The Human purH Gene Product, 5-Aminomidazole-4-carboxamide Ribonucleotide Formyltransferase/IMP Cyclohydrolase

CLONING, SEQUENCING, EXPRESSION, PURIFICATION, KINETIC ANALYSIS, AND DOMAIN MAPPING*

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We report here the cloning and sequencing of the cDNA, purification, steady state kinetic analysis, and truncation mapping studies of the human 5-aminomidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (AICARFT/IMPChase). These enzyme activities catalyze the penultimate and the final steps of de novo purine biosynthesis, respectively. In all species of both prokaryotes and eukaryotes studied, these two activities are present on a single bifunctional polypeptide encoded on the purH gene. The human purH cDNA is 1776 base pairs in length encoding for a 591-amino acid polypeptide (M₅ = 64,425). The human and avian purH cDNAs are 75 and 81% similar on the nucleotide and amino acid sequence level, respectively. The Kₘ values for AICAR and (6R,6S)-10-formyltetrahydrofolate are 16.8 μM ± 1.5 and 60.2 μM ± 5.0, respectively, for the cloned, purified human enzyme. A 10-amino acid sequence within the COOH-terminal portion of human AICARFT/IMPChase has some degree of homology to a previously noted "folate binding site." Site-directed mutagenesis studies indicate that this sequence plays no role in enzymatic activity. We have constructed truncation mutants which demonstrate that each of the two enzyme activities can be expressed independent of the other. IMPChase and AICARFT activities are located within the NH₂-terminal 223 and COOH-terminal 406 amino acids, respectively. The truncation mutant possessing AICARFT activity displays steady state kinetic parameters identical to those of the holoenzyme.

Aminimidazole ribonucleotide formyltransferase (AICARFT) catalyzes the penultimate step of the de novo purine synthetic pathway (Fig. 1). Along with another enzymatic activity earlier in the pathway, glycinamide ribonucleotide formyltransferase (GARFT), it requires a reduced folate cofactor, 10-formyltetrahydrofolate. Interest in AICARFT stems in part from its potential as a chemotherapeutic target. Inhibitors of GARFT are currently in clinical trials as anti-neoplastic agents. Also, AICARFT inhibition is thought to be the origin of the anti-purine effects of anti-folates such as methotrexate whose primary target is dihydrofolate reductase.

In all organisms studied to date, AICARFT activity is accompanied by inosine monophosphate cyclohydrolase (IMPChase, also known as inosinicase) located on the same polypeptide encoded by the purH gene. IMPChase is the final step in the purine de novo pathway. The activities of the purine pathway in eukaryotes are frequently found on multifunctional proteins, whereas in bacteria, the enzymes are typically monofunctional proteins. Thus, AICARFT/IMPChase is an exception in being bifunctional throughout evolution. A question which arises is whether there is some particular advantage which favors the bifunctional arrangement. For example, there might be kinetic "channeling" of the intermediate, formamidimidazole ribonucleotide (FAICAR), between the penultimate and final catalytic centers. It is also possible that the enzyme could have a single binding site for the nearly complete purine ribonucleotide, which would be sequentially operated upon by two catalytic sites. Alternatively, there may be two functionally independent domains, similar to the situation with the other multifunctional proteins of the de novo purine pathway as it exists in eukaryotes.

AICARFT/IMPChase from Bacillus subtilis (1), Salmonella typhimurium (2), Escherichia coli (3), and from chicken (4) have been cloned and expressed. Since there are no monofunctional proteins for sequence comparison, nothing is known about potential domain structure for AICARFT/IMPChase from any species. Although GARFT and AICARFT/IMPChase utilize the same cofactor and carry out very similar reactions, there has been no recognized sequence homology between them. This report describes the cloning, sequence, expression, and purification to homogeneity of the human AICARFT/IMPChase. Comparison of the human sequence with those of other species revealed a high degree of sequence conservation and homology. In addition, an amino acid sequence can be located which corresponds to a "folate binding consensus sequence" (5) previously noted in other 10-formyltetrahydrofolate binding enzymes such as GARFT. However, in contrast to other such sequences, this does not help to locate the catalytically active amino acid residues in AICARFT/IMPChase and appears to be simply a common structural element. Truncation mutant studies identify a COOH-terminal fragment which possesses no IMPChase activity but does possess AICARFT activity kinetically equivalent to the holoenzyme. An NH₂-terminal fragment has also been identified which has IMPChase activity but lacks AICARFT activity. These results suggest that AICARFT and IMPChase activities exist as independent domains.
EXPERIMENTAL PROCEDURES

