosynthesis of histidine derives a carbon and a nitrogen equivalent from the purine ring of ATP, an important aspect of this metabolic link is represented by the production of 5-aminoimidazole-4-carboxamido-1-β-D-ribofuranosyl 5′-monophosphate (AICAR)[1] in a central step of the histidine pathway. The recycling of AICAR into the de novo purine biosynthetic pathway from the histidine pathway is dependent upon the functions of the *hisH* and *hisF* genes in eubacteria (Winkler, 1987). Despite the large body of genetic information regarding histidine biosynthesis, the characterization of the constituent proteins has been minimal.

The biosynthesis of histidine implicates an array of unusual metabolic transformations, and there has been a significant level of ambiguity regarding the nature of the catalytic events associated with the formation of the imidazole ring. As illustrated in Scheme I, the essential step for this process involves the ribosylated nucleotides N^1-[(5′-phosphoribosyl)-formimino]-5-aminoimidazole-4-carboxamide ribonucleotide (5′-ProFAR) and N^1-[(5′-phosphoribulosyl)formimino]-5-aminoimidazole-4-carboxamide ribonucleotide (PRFAR). These nucleotide intermediates were originally characterized by the elegant work of Ames and co-workers and were respectively named BBMII and BBMIII after their characteristic properties following derivatization (Smith & Ames, 1964). As shown in the original investigations, the *hisF* and *hisH* gene products are involved in the process that transforms the nucleotide PRFAR into AICAR and imidazole glycerol phosphate (IGP). Glutamine has been assumed to be the source of the final nitrogen atom in the imidazole ring of IGP, and a recent observation provides support for this role. The deduced amino acid sequence for the HisH protein bears a high degree of sequence similarity at the glutamine binding site with other *trpG* type glutamine amidotransferases (Carlomagno et al., 1988). This has led to the postulate that the protein is involved in the amination step, which is consistent with the original conclusion of Ames (Smith & Ames, 1964). However, the actual catalytic properties of each gene product have remained unknown. In this paper, we report the first purification and characterization of these two proteins.

MATERIALS AND METHODS

DEAE Sepharose FF, Q Sepharose, Sephacryl S-100, Superose 12, and NAP-10 chromatography supports were purchased from Pharmacia. Acetylpyridine adenine dinucleotide (APAD), ampicillin, Brilliant Blue G and R, egg

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[1] Abbreviations: AICAR, 5-aminoimidazole-4-carboxamido-1-β-D-ribofuranosyl 5′-monophosphate; APAD, acetylpyridine adenine dinucleotide; IPTG, isopropyl β-D-thiogalactopyranoside; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; IGP, imidazole glycerol phosphate; PCR, polymerase chain reaction; 5′-ProFAR, N^1-[(5′-phosphoribosyl)formimino]-5-aminoimidazole-4-carboxamide ribonucleotide; PRFAR, N^1-[(5′-phosphoribulosyl)formimino]-5-aminoimidazole-4-carboxamide ribonucleotide; SDS–PAGE; sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SPDP, *N*-succinimidyl 3(2-pyridyldithio)propionate; TEAA, triethylammonium acetate; TEA, triethanolamine; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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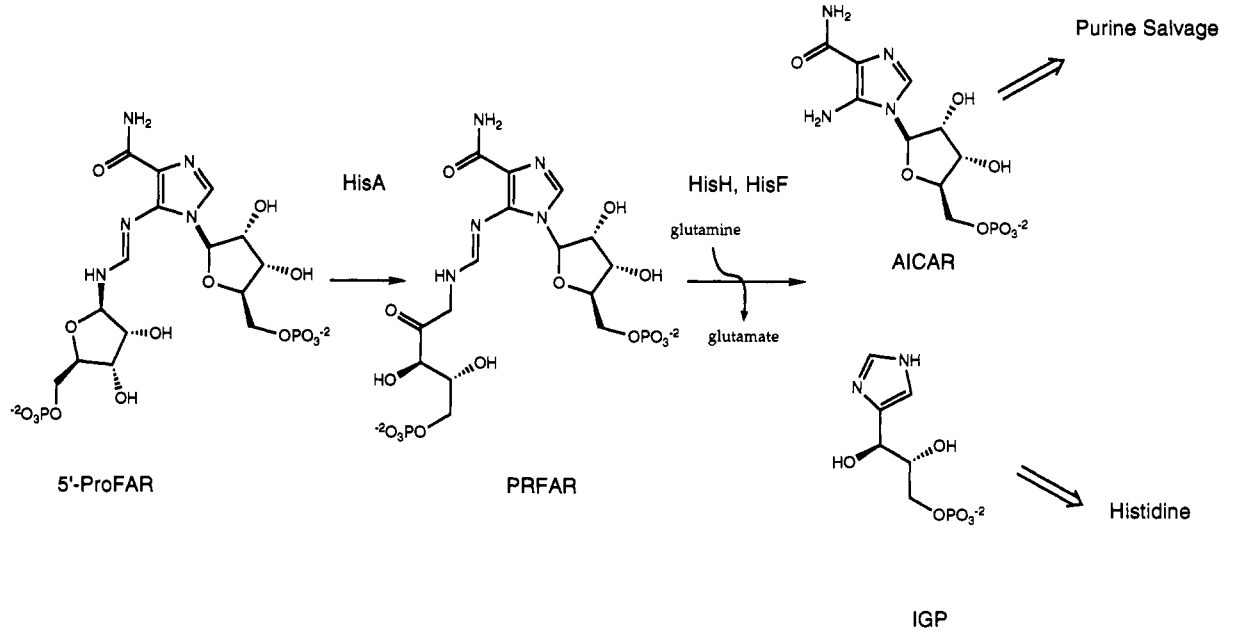
Scheme I



albumin, ribonuclease A, streptomycin sulfate, trypsinogen, 5-aminoimidazole-4-carboxamido 1-β-D-ribofuranoside, and 5-aminoimidazole-4-carboxamido-1-β-D-ribofuranosyl 5′-monophosphate (AICAR) were purchased from Sigma. Glutamate dehydrogenase was from Boehringer Mannheim. *N*-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) was from Pierce, and Dowex AG 50W-X8 (100 mesh), Bio-Gel HTP, and low molecular weight protein standards were from Bio-Rad Laboratories. Centriprep-10, Centricon-10, and PM-10 ultrafiltration membranes were from Amicon. Protein concentrations were determined by the modified Bradford method using BSA as a standard (Read & Northcote, 1981). SDS–PAGE was performed after the method of Laemmli as described (Laemmli, 1970). DNA sequencing was performed with the Sequenase version 2.0 kit from US Biochemicals or the TaqTrack sequencing system from Promega, and [α-^{35}S]-dATP was supplied by Amersham. The IGP was a gift from A. Parker (Purdue University). All oligonucleotides were prepared by the Purdue Univeristy Laboratory for Macromolecular Structure and purified by urea–PAGE before use. All UV-vis spectroscopy was performed on a Varian Cary 3 spectrophotometer interfaced with an Epson Equity 386 SX computer.

