J. Biochem. **122**, 309-313 (1997)

Characterization of Molecularly Cloned Human 5-Aminoimidazole-4-Carboxamide Ribonucleotide Transformylase

Takahisa Sugita,[1] Hideki Aya, Makoto Ueno, Tohru Ishizuka, and Keisuke Kawashima

Lead Generation Research Laboratory, Tanabe Seiyaku Co., Ltd., 3-16-89 Kashima, Yodogawa-ku, Osaka 532

Received for publication, February 5, 1997

The cDNA encoding human 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase has been cloned from a placenta cDNA library, utilizing a PCR-derived probe. It encodes a peptide of 592 amino acids. The amino (N)-terminal sequence of this enzyme, purified from HeLa cells and CCRF-CEM cells, was found to be APGQLALF-. Both sequencing results revealed a difference of six N-terminal residues when compared to the reported sequence of cloned cDNA from a hepatoma cDNA library. Northern-blot analysis of human AICAR transformylase mRNA showed the expression of a single 2.0 kb mRNA in all tissues examined. With the cloned cDNA fragment, we constructed expression vectors for mature and GST-fused AICAR transformylase. Both recombinant molecules possessing AICAR transformylase activity were overproduced in *Escherichia coli*. GST-AICAR transformylase can be purified to homogeneity by a single-step affinity procedure with glutathione Sepharose. Mutational analysis, utilizing this expression system, showed that His213 and His267 were essential for AICAR transformylase activity.

Key words: 5-aminoimidazole-4-carboxamide ribonucleotide transformylase, cDNA cloning, protein sequencing, purine biosynthetic enzyme, site-directed mutagenesis.

The purine biosynthetic enzyme 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase [EC 2.1.2.3] catalyzes formylation of AICAR by utilizing N^{10}-formyltetrahydrofolate as a formyl donor. AICAR transformylase and IMP cyclohydrolase [EC 3.5.4.10] are found on a single polypeptide and IMP biosynthesis is sequentially catalyzed by this bifunctional molecule. The *de novo* purine pathway is an important target for the development of chemotherapeutic agents, and the chemotherapeutic agent methotrexate exerts a potent inhibitory effect on AICAR transformylase through a polyglutamylated form (*1*). Understanding the molecular basis of AICAR transformylase activity is of great importance for the discovery of new anticancer drugs. Structural studies of AICAR transformylase have been conducted by cDNA cloning of human (*2*), avian (*3*), *Escherichia coli* (*4*), *Bacillus subtilis* (*5*), and *Salmonella typhimurium* (*6*) molecules. However, the mechanism of catalysis of AICAR transformylase is still not fully understood.

In the course of the characterization of human AICAR transformylase, we found that the amino (N)-terminal sequence of this molecule is different from the reported sequence, deduced from the cloned cDNA sequence (*2*). This prompted us to clarify the existence of isozymes of human AICAR transformylase. For this purpose, we have purified human AICAR transformylase and determined the N-terminal amino acid sequence. In addition, we have cloned and expressed the cDNA for human AICAR transformylase. Tissue distribution of AICAR transformylase mRNA and critical residues for enzyme activity were also determined.

[1] To whom correspondence should be addressed. Tel: +81-6-300-2571, Fax: +81-6-300-2593, E-mail: t-sugita@tanabe.co.jp

Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; GAR, glycinamide ribonucleotide; GST, glutathione S transferase; IPTG, isopropylthiogalactoside.

MATERIALS AND METHODS

Cell Lines, Bacterial Strains, Plasmids, and Media—HeLa (human cervical carcinoma cell line) and CCRF-CEM (human T lymphoblastoid cell line) were grown at 37°C in RPMI 1640 (GIBCO, Gaitherburg, MD) supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 50 μg/ml streptomycin under a 5% CO_2 atmosphere. HepG2 (human hepatocellular carcinoma cell line) was grown in Eagle's MEM (GIBCO) supplemented with non-essential amino acids, sodium pyruvate, and 10% heat-inactivated fetal calf serum. *E. coli* K12 *purH* mutant PCO207(F^-: *thi his tyrA trp proA purH fuaC lacY gal xyl mtl mal tonA tsx str phx*) was kindly provided by Dr. Nishimura (National Institute of Genetics, Genetic Stock Research Center, Mishima). PCO207 was grown in LB broth.