Cloning of the Human AICARFT/IMPChase cDNA—The human AICARFT/IMPChase (purH) cDNA was obtained from a human hepatoma / ZapI cDNA library (Statagene, Inc.) utilizing described polymerase chain reaction (PCR) methods with some modifications (6). Some partial 3’ nucleotide sequence information for the human purH cDNA had previously been obtained in the laboratories of Howard Zalkin and Jack Dixon,2 who very kindly provided it to us. Based on this information, we designed human purH-specific oligonucleotide primers to isolate a 3’ human purH cDNA fragment. Subsequently, PCR methods were used to produce fragments containing the 5’ end (7). The full-length human purH cDNA was assembled in the expression vector pET-14b (Novagen, Inc.) as shown in Fig. 2. The resulting recombinant was termed pETHATB-1800. A second full-length recombinant was constructed in the His-tag™ vector pET-23d (Novagen, Inc.), pETHAT-FLNN-1. The nucleotide sequence for each of the full-length clones was verified (8).

Expression of Human AICARFT/IMPChase—The full-length human AICAR formyltransferase/IMP cyclohydrolase, point mutants, and truncation mutants were expressed and prepared from Escherichia coli BL21(DE3) (Novagen, Inc.) transformed with recombinant expression plasmids. E. coli transformants were grown at 30 °C to an A600 of 1.0. Isopropyl-?D-thiogalactopyranoside (Sigma) was added to a final concentration of 0.1 mM and incubation of the culture was continued for an additional 3 h at 30 °C. The cells were subsequently harvested via centrifugation at 3500 × g for 20 min at 4 °C. Cell pellets were washed in ice-cold 0.85% NaCl and again pelleted via centrifugation. Pellets were either frozen at −70 °C or used immediately.

Purification of the Full-length Human AICAR Formyltransferase/IMP Cyclohydrolase—Purification of PurH was carried out at 4 °C. Fresh or frozen cell pellets (approximately 12 g wet weight) were resuspended in 20 ml of ice-cold Buffer HB (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 50 mM KCl, 20% glycerol). Lysosome enzyme was added to a final concentration of 10 mg/ml, and the suspension was incubated on ice for 20 min. Cells were subsequently sonicated on ice at 35% power for a total of 5 min using a Fisher Scientific Sonic Dismembrator model 300.

Cell lysates were clarified by centrifugation at 4 °C, 39,000 × g for 40 min in a Beckman T70 rotor. The clarified lysate was subsequently applied to a reactive red 120-agarose (Sigma) column (1.5 × 22.0 cm) equilibrated in Buffer HB. Buffer HB was then washed with 1 column volumes Buffer HB. Human AICARFT/IMPChase was eluted using 20 mM Tris-Cl, pH 7.5, 500 mM NaCl, 50 mM KCl, 20% glycerol. Fractions were assayed for either AICAR formyltransferase or IMP cyclohydrolase activity. Peak activity fractions were pooled and were diluted with an equal volume of ice-cold Buffer HB. The enzyme was subsequently concentrated using an Amicon ultrafiltration cell (250 ml) with a Diaflo YM30 ultrafiltration membrane (Amicon, Inc.). A buffer exchange into Buffer HB was then performed, and the enzyme was concentrated to a final volume of 10–15 ml. In order to further purify human AICARFT/IMPChase, the enzyme was desalted using a G25 coarse Sepharose (Pharmacia Biotech Inc.) column (1.5 × 22.0 cm) equilibrated in Buffer B (20 mM Tris-Cl, pH 7.5, 20% glycerol). AICARFT/IMPChase was immediately applied to an AICAR-Sepharose column (1.5 × 5.5 cm) equilibrated in Buffer B. AICAR-Sepharose was prepared according to the method of Smith et al. (9) using commercially available CNBr-activated Sepharose 4B (Pharmacia). The enzyme was eluted with 10 mM AICAR, 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 50 mM KCl, 20% glycerol. Fractions possessing IMPChase activity were pooled and concentrated using Centricon 30 concentrators. A buffer exchange was performed into Buffer B. Typically, the enzyme was passed over the AICAR-Sepharose column a second time in order to further purify the AICARFT/IMPChase. This second AICAR-Sepharose column elution was also followed by Centricron 30 enzyme concentration with a buffer exchange into Buffer HB. In order to determine if all AICAR had been removed, AICAR activity assays were performed in the presence and absence of additional AICAR.

