5-Aminoimidazole-4-carboxamide Ribotide Transformylase−IMP Cyclohydrolase from Human CCRF-CEM Leukemia Cells: Purification, pH Dependence, and Inhibitors†

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ABSTRACT: The bifunctional enzyme 5-aminoimidazole-4-carboxamide ribotide (AICAR) transformylase−IMP cyclohydrolase has been purified 780-fold to apparent homogeneity from human CCRF-CEM leukemia cells, completed with chromatography on Affi-Gel Blue followed by AICAR−Sepharose 4B. Using a sensitive radioassay, IMP cyclohydrolase has a K_s value for 5-formamidoimidazole-4-carboxamide ribotide (FAICAR) at pH 7.4 of 0.87 ± 0.11 μM. The following purine nucleotide derivatives were potent competitive inhibitors of IMP cyclohydrolase: 2-mercaptoinosine 5′-monophosphate (K_i = 0.094 ± 0.024 μM), xanthosine 5′-monophosphate (K_i = 0.12 ± 0.01 μM), 2-fluoroadenine arabinoside 5′-monophosphate (K_i = 0.16 ± 0.02 μM), 6-mercaptopurine riboside 5′-monophosphate (K_i = 0.20 ± 0.02 μM), adenosine N^1-oxide 5′-monophosphate (K_i = 0.28 ± 0.03 μM), and N^6-(carboxymethyl)adenosine 5′-monophosphate (K_i = 1.7 ± 0.42 μM). The pH dependencies of V_{max} and V_{max}/K_s values for IMP cyclohydrolase are consistent with a single ionizable amino acid residue (pK_a = 7.57 ± 0.09) of the enzyme which must be unprotonated for catalysis to occur and a residue (pK_a = 7.57 ± 0.14) which must be unprotonated for FAICAR to bind. The pK_a values of 5.81 ± 0.03 and 9.41 ± 0.04 determined for FAICAR indicate that ionization of the substrate does not contribute significantly to the pH effects observed. Chemical modification of IMP cyclohydrolase provides evidence for arginine and cysteine residues at the active site, and roles for these residues in the mechanism of catalysis are proposed.

The ninth and tenth reactions of the pathway for *de novo* biosynthesis of purine nucleotides are catalyzed by a bifunctional protein containing the enzymic activities AICAR[1] transformylase (EC 2.1.2.3) and IMP cyclohydrolase (EC 3.5.4.10) (Scheme 1). Mueller and Benkovic (1981) purified this enzyme 154-fold to apparent homogeneity from chicken liver in 5 steps, completed with elution from an AICAR−Sepharose column. Human AICAR transformylase−IMP cyclohydrolase has been purified 435-fold in 3 steps from human MCF-7 breast cancer cells (Allegra *et al.*, 1985). The enzyme, eluted from Affi-Gel Blue in the final step, contained three significant contaminating proteins. Birds excrete nitrogen as the purine uric acid, and consequently avian liver provides a rich source for purine pathway enzymes. By contrast, humans excrete urea and levels of purine pathway enzymes are much lower, and complete purification of the human bifunctional enzyme would require a more selective procedure.

For the avian enzyme, AICAR transformylase and IMP cyclohydrolase are contained in a single polypeptide which associates as a dimer (Mueller & Benkovic, 1981). The amino acid sequence deduced from the nucleotide sequence of the cDNA yields a molecular weight of 64.4 kDa for the monomer (Ni *et al.*, 1991). AICAR transformylase has K_s values of 21 μM for N^{10}-formyltetrahydrofolate and 15 μM for AICAR, but Mueller and Benkovic (1981) reported that the K_s value of IMP cyclohydrolase for FAICAR was less than 1 μM, too low to be measured spectrophotometrically. AICAR transformylase has been proposed as the site of inhibition of the *de novo* purine pathway induced by methotrexate in human MCF-7 breast cancer cells (Allegra *et al.*, 1985, 1987). Inhibition of dihydrofolate reductase by methotrexate results in accumulation of cellular dihydrofolate polyglutamates which are potent inhibitors of AICAR transformylase. Sant *et al.* (1992) confirmed that methotrexate induces blockade of AICAR transformylase, but showed that the primary site of inhibition of the purine pathway by dihydrofolate polyglutamates is at the first reaction catalyzed by amido phosphoribosyltransferase. Thus, the *de novo* purine pathway is an important target for development of inhibitors with potential use as chemotherapeutic agents. In this paper, we have purified human AICAR transformylase−IMP cyclohydrolase to apparent homogeneity. Using the purified bifunctional enzyme, the mechanism of catalysis of IMP cyclohydrolase has been investigated. A number of purine nucleotide derivatives are potent competitive inhibitors of this enzyme and provide information on the binding specificity of the catalytic site.