Strains, Plasmids, and Media. *Escherichia coli* DH5α [*recA1*] was used as the host strain for all isolations of recombinant DNA vectors, and XL-1 Blue (Stratagene) was used for propagation of all phagemid vectors. Templates for dideoxy DNA sequencing were single-stranded forms of the appropriate M13mp18/19 (Boehringer Mannheim) or Bluescript II (Stratagene) clones after superinfection with the phage VCSM13 (Stratagene). *E. coli* strains FB1 [Δ*his*(*GDCB-HAFIE*)*750*] and FB182 (*hisF892*) were obtained from C. Bruni (University of Naples). The host strain for all overexpression of the *his* genes was the FB1 strain, which was grown as shake cultures at 37 °C in LB media. Wild-type *E. coli* strain W3110 was obtained from the *E. coli* Genetic Stocks Center (Yale University). The expression vector pJF119EH was a gift from Erich Lanka (Max-Planck-Institut, Berlin). All other methods for recombinant DNA were patterned after standard protocols (Sambrook et al., 1989).

Subcloning and Overexpression of the hisH and hisF Genes. The coding regions for the *hisH* and *hisF* genes were subcloned by PCR using oligonucleotide primers based upon the published sequence of the *his* operon (Carlomagno et al., 1988). The sequences of the 5′ and 3′ PCR primers are listed below, and 70 pmol of each was used in 100-μL reaction mixtures (Scharf, 1990). Template DNA (1–10 ng) were *Hin*dIII fragments in the 5.0–4.0-kb range derived from *E. coli* W3110 chromosomes. Each PCR product was purified by agarose gel electrophoresis before digestion with *Eco*RI and *Xma*I restriction endonucleases. The fragments were cloned into the corresponding sites of M13 or Bluescript KS+. After analysis of the DNA sequences, the coding regions were cloned from these plasmid constructs into the *Eco*RI and *Bam*HI sites of the *E. coli* expression vector pJF119EH (Fürste et al., 1986). These DNA constructs were confirmed by DNA restriction digestion with diagnostic enzymes and were named p*hisF-tac* and p*hisH-tac*.

hisH 5′ PCR primer

5′-CTAGGAATTCATATGAACGTGGTGATCCTT-3′

hisH 3′ PCR primer

5′-CGTACCCGGGTTATCACATCTCCAGGAAGT-3′

hisF 5′ PCR primer

5′-AGGAATTCATATGCTGGCAAAACGCATAAT-3′

hisF 3′-PCR primer

5′-CGTACCCGGGTTAACATATCCTGATCTCCA-3′

All expression studies of the *hisH* and *hisF* genes were conducted in *E. coli* FB1 after transformation with the appropriate DNA expression vector. One-liter cultures were grown in the presence of ampicillin (75 μg/mL) to late logarithmic phase (OD_{550} = 1.0) and induced by addition of IPTG to a final concentration of 1 mM. Six to eight hours after induction, the cells were pelleted by centrifugation,

washed with 0.1 M Tris·HCl (pH 7.5)/1 mM EDTA, and stored as a paste at −80 °C until needed.

Enzyme Assays. All assays were performed in a total volume of 1 mL at 30 °C with 50 mM Tris·HCl, pH 8.0, 100 μM PRFAR, and 5 mM glutamine. Reactions were initiated by the addition of 10 pmol (0.5 μg) of IGP synthase. In the absence of purified PRFAR, 100 μM 5′-ProFAR and 0.15 nmol of 5′-ProFAR isomerase (HisA) were added to the initial mixture. Assays of the HisH and HisF subunits were performed under the same conditions, with 0.69 nmol of HisH or 0.74 nmol of HisF included in the initial mixture. After preincubation, a limiting amount of HisH (12 pmol) or HisF (18 pmol) was added to initiate the reaction. PRFAR consumption was followed by a decrease in absorbance at 300 nm over 5 min. All extinction coefficients were determined in 50 mM Tris·HCl, pH 8.0. Those for PRFAR were derived from 5′-ProFAR: ϵ_{219} = 16 100 M^{-1} cm^{-1}, ϵ_{270} = 8141 M^{-1} cm^{-1}, ϵ_{290} = 7700 M^{-1} cm^{-1} (Martin et al., 1971), and ϵ_{300} = 6069 M^{-1} cm^{-1}. There are no significant differences in the UV spectra for PRFAR and 5′-ProFAR in the region from 270 to 300 nm. The experimentally determined values for AICAR extinction coefficients used in this study were ϵ_{219} = 2868 M^{-1} cm^{-1}, ϵ_{270} = 12 300 M^{-1} cm^{-1}, and ϵ_{300} = 432 M^{-1} cm^{-1}. A value for $\Delta\epsilon_{300}$ = 5637 M^{-1} cm^{-1} was determined by subtracting the value for AICAR from the appropriate extinction coefficient for PRFAR. Extinction coefficients for IGP were established under identical conditions, and values of ϵ_{209} = 4558 M^{-1} cm^{-1} and ϵ_{219} = 2679 M^{-1} cm^{-1} were used in these studies.

Glutaminase Studies. In a total volume of 1 mL was incubated either IGP synthase (0.2–2 nmol), HisH (0.1–2 nmol), or HisF (0.1–0.74 nmol) with 50 mM Tris·HCl, pH 8.0, and varied concentrations of glutamine (1–10 mM) at 30 °C. Samples were collected at specified time points over 60 min, boiled for 1 min, and frozen at −80 °C. In experiments with the effectors, 5′-ProFAR was used at concentrations of 1 μM to 5 mM, or IGP was used at concentrations ranging from 0.5 to 2.5 mM with 0.6 nmol of IGP synthase in the incubation. Glutamate assays were performed similarly to the protocol of Buchanan et al. (1978). An appropriate volume of experimental sample was incubated in a total volume of 1 mL with 50 mM Tris·HCl, pH 8.0, 50 mM KCl, 1 mM EDTA, 0.5 mM APAD, and 100 μg of glutamate dehydrogenase. Samples were incubated along with a blank containing no experimental sample at 37 °C for 1.5 h and microfuged for 2 min before reading the absorbance. The concentration of APADH produced was determined by the sample absorbance at 363 nm (ϵ_{363} = 8900 M^{-1} cm^{-1}), and standard curves were prepared daily. The initial velocity for these end-point assays was calculated from the first 10 min over which a linear rate was observed. Rates observed in the absence of IGP synthase were at least 10^3 slower than the enzyme-mediated reaction.