Purified protein and protein fractions were resolved throughout the purification by discontinuous SDS-polyacrylamide gel electrophoresis as described by Laemmli (10). Typical running and stacking gels used were 12% (w/v) and 4% (w/v), respectively. An SDS reducing sample buffer (62.5 mM Tris-Cl, pH 6.8, 10% glycerol, 2% SDS, 0.71 mM β-mercaptoethanol, 0.0013 (w/v) bromphenol blue) was added to protein samples prior to electrophoresis. Samples were subsequently heated to 90 °C for 5 min, loaded onto the SDS-polyacrylamide gel, and electrophoresed. Gels were stained with Coomassie Blue R250.

Mass Spectroscopy of Purified Human AICARFT/IMPChase—Mass spectroscopy was performed at Yale University facilities. Samples were analyzed on a VG Quattro (Fisons Instruments, VG BioTech, Altrincham, Cheshire, UK) triple quadrupole mass spectrophotometer equipped with an electrospray ion source (Analytica of Branford, Branford, CT). Data were collected and processed (smoothed, centered, and transformed) using the VG Mass Lynx software. Samples were dissolved in l-propanol and water (1:1 v/v) plus 0.1% formic acid and were subsequently injected into the electrospray ion source using a Rhodyne valve (model 7125) at a flow rate of 5 μl/min. Nitrogen was used as a drying gas as well as to form the spray. The peak width at full width half-maximum for a singly charged ion was approximately 2 atomic mass units. Several scans of 10-s duration were recorded in the multiple computer averaging mode.

FPLC Superdex 75 Gel Filtration Analysis of Human AICARFT/IMPChase—Individual experiments were conducted using human PurH purified from three independent preparations. Purified human AICARFT/IMPChase was applied to an FPLC Superdex 75 column (Pharmacia) equilibrated in Buffer HB (described previously) and also in Buffer HB containing either 150 μM 10-formyl-FH4, 100 μM AICAR or both substrates at 4 °C. When FPLC experiments were conducted in the presence of the substrates, the enzyme samples were preincubated with the substrate(s) for 30 min prior to loading the column. The column was run at a flow rate of 0.4 ml/min collecting 0.25-ml fractions. Fractions were subsequently assayed for either AICARFT or inosinicase activities. These experiments were conducted with each of the three human PurH preparations.

Molecular mass standard curves for the Superdex 75 column were calibrated using the elution profiles of the standard molecular mass markers (Pharmacia): albumin (67.0 kDa), ovalbumin (43.0 kDa), chymotrypsin (25.0 kDa), and ribonuclease A (13.7 kDa) monitored by ultraviolet absorbance at λ = 280 nm. The column void volume was determined by the elution of dextran blue (2,000 kDa) from the column.

Enzyme Assays—Enzyme activity assays were carried out at room temperature. All assays were dependent on protein concentration and were initially linear with time. Protein concentration was determined by the Bradford protein assay (11). Required dye reagents were purchased from Bio-Rad. All assays were monitored using a Perkin-Elmer Lambda2 spectrophotometer employing the use of Perkin-Elmer PECS5 computerized spectroscopy software.

2 H. Zalkin and J. E. Dixon, personal communication.

3 Details of the cloning and assembly of the human purH cDNA may be obtained from the authors upon request.
extinction coefficient $1.53 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at A$_{260}$ nm at pH 13 (14).