AICAR Transformylase Assay—AICAR transformylase was assayed spectrophotometrically at 37°C by monitoring the conversion of N^{10}-formyltetrahydrofolate to tetrahydrofolate at 298 nm ($\Delta\varepsilon = 19{,}700\ M^{-1}\cdot cm^{-1}$) (*7*). The enzyme assay reaction mixture contained 30 mM Tris HCl (pH 7.4), 20 mM KCl, 10 mM 2-mercaptoethanol, 50 μM AICAR, and 100 μM N^{10}-formyltetrahydrofolate.

Purification of AICAR Transformylase—HeLa, CCRF-CEM, and HepG2 cells were used as sources of AICAR transformylase. Purification was performed as described previously (*8*) and included the following steps: streptomycin sulfate precipitation, ammonium sulfate precipitation,

Affi-Gel Blue column chromatography, and AICAR-Sepharose 4B column chromatography. The N-terminal amino acid sequence was determined using 1 μg of purified AICAR transformylase and a protein sequencer LF 3000 (Beckman, Fullerton, CA).

cDNA Cloning—A partial human AICAR transformylase cDNA was amplified by RT-PCR using degenerate oligonucleotide primers, designed according to the avian AICAR transformylase sequence (*3*). The sense and antisense strand oligonucleotide primer sequences were 5′-AC(AG)GG(GC)TTCCCTGAGATG-3′ and 5′-TGATG(AG)GG(AG)TTCATGCCATA-3′, respectively. The amplified 480 bp fragment was random-prime labeled and used as a hybridization probe to screen 10^6 plaques from a human placenta cDNA library (Clontech, HL5014b, Palo Alto, CA). Positively hybridizing plaques were purified and phage DNA was isolated by a cleared lysate method. *Eco*RI cDNA inserts were subcloned into pBluescript II SK (Stratagene, La Jolla, CA). Representative clones were subjected to complete directed sequencing of both cDNA strands, using 20-base oligonucleotide primers for each of the sense and antisense strands. Large-scale plasmid preparations were purified by anion-exchange chromatography (Qiagen, Chatsworth, CA).

Amplification of cDNA—cDNA fragments corresponding to the N-terminal portion of human AICAR transformylase were amplified by RT-PCR. One microgram of total RNA of HeLa or HepG2 cells was used for the template. The sense strand oligonucleotide primers for MAPGQL and MSSLS sequences were 5′-ATGGCTCCCGGCCAGCTC-3′ and 5′-ATGTCTTCTCTCTCA-3′, respectively. The anti-sense strand oligonucleotide primer was 5′-AAGGGATAGAGATTGCAGGC-3′. The amplification reaction for the MAPGQL sequence-specific cDNA fragment consisted of a pre-cycle at 94°C for 4 min followed by 30 cycles at 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min. The amplification reaction for the MSSLS sequence-specific cDNA fragment consisted of a pre-cycle at 94°C for 4 min followed by 30 cycles at 94°C for 1 min, 40°C for 2 min, and 55°C for 3 min.

Northern-Blot Analysis—A human multiple tissue Northern (MTN) blot (Clontech) containing 2 μg of poly-(A)$^+$ RNA from individual tissues was hybridized with a ^{32}P-labeled human AICAR transformylase cDNA fragment, which was the same probe as that used for cDNA cloning.

Construction of the Expression Vector—To amplify cDNA encoding the entire amino acid sequence of AICAR transformylase, primers spanning the initiation methionine (5′-TAAGGATCCCC**ATG**GCTCCCGGCCAGCTCG-3′) and downstream of the stop codon (5′-TATGGATCCCGGG**TCA**GTGGTGGAAGAGCCGAAGGTT-3′) containing a *Bam*HI site (underlined) were used. The PCR product was recovered and digested with *Nco*I and *Bam*HI for ligation into the plasmid vector pTrc99A (Pharmacia Biotech, Uppsala, Sweden) to yield pAT801. For ligation into pGEX-3X (Pharmacia Biotech), the PCR product was digested with *Bam*HI and subcloned to yield pAT901. The inserts were sequenced to confirm their authenticity.