Preparation of PRFAR. The preparations of 5′-ProFAR and 5′-ProFAR isomerase used modified versions of the original method of Martin et al. (1971), and the details will be published elsewhere. 5′-ProFAR (70 μmol) was incubated in 50 mM TEA, pH 7.5, and 0.2 M NaCl at 30 °C with 3 nmol of purified 5′-ProFAR isomerase. The reaction was monitored by reverse-phase chromatography on a Hamilton PRP-1 column (0.46 × 25 cm) using isocratic elution with 0.1 M triethylammonium acetate (TEAA), pH 7.5, at 0.75 mL min^{-1} and detection at 290 nm. Under these conditions, the 5′-ProFAR eluted at 9.5 min and the PRFAR eluted at 11.5 min. The crude PRFAR was lyophilized, redissolved in 5 mL of H_2O, and passed through a 0.45-μm filter before being loaded onto a column of Q Sepharose (1.5 × 20 cm) equilibrated in 25 mM NH_4HCO_3, pH 8.0, at 4 mL min^{-1}. The PRFAR was eluted with a linear gradient to 0.5 M NH_4HCO_3, pH 8.0. Further purification of the recovered PRFAR was achieved on a Rainin Dynamax C-18 column (1 × 25 cm). These runs were performed isocratically in 0.1 M TEAA, pH 7.0, at 2 mL min^{-1}; PRFAR elutes at 23 min under these conditions. The desired fractions were dried by lyophilization to yield a viscous liquid, which was diluted with 5 mL of H_2O. The soluble material was passed through a column of AG 50W-X8 resin (2.5 × 4 cm; Na^+ form). The sodium-PRFAR was dried by lyophilization, redissolved in H_2O, and stored in small aliquots at −80 °C. Samples of these PRFAR preparations were stable to repeated freeze–thaw cycles.

Protein Purification. HisH. All purification steps were performed at 4 °C. A frozen cell paste (30 g) was thawed and resuspended in 100 mL of 50 mM Tris·HCl, pH 7.5/1 mM EDTA (buffer A). The resuspension was sonicated for three 30-s bursts with 30-s rests on ice before being passed through a French pressure cell at 18 000 psi and centrifuged for 20 min at 17000*g*. Streptomycin sulfate solution (10% w/v) was added to the supernatant to 1% (v/v), and the mixture was kept on ice for 15 min with occasional inversion. After centrifugation at 17000*g*, the supernatant was diluted in buffer A to 2 mg mL^{-1} before being loaded at 10 mL min^{-1} onto a DEAE Sepharose FF column (5 × 5 cm) equilibrated in buffer A. After washing with buffer A, the protein was eluted with a linear gradient composed of 400 mL of buffer A and 400 mL of buffer A + 0.25 M KCl (buffer B). The HisH eluted at 250 mL into the gradient. Fractions of specific activity greater than 2 units mg^{-1} were pooled and diluted with 40 mM potassium phosphate, pH 7.0, to achieve a final phosphate concentration of 20 mM. This solution was passed through a Bio-Gel HTP column (2.5 × 15 cm) equilibrated in 20 mM potassium phosphate, pH 7.0, at 5 mL min^{-1}, followed by a wash with 400 mL of the same buffer. Protein that did not bind (450 mL) was diluted to 10% (v/v) glycerol and concentrated to 20 mL by ultrafiltration using a PM-10 membrane. Gel filtration was performed on 140-mg portions of protein using a Sephacryl S-100 column (1.5 × 69 cm) equilibrated in buffer A + 0.1 M KCl (buffer C) at 0.45 mL min^{-1}. A small amount of low specific activity HisH eluted as an aggregate in the void volume, and the protein-containing fractions with specific activity greater than 28 units mg^{-1} were pooled and stored at −80 °C in 50% glycerol (v/v). The volume of elution for HisH from the S-100 column was 81 mL.

HisF. Cell extracts and protein fractionation were identical to that for HisH except that 35 mM potassium phosphate, pH 7.0, was substituted for the Tris·HCl in the DEAE Sepharose chromatography. HisF eluted at 400 mL into the gradient, and fractions with a specific activity greater than 2 units mg^{-1} were pooled and concentrated as described above. The HisF was further purified in 80-mg portions on the same Sephacryl S-100 column using the conditions described above, and protein-containing fractions with a specific activity greater than 15 units mg^{-1} were pooled and stored at −80 °C in 50% (v/v) glycerol. The volume of elution from the S-100 column for the purified HisF protein was 61 mL.

Isolation of the Holoenzyme. Ten-milligram portions of the *hisH* and *hisF* gene products (0.46 μmol HisH, 0.35 μmol HisF), both in 50% glycerol (v/v), were mixed and diluted to 15 mL with buffer C. Glycerol was removed by ultrafiltration

in a Centriprep-10 at 900*g* at 5 °C. Fresh buffer C was added, and the process was repeated until glycerol was less than 20% (v/v). The final sample (≤5 mL) was kept on ice for 30 min before being loaded onto a Sephacryl S-100 column as described above. A single major protein peak that constituted all of the glutamine-dependent enzyme activity eluted at 54 mL and was stored at 4 °C. A second procedure utilized a Superose-12 column operating at 0.5 mL min^{-1} in buffer C. The individual subunits (40–50 μg each) or the holoenzyme (40 μg) was loaded onto this column and standardized in a manner similar to that for the S-100 chromatography. The following materials were run as molecular mass markers: blue dextran (>2 × 10^3 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), trypsinogen (24 kDa), and ribonuclease A (13.7 kDa).

Attempts To Isolate an Intermediate. Reactions were performed in 1-mL total volumes at 30 °C for 15 min in solutions containing 50 mM Tris·HCl, pH 8.0, 100 μM PRFAR, and either 0.17 nmol of HisF or 5 mM glutamine with 0.15 nmol of HisH. Protein was removed by ultrafiltration through a Centricon-10 at 3000*g* for 1 h at 4 °C. The filtrate was then incubated in the presence of the second protein as above. After each incubation, reactions were inspected by UV-vis spectroscopy to detect the presence of PRFAR or AICAR. The final step involved the addition of the original protein to verify the concentrations by assessing the absorbance at 300 nm.

Stability Studies. IGP synthase stock solution (100 μL, 1.7 mg) was dialyzed against 50 mL of 5 mM Tris·HCl, pH 7.6, at 4 °C for 2 h in a Spectra/Por MicroDialyzer using a 5000-MWCO Spectra/Por CE membrane with buffer continuously recycled through the apparatus. HisH (500 μL, 1.7 mg) and HisF (500 μL, 2.0 mg) samples in 50% glycerol were diluted with 1.5 mL of 5 mM Tris·HCl, pH 7.6, and concentrated in a Centricon-10 at 3000*g* at 4 °C. The process was repeated to reduce the glycerol content to ≤1% (v/v). All of the recovered samples were placed in 1.5-mL Eppendorf tubes and incubated at 27 °C for the extent of the study with aliquots taken for enzyme assay at specified time intervals.

Stoichiometry Analyses and Progress Curves. One-milliliter reaction mixtures containing 100 μM PRFAR and 5 mM glutamine in 50 mM Tris·HCl, pH 8.0, were incubated for 5 min at 30 °C before addition of 9.0 pmol of IGP synthase. This amount of enzyme converted all of the PRFAR to product in 30 min. Samples (80 μL) were collected for 1 h at 6-min intervals and processed for glutamate assays as described above. PRFAR consumption and the formation of products were quantified in the same reaction by the difference in absorbance values before and after the reaction. AICAR production was calculated using a value of $\Delta\epsilon_{270}$ = 4159 M^{-1} cm^{-1}, which was determined by subtracting the ϵ_{270} for PRFAR from the value for AICAR. IGP was quantified by using $\Delta\epsilon_{219}$ = 10 550 M^{-1} cm^{-1}, which was calculated by subtracting the contributions from AICAR and IGP. The time course of the reaction was monitored at 270 and 300 nm.