Reaction mixtures (500 µl = final reaction volume) contained a final concentration of 33 mM Tris-Cl, pH 7.4, 25 mM KCl, 5 mM 2-mercaptoethanol, 0.1 mM (6R,6S)-10-formyltetrahydrofolate and 0.05 mM AICAR as described by Mueller and Benkovic (15). The reaction mixture was mixed prior to the addition of the human AICARFT/IMPChase enzyme and subsequently mixed again. AICARFT activity was monitored by the formation of FH$_4$, A$_{248}$ nm, for 4 min according to the method of Black et al. (16) employing the use of a Perkin-Elmer Lambda2 spectrophotometer. The concentration of FH$_4$ formed was calculated by the difference in absorbance values between the extinction coefficients for 10-formyl-FH$_4$ and FH$_4$, 1.77 $\times 10^4$ M$^{-1}$ cm$^{-1}$ (16).

IMP Cyclohydrolase Activity—FAICAR was synthesized according to previously published procedures (15, 17). The concentration of FAICAR was determined using the extinction coefficient for FAICAR at A$_{260}$ nm and pH 7.4 (6.59 $\times 10^3$ M$^{-1}$ cm$^{-1}$) (15). IMP cyclohydrolase enzyme activity consisted of 100 µM Tris-Cl, pH 7.4, and 0.1 mM FAICAR. Following mixing within the cuvette, the enzyme was added to a final reaction volume of 500 µl. IMP cyclohydrolase activity was monitored by the appearance of IMP at A$_{248}$ nm for 4 min. The final concentration of IMP formed was determined by using the calculated value for the differences between the two extinction coefficients for IMP and FAICAR at A$_{260}$ nm and pH 7.4, 5.71 $\times 10^3$ M$^{-1}$ cm$^{-1}$ (15, 18).

Steady State Kinetic Analysis—The standard AICARFT assay was used for determining the steady state kinetic parameters for both the full-length human enzyme as well as the AICARFT activity-possessing truncation mutant. Experiments were performed varying concentrations of both AICARFT as well as (6S)-10-formyl-FH$_4$. The data were fitted to the Michaelis-Menton equation using the EnzFitter program (Elsevier Biosoft) to calculate $K_m$ and $V_{max}$ values and their standard errors.

Site-directed Mutagenesis—Site-directed mutagenesis was performed according to PCR methods described by Higuchi et al. (19) using plasmid pETHATFLNN-1 as the mutagenic substrate. Mutagenic primers (sense and antisense, respectively) used for generating each point mutation include: H469A, 5'-CTTAGACAGCTCCACAAGTGCTTTCG (H and I restriction sites, indicated in parenthesis, were underlined). The cellular extract was prepared from isopropyl-b-D-thiogalactopyranoside-induced transformants as described previously. The extract was loaded onto an AICAR-Sepharose column (1 x 4 cm) equilibrated in Buffer B (described previously) and subsequently washed with 5 column volumes of Buffer B. The truncation mutant expressed from pETHATNB-1200 was eluted with 10 mM AICAR, 20 mM Tris-Cl, pH 7.4, and subsequently mixed again. AICARFT activity was monitored by the appearance of IMP at A$_{248}$ nm for 4 min. The concentration of IMP formed was determined using the calculated extinction coefficient for FAICAR at A$_{260}$ nm and pH 7.4, 6.59 $\times 10^3$ M$^{-1}$ cm$^{-1}$ (15, 18).

Full length human purH coding region

FIG. 2. Human AICARFT/IMPChase cDNA clone. PCR was used to assemble the full-length 1776-bp human purH cDNA. Three overlapping partial human purH cDNA clones were obtained as presented under “Experimental Procedures.” A, 5’ purH cDNAs pHATB4 and pHAT4A were joined by PCR resulting in a 705-bp human 5’ purH cDNA fragment. B, the 705-bp cDNA fragment was used in a subsequent PCR reaction with the 3’ 1142-bp cDNA fragment. Unique Ncol and BamHI restriction sites (in brackets) were introduced via oligonucleotide design. A conservative amino acid replacement, S2A, was introduced for cloning purposes.