Preparation of Recombinant Enzyme—IPTG-induced *E. coli* cells were suspended in phosphate-buffered saline (PBS) (pH 7.4) containing 10 μg/ml aprotinin, 10 μM pepstatin, 10 μM leupeptin, 1 mM PMSF, 1 mM EDTA, and 2 mM DTT. Lysozyme was added to give a concentration of 0.2 mg/ml, then the mixture was allowed to stand for 15 min at 4°C and finally sonicated for 30 s. Triton X-100 (1%) was added and the whole was centrifuged for 10 min at 10,000 × *g*. The supernatant was subjected to affinity purification using Glutathione Sepharose 4B (Pharmacia Biotech). Briefly, the supernatant was mixed with Glutathione Sepharose 4B equilibrated with PBS, and rotated gently for 30 min at room temperature. The gel was washed four times with PBS containing 1% Triton X-100 and the bound material was eluted with 10 mM glutathione in 50 mM Tris HCl (pH 8.0).

Site-Directed Mutagenesis—Site-directed mutagenesis of His213 and His267 to Ala was performed by introducing mutant cDNA fragments amplified by PCR. For His213 mutation, a cDNA fragment was amplified with a primer spanning the initiation methionine (5′-TAAGGATCCCC**A-TG**GCTCCCGGCCAGCTCG-3′) and a mutant primer (5′-GGGAAGCTTGGGCTGCAGTGTGTACAGCTGGGCAG-GGGTCTG**AGC**TGGGT-3′). The PCR product was cut with *Nco*I and *Hin*dIII and the pAT901 *Nco*I-*Hin*dIII fragment was replaced with the mutant fragment to yield pHA213. For His267 mutation, a cDNA fragment was amplified with a primer spanning the initiation methionine (5′-TAAGGATCCCC**ATG**GCTCCCGGCCAGCTCG-3′) and a mutant primer (5′-GTGGAATTCCAACAGCAGC-ACCTGCTGGGCTGAC**AGC**TTTGAAAG-3′). The PCR product was cut with *Bam*HI and *Eco*RI and the pAT901 *Bam*HI-*Eco*RI fragment was replaced with the mutant fragment to yield pHA267.

Inactivation of GST-AICAR Transformylase by Diethyl Pyrocarbonate—Purified GST-AICAR transformylase was incubated with or without 5 mM diethyl pyrocarbonate in 50 μl of 50 mM potassium phosphate buffer (pH 7.4) at 20°C and 4 samples were taken at 3 min intervals for enzyme assay.