Steady-State Kinetic Analyses. Kinetic parameters for the IGP synthase were determined in the assay conditions described above using 9 pmol of enzyme in each experiment. PRFAR concentrations were varied from 2 to 25 μM while glutamine was held constant at 5 mM. Glutamine concentrations were varied from 50 to 500 μM, and NH_4Cl concentrations were varied from 40 to 400 mM, while PRFAR was kept at 100 μM. Thirty-five picomoles of HisF was used in experiments designed to determine the K_m of this enzyme for NH_4Cl. The Michaelis constants were calculated from fitting the data to $v = VA/[K_A + A]$ as executed by the program Enzyme Kinetics (Trinity Software).

RP-HPLC Analyses. HisH (0.46 nmol), HisF (0.35 nmol), and IGP synthase (0.40 nmol) samples were analyzed for purity by chromatography on a Rainin Microsorb-MV C4 column using a Beckman HPLC system. The column was equilibrated in 20% acetonitrile/0.1% trifluoroacetic acid (TFA), and the proteins were eluted with a linear gradient to 70% acetonitrile/0.1% (TFA) over a 20-min period at 1 mL min^{-1}. Under these conditions, HisH eluted at 14.5 min and HisF eluted at 15.4 min. The IGP synthase sample repeatedly eluted as the two subunits.

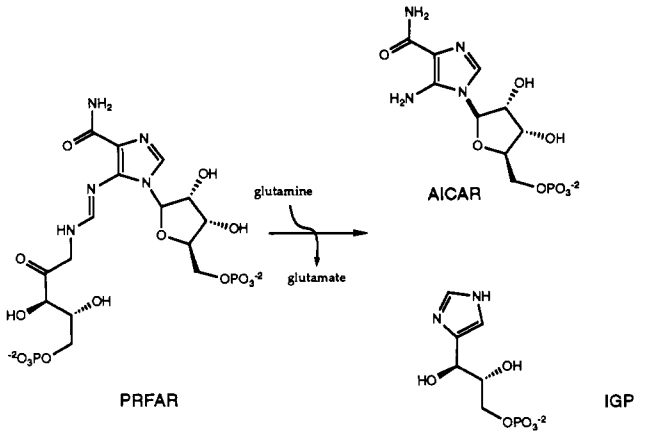
Cross-Linking Studies. The IGP synthase isolated from the S-100 gel filtration column was incubated with the heterobifunctional cross-linking reagent SPDP. For the SDS–PAGE analysis, 2 mM reagent was incubated with 20 μM protein in 50 mM Hepes, pH 8.0, at ambient temperature for 5 min (Carlsson et al., 1978). A series of incubations were conducted with varied concentrations of SPDP from 50 μM to 2 mM to establish the reagent and time dependence of the cross-linking reaction. Aliquots (4 μL) were taken at various time points and subjected to SDS–PAGE under reducing and nonreducing conditions.

Subunit Titrations. Under the first conditions, dilute solutions of the HisH (68 μg mL^{-1}) and HisF (120 μg mL^{-1}) proteins were freshly prepared, and the purified HisH subunit (175 pmol) was incubated with varied amounts of HisF (44–700 pmol) in a total volume of 500 μL of buffer A. The specific activity of the IGP synthase was measured after each mixture was allowed to stand for >30 min on ice and was calculated upon the basis of the amount of HisH in each assay. For the second case, undiluted HisF (10 μL, 1.2 nmol) was mixed with varied amounts of HisH (0.3–4.8 nmol) in a total volume of 40 μL of assay buffer, incubated for 30 min on ice, and diluted to 1 mL before a 10-μL sample was taken for an activity assay. The specific activity calculation was based upon the amount of HisF in each assay.

RESULTS

Cloning of Genes and Protein Purification. As a general approach for studies of the histidine biosynthetic enzymes, overexpression systems using a *tac* promoter vector have been developed for each of the target genes. A rapid and efficient approach was adopted that used primer-adapted PCR to modify the flanking noncoding regions of the desired DNA and incorporate unique restriction enzyme recognition sites. This allowed for optimal positioning of the start codon in the selected *E. coli* expression vector. The independently cloned genes were analyzed by DNA sequencing and found to corroborate those previously reported (Carlomagno et al., 1988). The proteins encoded by the *hisH* and *hisF* genes of *E. coli* have been overexpressed to levels > 10% of the soluble protein extract. An *E. coli* strain that is devoid of the histidine operon was used in these expression studies which facilitated our efforts to purify each protein upon the basis of specific catalytic properties. As illustrated in Figure 1, the enzyme-mediated conversion of the ketose nucleotide PRFAR can be continuously monitored at 300 nm and is based upon the original observations of Smith and Ames (1964). In Tables I and II are summarized data for each of the purification schemes developed for the *hisH* and *hisF* gene products. This method of analysis was used to establish that extracts of *E. coli* overexpressing the *hisH* and *hisF* genes were both required to convert PRFAR to AICAR in the presence of glutamine.

The preparation of the required substrate PRFAR was made possible with purified samples of 5′-ProFAR isomerase. This



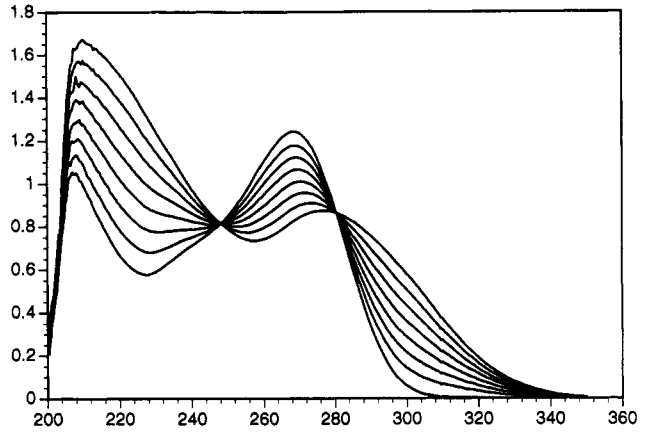


FIGURE 1: Time-dependent UV-vis spectra (bottom) of the HisH–HisF-mediated conversion of PRFAR to AICAR and IGP (top). The reaction conditions included 100 μM PRFAR, 5 mM glutamine, 50 mM Tris·HCl, pH 7.8, and 0.6 μg (0.005 unit) of IGP synthase. Scans were taken every 2 min and indicate decreases in absorbance at 300 and 210 nm and an increase at 270 nm.

Table I: Purification of *hisH* Gene Product

step	volume (mL)	protein (mg)	total units	units/mg	% yield
streptomycin	120	2736	7716	2.8	100
DEAE Sepharose	190	808	5951	7.4	77
BioGel HTP	450	545	5580	10	72
Sephacryl S-100	14	109	3740	34	48

enzyme has been found to catalyze an irreversible isomerization of the ribosyl form of the substrate to the ribulosyl nucleotide.[2] Reverse-phase HPLC conditions have been developed to achieve reproducible preparations of PRFAR that are of suitable purity; these preparations have been stable upon storage as the sodium salt in H_2O. Precautions were taken to eliminate the possible contamination of the substrate with ammonium ions because they were discovered to be a source for a low background turnover of PRFAR that was mediated by HisF.