1800 and were directionally subcloned and expressed in pET (Novagen, Inc.) vectors in order to identify possible functional activity domains. Three truncation mutants were generated: two amino-terminal truncation mutants expressing the first 223 and 230 amino acid residues of human purH, respectively, and a carboxy-terminal truncation mutant expressing the last 406 amino acid residues.

The first NH$_2$-terminal AICARFT/IMPChase cDNA truncation mutant, pETHAT-IMP, was constructed by PCR amplification of the first 669 nucleotides using the upstream and downstream primers 5’-CG-G(CATGG)CTCTCTTACGCCGTTTAG and 5’-GAGAT(GCG-GCCG)GGGTGAGTGGTACAGG, respectively (unique Ncol and NotI restriction endonuclease sites are in parentheses and the start codon is underlined). A conservative amino acid change S2A, indicated in bold, was introduced to facilitate cloning and expression of human AICARFT/IMPChase. The PCR product was resolved on a 1.2% agarose gel, gel-purified, and cloned into pCR™II (Invitrogen). The resulting plasmid, pTAIMP, was digested with Ncol and NotI, and the 669-bp 3’ purH fragment was subsequently directionally subcloned into the Ncol and NotI polylinker sites of expression vector pET-23d creating plasmid pETHAT-IMP.

A second truncation mutant expressing the amino-terminal 230 amino acid residues was constructed. However, rather than cloning into the His-tag™ vector, pET-23d, we used vector pET-14b. Unique Ncol and BamHI restriction sites (designated in parentheses) were introduced into the 5’ and 3’ ends via oligonucleotide design. Truncation mutant pETHUIMP-230 was constructed using the upstream primer 5’-CGG(CATGG)CTCTCTTACGCCGTTTAG (described previously) and the downstream primer 5’-CCCAAGATCCAGCTTATGTTGATGGGGAGAG. Again, start and stop (antisense) codons are underlined.

The carboxy-terminal truncation mutant, pETHATNB-1200, was generated using a similar strategy. The upstream and downstream primer combinations used include: 5’-GGA(CATGG)GAAATTTAGATGATGTTGGGAACG (unique Ncol and BamHI restriction sites, indicated in parenthesis, were introduced into the 5’ and 3’ ends via oligonucleotide design. Start and stop (antisense orientation) codons are underlined. This recombinant was similarly constructed in vector pET-14b.

Partial Purification of Carboxy-terminal Truncation Mutant Possessing AICARFT Activity—Plasmid DNA encoding PurH truncation mutant, pETHATNB-1200, was transformed into E. coli BL21(DE3). The cell lysate was prepared from isopropyl-β-D-thiogalactopyranoside-induced transformants as described previously. The extract was loaded onto an AICAR-Sepharose column (1 x 4 cm) equilibrated in Buffer B (described previously) and subsequently washed with 5 column volumes of Buffer B. The truncation mutant expressed from pETHATNB-1200 was eluted with 10 mM AICAR, 20 mM Tris-Cl, pH 7.4, and subsequently mixed again. AICARFT activity was monitored by the appearance of IMP at A$_{248}$ nm for 4 min. The concentration of IMP formed was determined using the calculated extinction coefficient for FAICAR at A$_{260}$ nm and pH 7.4, 6.59 $\times 10^3$ M$^{-1}$ cm$^{-1}$ (15, 18).
7.5, 150 mM NaCl, 50 mM KCl collecting 0.5-ml fractions. Fractions were analyzed by gel banding as well as AICARFT enzyme activity assays. Fractions determined to contain the truncation protein were subsequently pooled, concentrated using Centricon-10 filters (Amicon), and exchanged into Buffer HB.