RESULTS AND DISCUSSION

Protein Sequencing and cDNA Cloning of Human AICAR Transformylase—To characterize human AICAR transformylase, we purified the enzyme from HeLa cells and determined that the N-terminal amino acid sequence was APGQLALF-. This sequence is different from the reported sequence (MSSLS-) of human AICAR transformylase, which was based on the cloned cDNA from a hepatoma cDNA library (*2*). To clarify the existence of isozymes of AICAR transformylase, we cloned human AICAR transformylase cDNA using the human placenta cDNA library. We used as a probe a PCR-generated partial human AICAR transformylase cDNA fragment, which was specifically amplified with oligonucleotide primers designed on the basis of the amino acid sequence of avian AICAR transformylase. A representative clone consisting of 1,958 bp nucleotides with an open reading frame of 592 amino acids is shown (Fig. 1). The sequence has been deposited in the DDBJ/EMBL/GenBank DNA databases (accession number D89976). The deduced amino acid sequence of the N-terminal portion showed complete agreement with the N-terminal sequence of purified human AICAR transformylase. Comparison of the above open reading frame with the reported sequence of human AICAR transformylase revealed that six amino acids of the N-terminal portion and the 165th residue were different. To see whether this

```
1     CGCACGTGGTGCCGCCGCTGCTGCCTCCCGCTCGCCCTGAACCCAGTGCCTGCAGCCATG
                                                              M      1
61    GCTCCCGGCCAGCTCGCCTTATTTAGTGTCTCTGACAAAACCGGCCTTGTGGAATTTGCA
        A  P  G  Q  L  A  L  F  S  V  S  D  K  T  G  L  V  E  F  A   21
121   AGAAACCTGACCGCTCTTGGTTTGAATCTGGTCGCTTCCGGAGGGACTGCAAAAGCTCTC
        R  N  L  T  A  L  G  L  N  L  V  A  S  G  G  T  A  K  A  L   41
181   AGGGATGCTGGTCTGGCAGTCAGAGATGTCTCTGAGTTGACGGGATTTCCTGAAATGTTG
        R  D  A  G  L  A  V  R  D  V  S  E  L  T  G  F  P  E  M  L   61
241   GGGGGACGTGTGAAAACTTTGCATCCTGCAGTCCATGCTGGAATCCTAGCTCGTAATATT
        G  G  R  V  K  T  L  H  P  A  V  H  A  G  I  L  A  R  N  I   81
301   CCAGAAGATAATGCTGACATGGCCAGACTTGATTTCAATCTTATAAGAGTTGTTGCCTGC
        P  E  D  N  A  D  M  A  R  L  D  F  N  L  I  R  V  V  A  C  101
361   AATCTCTATCCCTTTGTAAAGACAGTGGCTTCTCCAGGTGTAACTGTTGAGGAGGCTGTG
        N  L  Y  P  F  V  K  T  V  A  S  P  G  V  T  V  E  E  A  V  121
421   GAGCAAATTGACATTGGTGGAGTAACCTTACTGAGAGCTGCAGCCAAAAACCACGCTCGA
        E  Q  I  D  I  G  G  V  T  L  L  R  A  A  A  K  N  H  A  R  141
481   GTGACAGTGGTGTGTGAACCAGAGGACTATGTGGTGGTGTCCACGGAGATGCAGAGCTCC
        V  T  V  V  C  E  P  E  D  Y  V  V  V  S  T  E  M  Q  S  S  161
541   GAGAGTAAGGACACCTCCTTGGAGACTAGACGCCAGTTAGCCTTGAAGGCATTCACTCAT
        E  S  K  D  T  S  L  E  T  R  R  Q  L  A  L  K  A  F  T  H  181
601   ACGGCACAATATGATGAAGCAATTTCAGATTATTTCAGGAAACAGTACAGCAAAGGCGTA
        T  A  Q  Y  D  E  A  I  S  D  Y  F  R  K  Q  Y  S  K  G  V  201
661   TCTCAGATGCCCTTGAGATATGGAATGAACCCACATCAGACCCCTGCCCAGCTGTACACA
        S  Q  M  P  L  R  Y  G  M  N  P  [H] Q  T  P  A  Q  L  Y  T  221
721   CTGCAGCCCAAGCTTCCCATCACAGTTCTAAATGGAGCCCCTGGATTTATAAACTTGTGC
        L  Q  P  K  L  P  I  T  V  L  N  G  A  P  G  F  I  N  L  C  241
781   GATGCTTTGAACGCCTGGCAGCTGGTGAAGGAACTCAAGGAGGCTTTAGGTATTCCAGCC
        D  A  L  N  A  W  Q  L  V  K  E  L  K  E  A  L  G  I  P  A  261
841   GCTGCCTCTTTCAAACATGTCAGCCCAGCAGGTGCTGCTGTTGGAATTCCACTCAGTGAA
        A  A  S  F  K  [H] V  S  P  A  G  A  A  V  G  I  P  L  S  E  281
901   GATGAGGCCAAAGTCTGCATGGTTTATGATCTCTATAAAACCCTCACACCCATCTCAGCG
        D  E  A  K  V  C  M  V  Y  D  L  Y  K  T  L  T  P  I  S  A  301
961   GCATATGCAAGAGCAAGAGGGGCTGATAGGATGTCTTCATTTGGTGATTTTGTTGCATTG
        A  Y  A  R  A  R  G  A  D  R  M  S  S  F  G  D  F  V  A  L  321
1021  TCCGATGTTTGTGATGTACCAACTGCAAAAATTATTTCCAGAGAAGTATCTGATGGTATA
        S  D  V  C  D  V  P  T  A  K  I  I  S  R  E  V  S  D  G  I  341
1081  ATTGCCCCAGGATATGAAGAAGAAGCCTTGACAATACTTTCCAAAAAGAAAAATGGAAAC
        I  A  P  G  Y  E  E  E  A  L  T  I  L  S  K  K  K  N  G  N  361
1141  TATTGTGTCCTTCAGATGGACCAATCTTACAAACCAGATGAAAATGAAGTTCGAACTCTC
        Y  C  V  L  Q  M  D  Q  S  Y  K  P  D  E  N  E  V  R  T  L  381
1201  TTTGGTCTTCATTTAAGCCAGAAGAGAAATAATGGTGTCGTCGACAAGTCATTATTTAGC
        F  G  L  H  L  S  Q  K  R  N  N  G  V  V  D  K  S  L  F  S  401
1261  AATGTTGTTACCAAAAATAAAGATTTGCCAGAGTCTGCCCTCCGAGACCTCATCGTAGCC
        N  V  V  T  K  N  K  D  L  P  E  S  A  L  R  D  L  I  V  A  421
1321  ACCATTGCTGTCAAGTACACTCAGTCTAACTCTGTGTGCTACGCCAAGAACGGGCAGGTT
        T  I  A  V  K  Y  T  Q  S  N  S  V  C  Y  A  K  N  G  Q  V  441
1381  ATCGGCATTGGAGCAGGACAGCAGTCTCGTATACACTGCACTCGCCTTGCAGGAGATAAG
        I  G  I  G  A  G  Q  Q  S  R  I  H  C  T  R  L  A  G  D  K  461
1441  GCAAACTATTGGTGGCTTAGACACCATCCACAAGTGCTTTCGATGAAGTTTAAAACAGGA
        A  N  Y  W  W  L  R  H  H  P  Q  V  L  S  M  K  F  K  T  G  481
1501  GTGAAGAGAGCAGAAATCTCCAATGCCATCGATCAATATGTGACTGGAACCATTGGCGAG
        V  K  R  A  E  I  S  N  A  I  D  Q  Y  V  T  G  T  I  G  E  501
1561  GATGAAGATTTGATAAAGTGGAAGGCACTGTTTGAGGAAGTCCCTGAGTTACTCACTGAG
        D  E  D  L  I  K  W  K  A  L  F  E  E  V  P  E  L  L  T  E  521
1621  GCAGAGAAGAAGGAATGGGTTGAGAAACTGACTGAAGTTTCTATCAGCTCTGATGCCTTC
        A  E  K  K  E  W  V  E  K  L  T  E  V  S  I  S  S  D  A  F  541
1681  TTCCCTTTCCGAGATAACGTAGACAGAGCTAAAAGGAGTGGTGTGGCGTACATTGCGGCT
        F  P  F  R  D  N  V  D  R  A  K  R  S  G  V  A  Y  I  A  A  561
1741  CCCTCCGGTTCTGCTGCTGACAAAGTTGTGATTGAGGCCTGCGACGAACTGGGAATCATC
        P  S  G  S  A  A  D  K  V  V  I  E  A  C  D  E  L  G  I  I  581
1801  CTCGCTCATACGAACCTTCGGCTCTTCCACCACTGATTTTACCACACACTGTTTTTTGGC
        L  A  H  T  N  L  R  L  F  H  H  *
1861  TTGCTTATGTGTAGGTGAACAGTCACGCCTGAAACTTTGAGGATAACTTTTTAAAAAAAT
1921  AAAACAGTATCTCTTAAAAAAAAAAAAAAAAAAAAAAA
```