Gel filtration studies were conducted to estimate the native molecular weight of the HisH and HisF proteins. Under the specified conditions, both proteins were found to elute in volumes that were not consistent with calculated monomeric or multimeric molecular weights. On Sephacryl S-100 and Superose 12, the respective observed molecular masses were 11.6 and 11.8 kDa for HisH and 31.6 and 33.9 kDa for HisF. The predicted monomeric molecular weights are 21 655 for HisH and 28 457 for HisF (Carlomagno et al., 1988). The purified proteins were subjected to amino acid sequence analyses to verify the N-terminal regions. In each case, the observed amino acid sequence for the first 20 residues matched that predicted from the DNA sequence. The only exceptions were for the cysteine residues at position 9 of HisF and position 10 of HisH, which were not characterized.

[2] This work is not published and will be the subject of a forthcoming manuscript by S. E. Hamilton and V. J. Davisson.

Table II: Purification of *hisF* Gene Product

step	volume (mL)	protein (mg)	total units	units/mg	% yield
streptomycin	135	2781	4922	1.8	100
DEAE Sepharose	40	632	3602	5.8	73
Sephacryl S-100	16	128	2482	19	50

Catalytic Properties of the hisF and hisH Gene Products. There were several attempts to characterize the catalytic properties of the two purified proteins. In the case of HisF, the protein catalyzes the conversion of PRFAR to AICAR and IGP in the presence of ammonium salts, but not in the presence of glutamine. Thiol reducing agents were not required for the full catalytic activity, and the pH optimum with ammonium was found in the range of 8.0–9.0. For HisH, no catalytic activities associated with PRFAR consumption could be detected. No glutaminase activity was detected for either protein in the presence or absence of the nucleotide substrate PRFAR.

In order to establish the biochemical role of each of these proteins, an experiment was conducted to test for the presence of a free intermediate that may be produced by an undetected enzymatic activity of HisH or HisF (Ye et al., 1990). These proteins were independently incubated with the nucleotide substrate PRFAR and glutamine before ultrafiltration. The protein-free filtrates were then treated with the second protein and analyzed for the conversion of PRFAR to AICAR and IGP. In both cases, there was no detectable formation of products. To analyze for the consumption of the nucleotide substrate, the original protein was added back to the filtrates, and quantitative conversion of the PRFAR was detected by the continuous UV assay. Therefore, both HisH and HisF are required for the glutamine-dependent conversion of PRFAR. As stated above, there is no glutaminase activity detected for either protein in the presence of the nucleotide substrate. In total, this is the first direct evidence that a freely diffusing, stable intermediate does not exist at the juncture of HisH and HisF in the histidine biosynthetic pathway from *E. coli*.

Isolation of a Dimeric Protein. The results from the purification of HisF and HisH, and the characterization of their enzymatic properties, indicated that noncovalent associations of these proteins are essential for catalytic activity. As a means of determining the nature of the protein–protein interactions, purified HisH and HisF were combined before analysis by gel filtration chromatography. A single major protein fraction eluted that contained the enzymatic activity and both the HisF and HisH proteins as detected by SDS–PAGE. In Figure 2 are shown the relative elution profiles of HisH, HisF, and the mixture from the gel filtration column. The observed elution volumes and the estimated molecular masses of the HisH and HisF proteins are distinct from the corresponding values for the HisH–HisF combination, indicating their ability to form a stable complex. The observed molecular mass of the complex (47.5 kDa) is consistent with the calculated molecular weight for a HisH–HisF dimer (50 112). To further investigate the ratio of monomeric units in the complex, a 2:1 molar mixture of HisH and HisF was

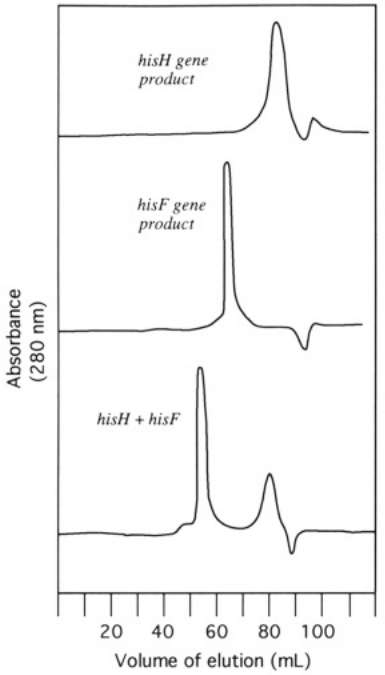


FIGURE 2: S-100 gel filtration chromatograms of the proteins HisH and HisF and the mixture of HisH and HisF. Observed masses: HisH, 11 kDa; HisF, 22.1 kDa; HisH–HisF mixture, 48 kDa.

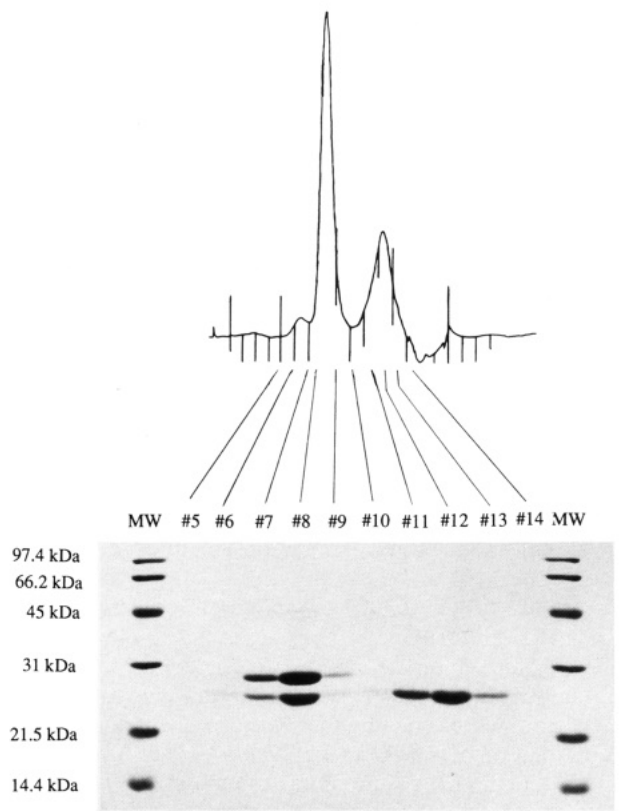


FIGURE 3: (Top) S-100 gel filtration chromatogram (absorbance at 280 nm) for a mixture of HisH (140 nmol) and HisF (70 nmol). (Bottom) Equal-volume aliquots of each fraction analyzed by 12% SDS–PAGE and stained with Coomassie blue after electrophoresis.

analyzed by gel filtration chromatography. All of the protein-containing peaks eluted from the column were analyzed for enzymatic activity and by SDS–PAGE (Figure 3), and the major peak was composed of both HisH and HisF. At a larger retention volume, the molar excess of HisH eluted in a presumed monomeric form which can participate in the catalytic turnover of PRFAR with glutamine and HisF.