RESULTS

Cloning and Sequence Analysis of the Human AICARFT/IMPCHase cDNA—The human AICARFT/IMPCHase cDNA clone, pETHATNB-1800, was obtained as described under "Experimental Procedures" and is shown in Fig. 2. The human purH cDNA nucleotide sequence has been submitted to GenBank™ with accession number U37436. The coding region of the human cDNA clone is 1776 nucleotides in length encoding for 591 amino acid residues (Mr, 564,425) and is presented in Fig. 3. Fig. 4 represents an amino acid sequence alignment of all reported full-length PurH sequences.

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**Fig. 3.** Nucleotide sequence of human AICARFT/IMPCHase cDNA and derived amino acid sequence of the protein. The nucleotide sequence is numbered from the initiator codon in the cDNA.
Enzyme Purification and Kinetic Analysis—Human AICAR Formyltransferase/IMP Cyclohydrolase

The human AICARFT/IMPCHase (Mr = 64,425) was purified essentially to homogeneity as described under "Experimental Procedures." The human AICARFT/IMPCHase was expressed at high levels using the pET-expression system and was purified over a series of four column steps. A typical purification is summarized in Table I. The initial reactive red 120-agarose column step typically resulted in a 2.5–6.2-fold purification. An apparent increase in total enzyme activity was consistently seen following the Amicon concentration step. Human AICAR Formyltransferase/IMP Cyclohydrolase

FIG. 4. Comparison of the amino acid sequences of eukaryotic and prokaryotic AICARFT/IMPCHase proteins. The aligned sequences are human (HU), avian (AV), E. coli (EC), S. typhimurium (ST), and B. subtilis (BS). The alignment was made using the PILEUP program (Genetic Computer Group, Madison, WI). Darkly shaded regions represent amino acid residues that are strictly conserved. Lighter shaded areas represent those residues having a single base change on the nucleic acid level and are, therefore, noted as representing "mutationally" conservative amino acid changes.
demonstrates higher activity in buffers containing 150 mM NaCl or in buffers containing 50 mM KCl perhaps as a result of stabilizing the enzyme conformation. The ionic strength of the enzyme buffer appears to have a greater effect on inosinicase activity. Removal of salt from the enzyme sample using a G25 coarse resin results in a 39 and 67% loss of AICARFT and IMPCHase specific activity, respectively. However, removal of salt is required for binding of the enzyme to the AICAR-Sepharose column. Two successive cycles of elution from an AICAR-Sepharose were required to further purify the human AICARFT/IMPCHase to near homogeneity (Fig. 5). Since it has been well established that both enzyme activities reside on a single polypeptide, we typically followed inosinicase activity throughout the purification. A typical purification of the AICARFT/IMPCHase expressed from the cloned human AICARFT/IMPCHase cDNA. The first truncation mutant, pETHAT-IMP, expressing the first 223 amino acids of the NH2-terminal portion of the full-length human clone in vector pET-23d. We also constructed an additional AICARFT/IMPCHase amino-terminal truncation mutant in vector pET-14b. The resulting plasmid, pETHUIMP-230, expresses the first 230 amino acid residues of the human AICARFT/IMPCHase coding region. A third truncation mutant was designed to express the final 406 amino acids of the COOH-terminal region of the human PurH protein. This COOH-terminal truncation mutant, encoded by plasmid pETHATNB-1200, overlaps with pETHAT-IMP by 111 nucleotides (37 amino acid residues) and with pETHUIMP-230 by 132 nucleotides (44 amino acid residues) (Fig. 6). Each of the truncation mutants as well as the wild type full-length human AICARFT/IMPCHase clone were transformed into E. coli. AICARFT and IMPCHase enzyme activity assays were carried out on extracts prepared from each of the transformants (Fig. 6). Extracts prepared from both pETHAT-IMP and pETHUIMP-230 transformants possessed IMPCHase activity, but lacked AICARFT enzyme activity. AICARFT enzyme activity was detected in extracts prepared from pETHATNB-1200 transformants and lacked IMPCHase activity. Both AICARFT and IMPCHase enzyme activities were detected in extracts prepared from transformants harboring the full-length human cDNA, pETHATNB-1800. Neither AICARFT nor IMPCHase activity was detected in E. coli harboring either vector pET-14b or pET-23d alone. These results demonstrate that the two enzyme activities are separable. In attempts to delineate the smallest peptides possessing either AICARFT or IMPCHase activity, truncation mutants expressing smaller portions of the AICARFT/IMPCHase carboxyl terminus or amino terminus were constructed (data not shown). SDS-polyacrylamide gel electrophoresis analysis of extracts prepared from these smaller constructs expressed in E. coli indicated that the peptides were expressed. However, enzyme activity assays indicated that these smaller polypeptides lacked both AICARFT as well as IMPCHase activity.