Fig. 1. **The nucleotide and deduced amino acid sequence of the human AICAR transformylase cDNA.** The numbering at the right refers to the peptide position, and that at the left, to the nucleotide position. A 480 bp cDNA fragment covering from the 220th to 698th nucleotide was amplified using degenerate oligonucleotide primers and used for cDNA cloning and Northern-blot analysis. Underlined peptide sequences are different from the reported sequence (*2*). A homologous region with the proposed N^{10}-formyltetrahydrofolate binding site is indicated by a dotted line (*12*). Boxed His residues are critical for AICAR transformylase activity (Table I).

difference was limited to hepatoma cells, we purified human AICAR transformylase from another cell line, CCRF-CEM. The N-terminal sequence of the enzyme from CCRF-CEM cells was the same as that of HeLa cells. We further analyzed the expression of two types of mRNA for AICAR transformylase by RT-PCR (Fig. 2). Two independent oligonucleotide sense primers corresponding to MAPGQL and MSSLS N-terminal-divergent sequences and a common anti-sense primer based on an internal sequence were used for the amplification. Total RNAs from HeLa cells and hepatoma HepG2 cells were used for the templates. A 320 bp cDNA fragment was amplified with the sense primer corresponding to the MAPGQL sequence from both HeLa and HepG2 RNAs. However, when the sense primer

encoding the MSSLS sequence was used, only a small amount of the 320 bp cDNA fragment was amplified from HepG2 RNA, but not from HeLa RNA. N-terminal amino acid sequencing of purified AICAR transformylase from HepG2 cells was also performed and only the signals of the APGQL sequence were detected. These results suggest that AICAR transformylase containing the APGQL N-terminal sequence is the predominant type of AICAR transformylase expressed in human cells. However, there may also be a divergent molecule with a different N-terminal sequence, whose expression is weak and perhaps limited to certain cell types. Examination of the molecular mechanism of the expression of such divergent molecules of AICAR transformylase must await further genomic structural analysis.