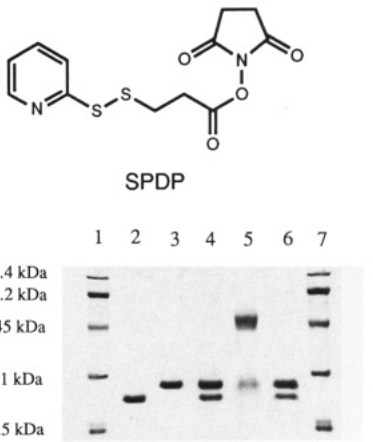


FIGURE 4: Structure of SPDP (top) and 12% SDS–PAGE of samples from the cross-linking of IGP synthase with SPDP (bottom). Lanes 1 and 7, molecular weight markers; lane 2, HisH; lane 3, HisF; lane 4, IGP synthase; lane 5, cross-linked IGP synthase not treated with reducing agent; lane 6, cross-linked IGP synthase treated with 2-mercaptoethanol. Equal amounts of protein were added to each of the lanes.

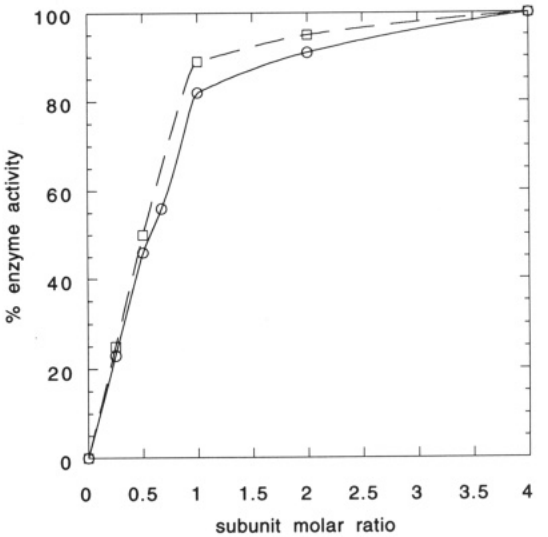


FIGURE 5: Effect of subunit stoichiometry on the specific activity of IGP synthase. Constant amounts of HisH (○) or HisF (□) were incubated on ice with varied molar amounts of the second subunit. Samples of each incubation were analyzed for IGP synthase activity under the standard assay conditions.

In order to further substantiate the native molecular weight of the IGP synthase, the isolated complex from the gel filtration study was subjected to chemical derivatization with SPDP. Under nonreducing conditions on SDS–PAGE, the complex is dissociated and the monomeric proteins appear as an apparent 1:1 mixture. After cross-linking, the derivatized protein migrates as a major band with a molecular mass of 52 kDa (Figure 4) which is consistent with a 1:1 complex of HisH and HisF. The degree of cross-linking was shown to be dependent upon the time of incubation and the concentration of the reagent (data not shown). Addition of a thiol reducing agent to the cross-linked sample before electrophoresis showed a complete dissociation of the complex and the appearance of the monomeric HisH and HisF.

The effect of the ratio of HisH and HisF upon the glutamine-dependent catalytic activity was studied by preincubating a constant amount of one subunit with varied amounts of the second subunit. As displayed in Figure 5, the maximal specific activity was achieved at a protein ratio consistent with a 1:1 complex of HisH and HisF. An increase (10–15%) in the

Table III: Steady-State Kinetic Properties of IGP Synthase and HisF

enzyme	substrate fixed	substrate varied	K_m[a]	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
IGP synthase	glutamine	PRFAR	1.5 μM	8.5	5.7×10^6
IGP synthase	PRFAR	glutamine	240 μM	9.1	3.8×10^4
IGP synthase	NH_4Cl	PRFAR	23 μM[b]	6.1	2.6×10^5
IGP synthase	PRFAR	NH_4Cl	291 mM	8.8	3×10^1
		(NH_3)[c]	(16 mM)		(5.5×10^2)
hisF subunit	NH_4Cl	PRFAR	21 μM[b]	5.7	2.7×10^5
hisF subunit	PRFAR	NH_4Cl	266 mM	8.6	3.2×10^1
		(NH_3)[c]	(15 mM)		(5.7×10^2)

[a] These values are apparent K_m constants at saturating concentrations of the second substrate. [b] Saturating concentrations of ammonium chloride could not be achieved due to inhibition of the enzyme activity at chloride ion concentrations >0.4 M. [c] The K_m for ammonia was calculated from the Henderson–Hasselbach equation.

specific activity was observed for higher ratios of the two proteins. This has been attributed to a small amount of monomeric proteins present in the dilute solutions that are trapped by the addition of excess subunit. A similar effect has been observed when the isolated IGP synthase was treated with additional amounts of either subunit (data not shown). However, the trend in the titration curve indicates that a substantial leveling effect on the specific activity occurs when the subunit molar ratio exceeds 1:1.

The consequences of this dimeric species on catalytic activity and physical stability were further investigated by direct comparison with the separate subunits. The complex was stable throughout the time course of this study with a loss of only 50% of its activity after incubation at 27 °C for 30 days. In contrast, the HisF and HisH proteins under similar conditions exhibited half-lives of less than 48 h (data not shown) and a decrease in their solubilities. In total, these analyses formulate the basis for the IGP synthase as a dimeric protein composed of a single subunit of HisH and HisF.

In comparison to many of the other *trpG* glutamine amidotransferases, the effects of added metals, reducing agents, and pH upon IGP synthase activity were inspected (Zalkin, 1985). The addition of divalent metal ions resulted in inhibition of the enzyme. In the presence of 10 mM $MgCl_2$ or 10 mM $MnCl_2$, the specific activity of the enzyme was reduced by 22% and 58%, respectively. Thiol reducing reagents had no effect upon the enzyme activity despite the putative role of the conserved cysteine residues in the *trpG* type glutamine amidotransferases (Chaparian & Evans, 1991). The pH optimum for the IGP synthase with glutamine was broad over the range of values from 6.0 to 8.0 but diminished to 85% at pH 8.5 and 22% at pH 9.5.