Kinetic Characterization of Carboxyl-terminal Truncation Mutant Possessing AICARFT Activity—The truncation mutant expressed from plasmid pETHATNB-1200 was partially purified as described under “Experimental Procedures.” The steady state kinetic parameters of the partially purified truncation polypeptide were subsequently determined. Kinetic values were similar to those observed for the full-length human enzyme (Table II). The lower Vmax observed for the truncation mutant most likely results from impurities in the enzyme preparation. Refinement of the purification of this truncation mutant as well as evaluation of the enzyme mechanism and con-
Human AICAR Formyltransferase/IMP Cytohydrolase

Domain by Site-directed Mutagenesis

Sequence alignment of several 10-formyl-FH4-requiring enzymes reveal similarities among the two known eukaryotic PurH sequences but not among any of the known prokaryotic PurH sequences. Nevertheless, these similarities warranted investigation.

Human AICAR Formyltransferase/IMP Cytohydrolase (AICARFT/IMPCHase) Asp501 was also conserved and due to its proximity to Asp503 we also chose to study that residue as well. We investigated whether these residues were essential for either human AICARFT or IMPCHase activities using site-directed mutagenesis. Three separate point mutations resulting in conservative amino acid replacements H469A, D501N, and D503N were introduced into the human purH cDNA. Each of these mutants, pETHAT-H469A, pETHAT-D501N, and pETHAT-D503N, as well as the wild type cDNA clone pETHATFLNN-1 and the vector pET-23d were expressed in E. coli. Protein expression was analyzed by SDS-polyacrylamide gel electrophoresis. Extracts prepared from each of the three mutants as well as the wild type purH cDNA clone indicated that AICARFT ha.

mutation results in conservative amino acid replacements H469A, D501N, and D503N were introduced into the human purH cDNA. Each of these mutants, pETHAT-H469A, pETHAT-D501N, and pETHAT-D503N, as well as the wild type cDNA clone pETHATFLNN-1 and the vector pET-23d were expressed in E. coli. Protein expression was analyzed by SDS-polyacrylamide gel electrophoresis. Extracts prepared from each of the three mutants as well as the wild type purH cDNA clone indicated.

The full-length cDNA (pETHATNB-1800), and each of the three truncation mutants (pETHUIMP-230, pETHAT-IMP, pETHATNB-1200) are depicted. The portions of the human purH coding region expressed in PET vectors are indicated by lines. The sizes of the DNA and the corresponding expressed polypeptides are indicated in parenthesis. Both AICARFT formyltransferase and IMP cytohydrolase enzyme activity assays were performed on extracts prepared from E. coli transformed each of the recombinants as described under "Experimental Procedures." Enzyme activity (+) and no enzyme activity (−) are indicated.
that a 64.3-kDa polypeptide was overexpressed corresponding to the size of full-length human PurH (data not shown). Both AICARFT and IMPCHase activity assays were performed using each of the extracts. AICARFT as well as IMPCHase activity was detected in extracts prepared from transformants possessing the wild type as well as each of the three mutant clones. No AICARFT or IMPCHase was detected in extracts prepared from the pET-23d transformant. These data indicate that conservative amino acid residue changes of amino acid residues His469, Asp501, and Asp503 do not inactivate either AICARFT or IMPCHase activity. The enzyme activity of mutant pETHAT-H469A diminished more rapidly with storage than the wild type or either of the other two mutants. While mutation H469A does not eliminate either of the two enzyme activities of human PurH, it may create structural perturbations that result in enzyme instability.