The physiological significance of each AICAR transformylase molecule is unclear. However, this difference should be taken into consideration when we design the anti-sense RNA/DNA of the AICAR transformylase gene to suppress the expression of this enzyme.

Tissue Distribution of AICAR Transformylase mRNA—Northern-blot analysis of total RNA from human tissues is shown in Fig. 3. A single hybridizing band of approximately 2.0 kb was detected in all of the tissues. The transcript was similar in size to the 1.8 kb coding sequence. This indicated that the AICAR transformylase mRNA contains small 3′ and 5′ untranslated regions. In fact, the 3′ untranslated region of cloned cDNA was 122 bp long. We could not detect alternatively spliced forms of the transcripts of different size, which might explain the differences between the N-terminal sequences of our clone and the clone from the hepatoma cDNA library.

Expression of Recombinant Protein—The expression vectors for mature AICAR transformylase (pAT801) and GST-fused AICAR transformylase (pAT901) were constructed by introducing AICAR transformylase cDNA into pTrc99A and pGEX-3X, respectively. *E. coli* AICAR transformylase mutant PCO207 was transformed by either pAT801 or pAT901 and logarithmic phase cultures were incubated with IPTG for further 4 h. Cell lysates were subjected to the AICAR transformylase enzyme assay, and significant activities were detected in cell lysates of both PCO207[pAT801] and PCO207[pAT901] (Table I, Exp. A). The protein was further analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie Blue staining (Fig. 4). *E. coli* PCO207[pAT801] overproduced AICAR transformylase with a molecular weight of 65 kDa. GST-fused AICAR transformylase, with a molecular weight of 90 kDa, was overproduced in *E. coli* PCO207[pAT901].

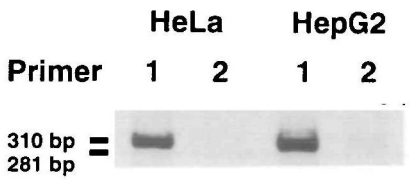

Fig. 2. **Amplification of cDNA fragments corresponding to the N-terminal portion of human AICAR transformylase.** N-terminal MAPGQL and MSSLS sequence-specific cDNA fragments were amplified by RT-PCR. Total RNAs of HeLa and HepG2 cells were used as the templates. Primer 1 and primer 2 encoded MAPGQL and MSSLS sequences, respectively.

The recombinant GST-AICAR transformylase, possessing enzyme activity, has many useful properties. This protein is highly expressed in *E. coli* cells, remains soluble and can be highly purified by a single-step affinity purifica-

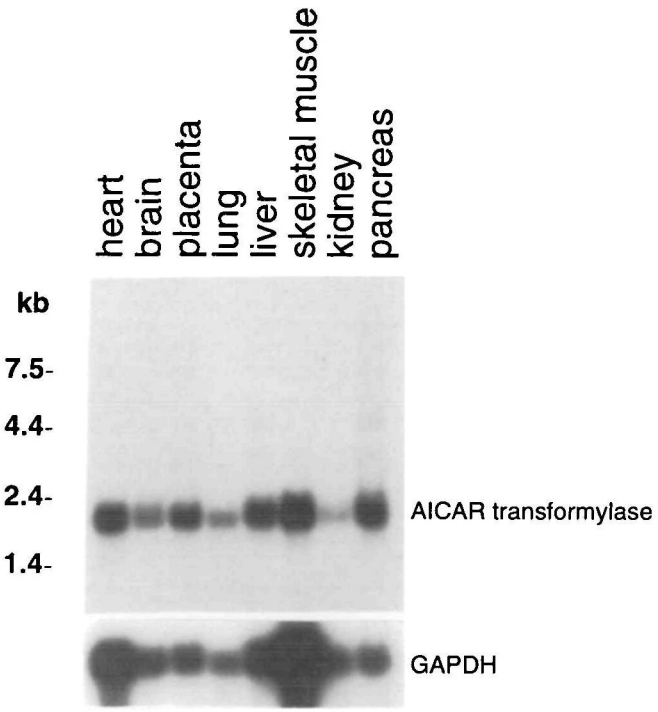

Fig. 3. **Northern-blot analysis of AICAR transformylase mRNA.** A human multiple tissue Northern (MTN) blot containing 2 μg of poly(A)$^+$ RNA was hybridized with ^{32}P-labeled human AICAR transformylase cDNA fragment. The blot was washed under stringent conditions (2×NaCl/Cit and 0.1% SDS, 60°C). The same blot was then stripped and re-probed with a human GAPDH cDNA probe.