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[1] Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide ribotide; FAICAR, 5-formamidoimidazole-4-carboxamide ribotide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high pressure liquid chromatography; SDS, sodium dodecyl sulfate.

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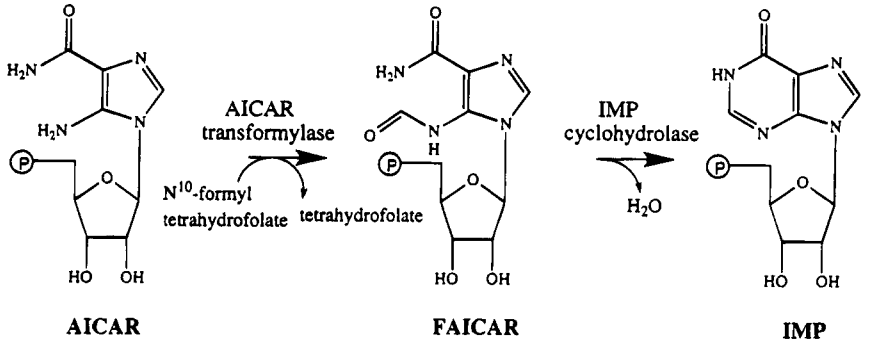
Scheme 1



EXPERIMENTAL PROCEDURES

Purine Intermediates, Nucleoside Derivatives, and Reagents for Chemical Modifications. Phenylglyoxal, citraconic anhydride, *N*-acetylimidazole, *N*-ethylmaleimide, and diethyl pyrocarbonate were obtained from the Sigma Chemical Co. (St. Louis, MO). AICAR, IMP, *N*-succino-AMP, AMP, XMP, GMP, 2′-deoxyadenosine 5′-monophosphate, 6-chloropurine ribotide, 8-azidoadenosine 5′-monophosphate, 8-bromoguanosine 5′-monophosphate, N^6-methyladenosine 5′-monophosphate, N^2-acetyl-2′-deoxyguanosine 5′-monophosphate, 6-mercaptopurine ribotide, adenosine N^1-oxide 5′-monophosphate, N^6-(carboxymethyl)adenosine 5′-monophosphate, xanthosine, 6-mercaptopurine riboside, adenosine N^1-oxide, and N^5-formyltetrahydrofolate were also from Sigma. 2-Fluoroadenine arabinoside 5′-monophosphate was from the National Cancer Institute (Bethesda, MD). Purine ribotide and 6-methylmercaptopurine ribotide were synthesized by the method of Yoshikawa *et al.* (1969). N^6-(Carboxymethyl)adenosine and 2-fluoroadenine arabinoside were prepared by hydrolysis of the corresponding nucleotide with potato phosphatase (Sigma) in 10 mM potassium acetate (pH 5.0) at 37 °C. Reaction mixtures were analyzed by HPLC on a Partisil 10-SAX column (25 × 0.46 cm, Whatman, Maidstone, U.K.) eluted with 7.0 mM KH_2PO_4 (pH 3.0) at a flow rate of 2 mL/min. The N^6-(carboxymethyl)adenosine and 2-fluoroadenine arabinoside produced eluted with retention times of 3.5 and 1.9 min and were 99% and 96% pure, respectively, based on absorbance at 260 nm.

N^{10}-Formyltetrahydrofolate was synthesized from N^5-formyltetrahydrofolate by a modification of the method of Rabinowitz (1963) and standardized using an extinction coefficient of 2.2×10^4 M^{-1} cm^{-1} at 258 nm (Robinson, 1971). FAICAR was prepared chemically from AICAR as the barium salt (Flaks *et al.*, 1957). [^{3}H]FAICAR (15 000 Ci/mol) was prepared by tritium exchange of unlabeled FAICAR by Moravek Biochemicals Inc. (Brea, CA). [^{3}H]-AICAR (15 000 Ci/mol) was synthesized enzymically from ^{3}H-labeled 5-aminoimidazole-4-carboxamide riboside (Moravek Biochemicals Inc. Brea, USA) using a 5′-specific nucleoside phosphotransferase extracted from *Serratia marcescens* (ATCC 14227) with *p*-nitrophenyl phosphate as the phosphate donor (Fyfe *et al.*, 1978). [^{14}C]Phenylglyoxal (24 Ci/mol, obtained as a solid) and [^{14}C]*N*-ethylmaleimide (13.5 mM, 7.4 Ci/mol) were from Amersham International plc (Amersham, U.K.).