Steady-State Kinetics of IGP Synthase. The catalytic properties of the IGP synthase complex were also studied by comparing the steady-state kinetic constants with those for the HisF protein. A summary of these values is shown in Table III. IGP synthase exhibits a low K_m value (1.5 μM) for the nucleotide substrate PRFAR in the presence of saturating glutamine concentrations. Catalytic turnover in the presence of 0.4 M NH_4Cl reaches 80% of the value for the glutamine-dependent process. This NH_4Cl-dependent reaction appears to be completely independent of the HisH domain as reflected in the K_m and k_{cat}/K_m values for the HisF subunit and the IGP synthase complex. Unfortunately, an apparent K_m value for PRFAR with the HisF subunit could not be determined due to an apparent substrate inhibition caused by NH_4Cl concentrations greater than 0.4 M. This inhibition was also observed with similar concentrations of

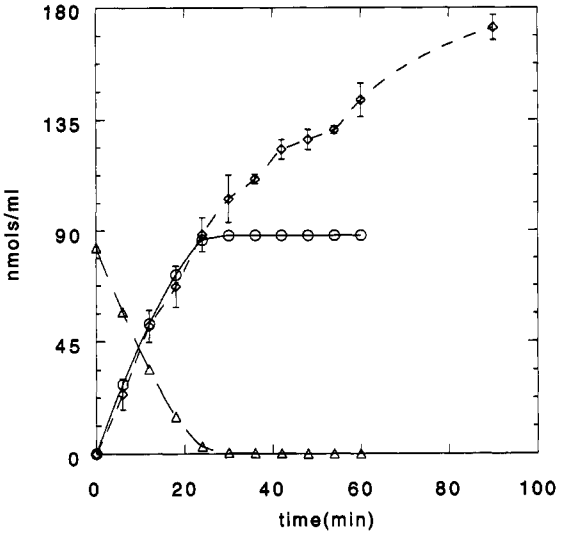


FIGURE 6: A time course of the reaction catalyzed by IGP synthase for the consumption of substrate PRFAR (△) and the production of AICAR (○) and glutamate (◇). The conditions and assays are described in the Materials and Methods section.

$(NH_4)_2SO_4$ and could be due to inhibitory concentrations of anionic species. However, it is clear from the high K_m value for NH_4Cl (NH_3) and the reduced values for k_{cat}/K_m that glutamine is the preferred substrate for the in vivo amination event.

Stoichiometry and Time Course of the IGP Synthase Reaction. In order to confirm the actual chemical turnover event catalyzed by the IGP synthase complex, the stoichiometry of the reaction was determined. This study relied on the differences in the absorbance spectra for the components of the substrate and the products. Consumption of 96 μmol of PRFAR was found to produce 101 μmol of AICAR and 87 μmol of IGP, demonstrating a 1:1:1 stoichiometric relationship in the glutamine-dependent reaction catalyzed by IGP synthase. Under the attempted experimental conditions, there was no apparent back reaction to form the substrate nucleotide. A time course for the consumption of PRFAR and glutamine was also performed that monitored the production of AICAR and glutamate. As shown in Figure 6, under conditions where nucleotide substrate was present from 0 to 30 min, a stoichiometric production of glutamate was observed. However, following the consumption of PRFAR, a continued hydrolysis of glutamine was detected over the next 1 h that was found to be enzyme mediated. This result prompted a study of the glutaminase property associated with the IGP synthase.

Glutaminase Activity of IGP Synthase. A catalytic property common to glutamine amidotransferases is a hydrolytic activity toward glutamine to form glutamate in the absence of additional substrate(s) (Zalkin, 1993). This glutaminase activity has been studied in some detail for other glutamine amidotransferases and used as a basis for mechanistic interpretations (Chaparian & Evans, 1991). Sequence homologies between the active sites of HisH and the *trpG* glutamine amidotransferases suggest a possible mechanistic link and provide further impetus for examining the glutaminase properties of IGP synthase. As shown in Table IV, the separate HisH and HisF domains do not exhibit any detectable glutaminase activity in the presence or absence of the nucleotide substrate. However, the IGP synthase does have a glutaminase property that is 0.8% of the turnover in the normal amidotransferase reaction and exhibits a K_m for glutamine that is 20-fold higher than for IGP synthesis. A

Table IV: Glutaminase Properties of HisF, HisH, and IGP Synthase

protein[a]	glutamine (mM)	modifier (concn)	K_m glutamine (mM)	k_{cat} (s^{-1})[b]
HisH	5			0
HisH	5	5′-ProFAR		0
HisF	5			0
HisF	5	5′-ProFAR		0
IGP synthase	varied		4.8	0.07
IGP synthase	varied	5′-ProFAR (2 mM)[c]	2.8	2.6
IGP synthase	varied	IGP (9 mM)	1.9	2.7
IGP synthase	5			0.034
IGP synthase	5	AICAR (0.5 mM)		0.034

[a] All reactions with the subunits were performed with up to 2 μM protein. [b] Under the conditions of these assays, the lower limit of detection for the K_{act} analysis is 2×10^{-4} s^{-1}. [c] The K_{act} for 5′-ProFAR was 0.43 mM; for IGP, 2.4 mM.

second feature common to amidotransferases is a glutaminase activity stimulated by substrate (Zalkin, 1993). For IGP synthase, the products were tested for their ability to modulate the glutaminase property. While the inclusion of AICAR had no effect on the glutaminase activity, IGP addition resulted in a 39-fold increase in the catalytic turnover and a 200-fold increase in the k_{cat}/K_m value. There is no synergistic effect from including both products in the glutaminase assay (data not shown). A second effector was discovered to be 5′-ProFAR, a nucleotide biosynthetic precursor to the normal substrate. These stimulatory effects were saturable and the K_{act} values estimated for IGP, 2.4 mM, and 5′-ProFAR, 0.43 mM, represent the concentrations that achieve 50% of V_{max}. There was no detectable turnover of the 5′-ProFAR during the course of the glutaminase assay, judged by HPLC analysis of the incubation mixtures.

DISCUSSION

Although the original characterization of histidine biosynthesis in *Salmonella typhimurium* established the order of the genes in the pathway, an ambiguity remained concerning the biochemical events leading to the production of AICAR and IGP. Two biosynthetic genes were known to be involved in the overall process, but the catalytic activities of each protein had not been established. One possibility included the existence of an intermediate that had eluded identification as originally suggested (Martin et al., 1971). To resolve this uncertainty, the constituent proteins have been isolated and their enzymatic properties have been characterized. A general system was developed for overexpression of the *E. coli* HisH and HisF proteins, and a similar strategy has been extended to other histidine biosynthetic genes. This recombinant DNA approach also assisted in the preparation of the required substrates for in vitro characterization of the catalytic properties of each protein.

The protein encoded by the *hisF* gene had previously been described as a cyclase with the capacity to catalyze conversion of the nucleotide substrate PRFAR to AICAR and IGP only in the presence of high ammonia concentrations at pH 8.5 (Smith & Ames, 1964). The purified protein from *E. coli* described above was also found to have an ammonia-dependent activity under similar conditions. An original proposed sequence of chemical events was first a nonenzymatic amination of the nucleotide substrate PRFAR in the presence of high ammonium salt to provide a substrate for the HisF-catalyzed cyclization process (Martin et al., 1971). The rate of the ammonium chloride dependent reaction of HisF appears to have a pH optimum at 8.5, in contrast to the holoenzyme, which has a broad pH optimum between 6 and 8 with glutamine. A consistent interpretation of these results is that the HisF-mediated process is actually dependent upon ammonia concentrations which increase at the elevated pH levels. In the case of the CarA subunit of carbamyl phosphate synthetase, a similar argument has been applied (Rubino et al., 1986). The HisF subunit exhibits a low degree of catalytic efficiency with ammonia as reflected in the high k_{cat}/K_m value with ammonium chloride. All the current evidence shows that HisF catalyzes a multistep process presumably within one active site of the monomeric protein. These activities include an ammonia-dependent carbon–nitrogen ligase, a carbon–nitrogen lyase, and a carbon–nitrogen cycloligase; the sequence of these events has yet to be defined and must await future studies.