**DISCUSSION**

The sequence of the human purH cDNA bears marked similarity to the previously reported avian sequence (4) with 75 and 81% identity on the nucleotide and amino acid sequence levels, respectively. The human sequence also demonstrates similarity to purH from prokaryotes, with amino acid identities ranging from 31 to 36% among the three bacterial sequences reported (1–3). While these sequences are similar, it should be noted that there appear to be distinct differences that may be characterized as being either more eukaryotic-like (human PurH amino acids 915–951) or characterized as being either more eukaryotic-like (human not yet described) or more prokaryotic-like (Gap trinucleotide human PurH amino acids 915–951). The 10-formyl-FH₄ binding site hypothesized by Cook et al. (5) is indicated in brackets (HU GARFT amino acids 915–924). GARFT active site residues, His₉¹⁵ and Asp₉₅₁, are indicated by asterisks. Corresponding amino acids in the human AICARFT/IMPCHase chosen for mutagenic studies include His₄₆⁹, Asp₅₀₁, and Asp₅₀₃.

A further intriguing question is whether or not the two domains are functionally independent or whether the intermediate, FAICAR (Fig. 1), might be “channeled” to the IMPCHase domain. The COOH-terminal truncation mutant demonstrated independent function with kinetic properties highly similar to those of the holoenzyme. The available spectrophotometric assay for IMPCHase activity lacks the sensitivity necessary to permit comparison of activities between the truncation mutants and the holoenzyme. Thus, the question of potential channeling must await the development of a more sensitive assay.

We investigated a region of the human AICARFT/IMPCHase sequence corresponding to the putative 10-formyl-FH₄ binding domain of human GARFT and other 10-formyl-FH₄-requiring enzymes using site-directed mutagenesis. Conservative amino acid replacements were introduced into the human purH cDNA by site-directed mutagenesis. These amino acid replacements had no effect on either AICARFT or IMPCHase activity for any of the mutant enzymes. While the residues His₉¹⁵ and Asp₉₅₁ are essential for GARFT activity, the corresponding residues in the human AICARFT/IMPCHase are not essential. Therefore, this region may represent a common structural motif among these 10-formyl-FH₄-requiring enzymes but is not involved in the single carbon transfer reaction nor in the binding of the folate substrate for AICARFT/IMPCHase. The crystal structure of E. coli GARFT shows that this region is near the folate binding site and may be important in the enzyme mechanism (25–27). Since no additional regions of similarity of the primary amino acid sequence between these two 10-formyl-FH₄-requiring enzymes are apparent, the actual site of 10-formyl-FH₄ binding for AICARFT/IMPCHase remains to be elucidated. Further investigation utilizing active site irreversible...
ible inhibitors against either AICARFT activity or inosinase activity will aid in the identification of residues essential for these activities.

Chemical modification studies by Szabados et al. (21) suggest that the IMPCHase activity of the human PurH requires an essential cysteine as well as an essential arginine residue. Amino acid sequence alignment of all known PurH sequences indicates that there are no cysteine residues conserved among all of the sequences (Fig. 4). There are, however, a total of 9 cysteine residues in the human AICARFT/IMPCHase, each of which is conserved in the avian AICARFT/IMPCHase sequence as well. Two of these cysteine residues, Cys100 and Cys145, are localized within the amino-terminal truncation mutant expressing human IMPCHase activity. Based upon our truncation mutant studies, three arginine residues serve as potential candidates essential for IMPCHase activity. These arginine residues, Arg132, Arg132, and Arg206 (numbered relative to the human sequence), are conserved among all known PurH sequences and are localized in large conserved “blocks” within the putative IMPCHase activity domain (Fig. 4). Residue Arg206 is located within the 37 amino acid overlap between our two truncation mutants possessing either IMPCHase activity or AICARFT activity and may be a candidate for mutational studies. Other potential candidates include Arg76 and Arg170 which are conserved in all PurH sequences with the exception of Bacillus subtilis where there is a proximal arginine residue.

Expression of the cloned human purH cDNA in E. coli has permitted production of large quantities of human AICARFT/IMPCHase. Further investigations of the structure and mechanism of this enzyme are now feasible.

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