Synthesis of 2-Mercaptoinosine and the 5′-Monophosphate. The nucleoside was synthesized from 5-aminoimidazole-4-carboxamide riboside (0.78 mmol) and carbon disulfide by the method of Yamazaki *et al.* (1967). The reaction was carried out in dry dioxane in a calorimetric bomb, and the product was initially crystallized from hot water with charcoal and was then recrystallized from methanol/benzene/hexane, 1:4:1 (v/v). The yield was 19%, and the product was pure when analyzed by reverse-phase HPLC and had λ_{max} values of 218 and 276 nm. A ^{1}H NMR spectrum was recorded in DMSO-d_6 at 600 MHz on a Bruker AMX 600 spectrometer: δ 8.04 (s, $1H_8$), 5.91 (d, $1H'_1$), 4.23 (m, $1H'_2$), 4.06 (m, $1H'_3$), 4.01 (m, $1H'_4$), 3.64 (m, $2H'_5$). The coupling of these resonances was not first-order, and coupling constants were not determined. 2-Mercaptoinosine 5′-monophosphate was synthesized from AICAR (0.11 mmol) and phenyl isothiocyanate by the method of Imai *et al.* (1971). The yield was 50%, and the product was pure when analyzed by gradient anion exchange HPLC and had λ_{max} values of 198 and 284 nm. A ^{1}H NMR spectrum was recorded in D_2O at 400 MHz on a Bruker AMX 400 wide-bore spectrometer: δ 8.14 (s, $1H_8$), 5.89 (d, $1H'_1$), 4.57 (m, $1H'_2$), 4.34 (m, $1H'_3$), 4.20 (m, $1H'_4$), 3.89 (m, $2H'_5$).

Growth of Cells. Human CCRF-CEM leukemia cells were grown in RPMI 1640 medium (Flow Laboratories, Sydney, Australia) containing 20 mM K−Hepes, 10% (v/v) fetal calf serum (Commonwealth Serum Laboratories, Melbourne, Australia), and 50 μg/mL gentamycin (Flow Laboratories), and the final pH was 7.2. Cells were harvested in late exponential phase (10^6 cells/mL), washed twice in Hank's basal salts solution, and stored at −80 °C.

Purification of AICAR Transformylase−IMP Cyclohydrolase. Human CCRF-CEM leukemia cells (2 L) were grown to late exponential phase (10^6 cells/mL) and harvested by centrifugation (100*g*, 30 min, 4 °C). All subsequent steps were at 0−4 °C except for the chromatography on Affi-Gel Blue which was at room temperature. The cells were washed twice in Hank's basal salts solution, resuspended in 10 mM KH_2PO_4 (pH 7.4) and 10 mM 2-mercaptoethanol, and lyzed by sonication (30 W, 3 × 20 s), and cellular debris was removed by centrifugation (100000*g*, 90 min). Streptomycin sulfate (20% w/v) was added to the cell-free extract to a final concentration of 1.0% (w/v), and after stirring for 10 min, precipitated nucleic acids and phospholipids were removed by centrifugation (20000*g*, 20 min). The clear supernatant was brought to 40% of saturation with solid ammonium sulfate and stirred for 10 min, and precipitated proteins were removed by centrifugation (20000*g*, 20 min). AICAR transformylase−IMP cyclohydrolase was then precipitated by increasing the ammonium sulfate concentration to 60% of saturation. After centrifugation (20000*g*, 20 min), the protein pellet was dissolved in 10 mM KH_2PO_4 (pH 7.4) and 10 mM 2-mercaptoethanol (5 mL) and dialyzed against

this buffer (2 L) overnight. The bifunctional enzyme was then purified by chromatography on a column of Affi-Gel Blue (1.4 × 19 cm; Allegra *et al.*, 1985) equilibrated with 10 mM KH_2PO_4 (pH 7.4), 100 mM KCl, and 10 mM 2-mercaptoethanol. The enzyme was eluted with a linear gradient (100 mL) from 100 to 1000 mM KCl in 10 mM KH_2PO_4 (pH 7.4) and 10 mM 2-mercaptoethanol at a flow rate of 40 mL/h. AICAR transformylase−IMP cyclohydrolase eluted with approximately 460 mM KCl. Fractions containing IMP cyclohydrolase activity were pooled and dialyzed against 7.5 mM KH_2PO_4 (pH 7.5) and 1 mM dithiothreitol (2 × 2 L) overnight. Purification of the bifunctional enzyme was completed by chromatography on a column of AICAR−Sepharose 4B (0.9 × 8.8 cm; Smith *et al.*, 1980) using a modification of the procedure of Mueller and Benkovic (1981). The column was equilibrated with 7.5 mM KH_2PO_4 (pH 7.5) and 1 mM dithiothreitol, and after application of the sample, it was washed with 7.5 mM Tris·HCl (pH 7.5) and 1 mM dithiothreitol (20 mL), and then 75 mM Tris·HCl (pH 7.5) and 1 mM dithiothreitol. The bifunctional enzyme was eluted with 75 mM Tris·HCl (pH 7.5), 10 mM AICAR, and 1 mM dithiothreitol from 4.5 to 9.0 mL. Fractions containing IMP cyclohydrolase activity were combined into three pools. Pool I, which eluted from 4.5 to 5.6 mL, was essentially homogeneous as indicated by SDS−polyacrylamide gel electrophoresis. The pure AICAR transformylase−IMP cyclohydrolase was concentrated, and the buffer was changed to 75 mM Tris·HCl, 75 mM KH_2PO_4 (pH 7.5), and 1 mM dithiothreitol using a Centricon 10 microconcentrator (Amicon Corp., Beverly, MA).