TABLE I. **Expression of mature and GST-fused human AICAR transformylase in *E. coli* PCO207.** *E. coli* transformants were incubated with 0.2 mg/ml lysozyme for 15 min at 4°C in PBS (pH 7.4) containing 10 μg/ml aprotinin, 10 μM pepstatin, 10 μM leupeptin, 1 mM PMSF, 1 mM EDTA, 2 mM DTT. Cells were sonicated for 30 s, then centrifuged for 10 min at 10,000×g, and cell lysates were subjected to AICAR transformylase assay.

Vector	IPTG	AICAR transformylase Specific activity (munits/mg protein)
Exp. A		
pTrc99A	−	0.2
	+	0.2
pAT801	−	0.3
	+	9.5
pGEX-3X	−	0.2
	+	0
pAT901	−	1.0
	+	25.2
Exp. B		
pGEX-3X	−	1.4
	+	1.7
pAT901	−	11.2
	+	29.1
pHA213	−	1.4
	+	1.7
pHA267	−	0.9
	+	1.6

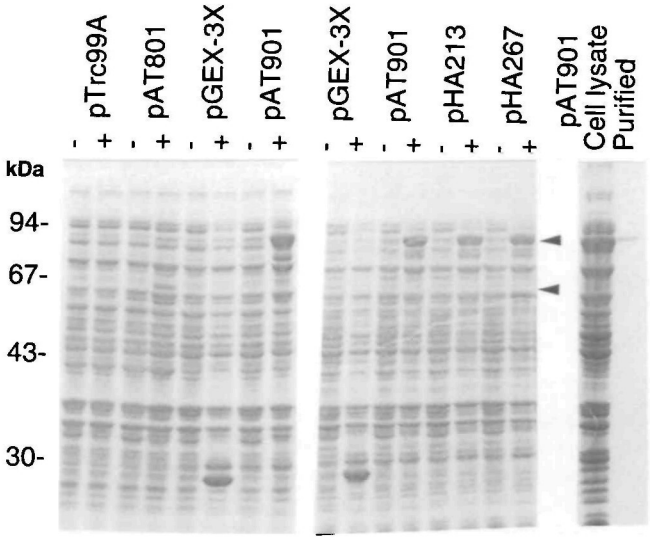

Fig. 4. **SDS-PAGE of recombinant human AICAR transformylase.** Samples were analyzed by SDS-PAGE followed by Coomassie Blue staining. Arrowheads indicate 65 kDa AICAR transformylase and 90 kDa GST-AICAR transformylase.

tion with glutathione Sepharose, as shown in Fig. 4. The apparent K_m value for N^{10}-formyltetrahydrofolate was determined to be 110 μM using purified GST-AICAR transformylase. This was similar to the reported K_m value of 118 μM, which was determined with avian AICAR transformylase (*9*).

Requirement of His Residues for Enzyme Activity—AICAR transformylase catalyzes transformylation of AICAR and utilizes N^{10}-formyltetrahydrofolate as a formyl donor. AICAR transformylase and GAR transformylase [EC 2.1.2.2] catalyze two steps in *de novo* purine biosynthesis by utilizing reduced folate as a cofactor. The study of GAR transformylase suggested that His and Asp residues constitute the active site of this enzyme. While His915 and Asp951, and His119 and Asp144 are essential for human and *E. coli* GAR transformylase activity (*10, 11*), respectively, site-directed mutagenesis studies showed that the corresponding residues His470 and Asp504 in the human AICAR transformylase were not essential for enzyme activity (*2*). The proposed N^{10}-formyltetrahydrofolate binding site spanning from His470 to Gly481 was not conserved in the prokaryotic molecules. Therefore, these residues are not involved in the single carbon transfer reaction or in folate binding of AICAR transformylase.

To determine whether His residues are essential for AICAR transformylase activity, purified GST-AICAR transformylase was incubated with diethyl pyrocarbonate, which can chemically modify His residues. Time-dependent inactivation of AICAR transformylase by diethyl pyrocarbonate was observed, suggesting that His residues are essential for catalytic activity. The first-order rate constant was determined to be 0.062 (min^{-1}) for decay of the enzyme activity.