Glutamine is required for the biosynthesis of IGP, and the role of the *hisH* gene product had been ascribed to that of a glutamine amidotransferase (Martin et al., 1971). Further evidence for this role was derived from recent investigations that led to a deduced amino acid sequence for HisH (Carlomagno et al., 1988). A high degree of sequence homology exists in a region of HisH that had been identified as part of the catalytic site in *trpG* type glutamine amidotransferases (Zalkin et al., 1985; Schendel et al., 1989; Zalkin, 1993). Our initial studies with the HisH protein were designed to test for a free intermediate resulting from the amination of PRFAR which could serve as a substrate for HisF. We have not been able to detect any conversion of PRFAR or glutamine in the absence of HisF and conclude that no additional free intermediates exist in the histidine biosynthetic pathway. Therefore, the pathway in *E. coli* and *S. typhimurium* consists of eight distinct biosynthetic genes and nine intermediates, all of which have been described.

A striking feature of the HisH protein has been the absence of any catalytic activity in the glutaminase assay. This hydrolase property is retained by many of the *trpG* and *purF* type glutamine amidotransferases, even for those which are heteromultimeric proteins like the HisH–HisF complex. For example, the isolated glutamine amidotransferase subunits of anthranilate synthase from *Pseudomonas putida* and carbamyl phosphate synthetase both exhibit glutaminase activity in the absence of their respective substrates (Trotta et al., 1974; Goto et al., 1976). The exceptions to date have been the anthranilate synthase II subunit from *S. typhimurium* (Bauerle et al., 1987) and the recently described *E. coli* PabA subunit involved in *p*-aminobenzoate synthesis (Roux & Walsh, 1992). Despite the high degree of active site sequence homology with these other amidotransferase domains, the glutamine-dependent catalytic properties of HisH require the presence of HisF. The interdependence of two gene products for catalysis suggested a physical association of the two proteins. Also, insoluble forms of both proteins were encountered during their purifications, and the catalytic activities exhibited minimal thermal stability. Obtained by using a variety of physical and kinetic methods, our results with the reconstituted HisH–HisF complex establish that this protein is a stable, catalytically complete enzyme involved in the central step of histidine biosynthesis. The dimeric protein is composed of one subunit each of HisF and HisH and is proposed to be the form of IGP synthase found in vivo.

As a heteromultimeric glutamine amidotransferase, IGP synthase has a corollary with the related enzymes anthranilate synthase (Smith & Bauerle, 1969; Goto et al., 1976) and carbamyl phosphate synthetase (Trotta et al., 1974). There

is also evidence favoring a proposed heteromultimeric structure for the enzyme aminodeoxychorismate synthase which is composed of the proteins encoded by *pabA* and *pabB*, even though they do dissociate under conditions of the isolation (Ye et al., 1990; Green & Nichols, 1991). Like HisH, the isolated amidotransferase domain PabA has no glutamine-dependent catalytic activity but in the presence of PabB forms an apparent 1:1 complex under the assay conditions. These multimeric proteins are consistent with the general domain structure of the *trpG* glutamine amidotransferase family of enzymes encoded by a single gene in which the transferase domain is distinct from synthase (or synthetase) domain (Zalkin et al., 1985). Despite the divergence in the primary structure beyond the active site region, IGP synthase constitutes yet another example of the recruitment of a glutamine amidotransferase domain to serve in ammonia transfer from glutamine via a probable conserved catalytic mechanism. Additional functional significance of the amino acid conservation in the active site region of HisH will have to await further investigation.

There are several lines of evidence for critical subunit interactions in the IGP synthase holoenzyme that are responsible for an active glutamine amidotransferase. As an isolated subunit, HisH has no detectable catalytic properties. In the holoenzyme, there is a measurable glutaminase activity that has a low turnover in the absence of the nucleotide substrate. An enhancement of the glutaminase activity can be elicted by inclusion of the product IGP or the biosynthetic precursor to the substrate 5′-ProFAR. Although 5′-ProFAR binds to the synthase with an apparent reduced affinity, the stimulation of the glutaminase activity has been interpreted as a specific interaction on the HisF domain in a manner that mimics the substrate PRFAR. Noteworthy is the fact that the HisF bears a significant degree of amino acid sequence identity with the 5′-ProFAR isomerase encoded by the *hisA* gene. Also, a potential substrate recognition region in HisA and HisF has been proposed upon the basis of a common signature sequence in both of these proteins (Sheridan & Venkataraghavan, 1992). Modulation of the glutaminase activity by IGP is most significant with respect to the subunit interactions and suggests that the binding site on HisF is shared or in close proximity to the glutamine site on HisH, while the AICAR site is distinct. Alternatively, substrate or product binding on the HisF domain could influence the catalytic properties of HisH by inducing a protein conformational change. This study has established that, under turnover conditions with normal substrates, a 1:1 stoichiometric relationship exists for the consumption of glutamine and the formation of products. Therefore, no uncoupling of the glutaminase activity and the amination process could be detected when PRFAR was present. As might be expected, a specific type of structural environment would be necessary for coupling of the amidotransfer event and to insure against a wasteful expenditure of glutamine.

The results described here provide the first biochemical evidence for an essential role of the *hisH* gene in eubacteria. A similar conclusion has been derived from a series of genetic studies on frameshift mutations in the *hisH* gene (Pons et al., 1988). From our in vitro studies, glutamine is clearly the preferred nitrogen donor for IGP biosynthesis as it is some 10^3-fold more efficient as a substrate than ammonium ion at pH 8.0. However, the significance of the ammonia-dependent activity is not obvious from our studies. The k_{cat}/K_m values for ammonia and nucleotide are essentially identical for the synthase and the HisF subunit, indicating that HisH has no effect upon this process. Evidence for actual binding of ammonia to the enzyme has not been established, but the steady-state kinetics suggest a weak association at best. The implication is that the ammonia-dependent activity is a consequence of the mechanism of the amidotransfer reaction and not a process evolved for efficiency of metabolism. It does not, however, rule out the possibility that this function may have been derived from an ancestral origin that required the use of ammonia as a substrate. Strains that are deficient in HisH might be complemented only by exogenous (non-physiological) levels of ammonia or perhaps by overexpression of the HisF protein in analogy to the case with the *carB* gene (Rubino et al., 1987). All the current genetic evidence indicates that differential expression of the two proteins does not occur. Therefore, the physiological relevance of this activity is questionable on the basis of these in vitro results and cannot explain the leakiness often found for genetic mutations in the *hisH* gene (Johnston & Roth, 1979; Pons et al., 1988). In total, the combination of genetic and biochemical information points to the fact that inhibition of the IGP synthase should have a profound impact on purine metabolism.

CONCLUSION

The IGP synthase has multifunctional catalytic properties essential for the formation of the imidazole heterocycle of histidine. In *E. coli*, this enzyme is composed of two different proteins, each with unique functional properties. Glutamine serves as a nitrogen donor, as indicated by the stoichiometric relationship for the conversion of PRFAR and glutamine to form IGP, AICAR, and glutamate. The biochemical properties of the enzyme are consistent with an evolutionary relationship with the *trpG* type glutamine amidotransferases.

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