Truncation mutant analysis of human AICAR transformylase showed that AICAR transformylase and IMP cyclohydrolase are coded by separate functional domains and that only the 406-amino-acid carboxy portion is essential for AICAR transformylase activity (*2*). Considering these results, we tried to determine the critical His residues necessary for AICAR transformylase activity. We introduced point mutations in some of the His residues which are conserved in eukaryotes and prokaryotes within the 406 amino acid carboxy portion. Loss of enzyme activity was observed in both His213 and His267 mutants, in which each His was replaced by Ala (Table I, Exp. B, pHA213 and pHA267). As for the expression, comparable amounts of the mutant protein and the authentic protein were expressed (Fig. 4). Solubility of these fusion proteins was checked and a significant amount of fusion protein was found in the soluble fraction (data not shown). These results suggest that His213 and His267 are critical for AICAR transformylase activity. However, at this moment, it is not clear whether these residues are at the active site. Irreversible inhibitors of AICAR transformylase will provide more information about the active site residues.

We thank Dr. A. Nishimura for providing *E. coli* strain PCO207, and Dr. A. Southey for a critical reading of the manuscript.

REFERENCES

1. Allegra, C.J., Drake, J.C., Jolivet, J., and Chabner, B.A. (1985) Inhibition of phosphoribosylaminoimidazolecarboxamide transformylase by methotrexate and dihydrofolic acid polyglutamates. *Proc. Natl. Acad. Sci. USA* **82**, 4881-4885
2. Rayl, E.A., Moroson, B.A., and Beardsley, G.P. (1996) The human *purH* gene product, 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase. Cloning, sequencing, expression, purification, kinetic analysis, and domain mapping. *J. Biol. Chem.* **271**, 2225-2233
3. Ni, L., Guan, K., Zalkin, H., and Dixon, J.E. (1991) *De novo* purine nucleotide biosynthesis: cloning, sequencing and expression of a chicken *PurH* cDNA encoding 5-aminoimidazole-4-carboxamide-ribonucleotide transformylase-IMP cyclohydrolase. *Gene* **106**, 197-205
4. Aiba, A. and Mizobuchi, K. (1989) Nucleotide sequence analysis of genes *purH* and *purD* involved in the *de novo* purine nucleotide biosynthesis of *Escherichia coli*. *J. Biol. Chem.* **264**, 21239-21246
5. Ebbole, D.J. and Zalkin, H. (1987) Cloning and characterization of a 12-gene cluster from *Bacillus subtilis* encoding nine enzymes for *de novo* purine nucleotide synthesis. *J. Biol. Chem.* **262**, 8274-8287
6. Chopra, A.K., Peterson, J.W., and Prasad, R. (1991) Nucleotide sequence analysis of *purH* and *purD* genes from *Salmonella typhimurium*. *Biochim. Biophys. Acta* **1090**, 351-354
7. Black, S.L., Black, M.J., and Mangum, J.H. (1978) A rapid assay for 5-amino-4-imidazolecarboxamide ribotide transformylase. *Anal. Biochem.* **90**, 397-401
8. Szabados, E., Hindmarsh, E.J., Phillips, L., Duggleby, R.G., and Christopherson, R.I. (1994) 5-Aminoimidazole-4-carboxamide ribotide transformylase-IMP cyclohydrolase from human CCRF-CEM leukemia cells: purification, pH dependence, and inhibitors. *Biochemistry* **33**, 14237-14245
9. Baggott, J.E., Morgan, S.L., Ha, T., Vaughn, W.H., and Hine, R.J. (1992) Inhibition of folate-dependent enzymes by non-steroidal anti-inflammatory drugs. *Biochem. J.* **282**, 197-202
10. Kan, C.C., Gehring, M.R., Nodes, B.R., Janson, C.A., Almassy, R.J., and Hostomska, Z. (1992) Heterologous expression and purification of active human phosphoribosylglycinamide formyltransferase as a single domain. *J. Protein. Chem.* **11**, 467-473
11. Inglese, J., Smith, J.M., and Benkovic, S.J. (1990) Active-site mapping and site-specific mutagenesis of glycinamide ribonucleotide transformylase from *Escherichia coli*. *Biochemistry* **29**, 6678-6687
12. Cook, R.J., Lloyd, R.S., and Wagner, C. (1991) Isolation and characterization of cDNA clones for rat liver 10-formyltetrahydrofolate dehydrogenase. *J. Biol. Chem.* **266**, 4965-4973