Assay of AICAR Transformylase and IMP Cyclohydrolase. During purification of the bifunctional enzyme, IMP cyclohydrolase was assayed spectrophotometrically at 250 nm using a difference in the extinction coefficients of FAICAR and IMP of 5710 M^{-1} cm^{-1} (Mueller & Benkovic, 1981), but ^{3}H-labeled substrates were used for sensitive assays (Szabados & Christopherson, 1994). Reaction mixtures for AICAR transformylase contained 50 mM K−Hepes (pH 7.4), 1 mM dithiothreitol, 5% (v/v) glycerol, 15 mM KCl, 500 μM N^{10}-formyltetrahydrofolate, and [^{3}H]AICAR (50 μM, 200 Ci/mol). Assay mixtures for IMP cyclohydrolase contained 50 mM K−Hepes (pH 7.4), 1 mM dithiothreitol, 5% (v/v) glycerol, and appropriate concentrations of [^{3}H]FAICAR (15 000 Ci/mol). Assay mixtures were incubated at 37 °C, and the reaction was initiated with 2 μL of enzyme to give a final volume of 25 μL. Samples (7 μL) were taken at three appropriate times and spotted onto poly(ethylene imine)−cellulose chromatograms which were developed by ascending chromatography with 9.6% (v/v) formic acid/methanol, 19:1 (v/v), after standing overnight in a saturated atmosphere of this solvent (Szabados & Christopherson, 1994). ^{3}H-Labeled spots were located by fluorography, cut out, and counted in scintillation cocktail (3.0 g of 2,5-diphenyloxazole/L of toluene) with an efficiency of 18.3%. Enzymic activity was determined by linear regression to the three time points for product formed *versus* time.

Determination of pK_a Values for FAICAR. FAICAR was titrated in the presence of an initial excess of HCl. The titration mixture (1.4 mL) contained 22.8 μmol of FAICAR, 68.4 μmol of HCl and 806 μmol of NaCl. A total of 100 μL of 1.6 M NaOH was added in 1 μL aliquots. After each addition, the titration mixture was equilibrated to 37 °C before measuring the pH. The NaOH solution was standardized by titration against potassium hydrogen phthalate which had been dried over P_2O_5. The HCl solution was standardized by titration against the NaOH.

Effect of pH on IMP Cyclohydrolase Activity. A three-buffer mixture which gave a constant ionic strength of 0.1 at all pH values (Ellis & Morrison, 1982) was used, and the pH was adjusted with KOH. For the pH range from 6.0 to 8.5, a mixture of 50 mM acetate, 50 mM 2-(*N*-morpholino)-ethanesulfonic acid (Mes), and 100 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) was used, and from pH 8.5 to 9.5 the buffer mixture was 100 mM 2-[(carbamoylmethyl)amino]ethanesulfonic acid (Aces), 52 mM Tris, and 52 mM ethanolamine. K−Hepes (50 mM) was used over an abbreviated pH range for comparison. IMP cyclohydrolase activity at each pH was determined as described above at 10 concentrations of FAICAR (0.1−8 μM). The reaction was initiated with 16.1−129 pg of pure bifunctional enzyme, depending upon the pH of the assay. Values for K_s and V_{max} at each pH were obtained by nonlinear regression analysis to the Michaelis−Menten equation using the program DNRP53 (Duggleby, 1984).

Polyacrylamide Gel Electrophoresis. Fractions from the purification of the bifunctional enzyme were analyzed by SDS−polyacrylamide gel electrophoresis using the discontinuous buffer system described by Laemmli (1970) with 4% (w/v) stacking and 10% (w/v) resolving polyacrylamide gels. Protein samples (7 μL) were mixed with an equal volume of sample buffer [150 mM Tris·HCl (pH 8.8), 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol], electrophoresed, stained with Coomassie Blue R250, and then silver-stained (Gorg *et al.*, 1985). The purified bifunctional enzyme was also analyzed by native polyacrylamide gel electrophoresis. Duplicate samples of enzyme in 50 mM K−Hepes (pH 7.4), 20% (v/v) glycerol, and 2 mM dithiothreitol were electrophoresed through a 6% (w/v) resolving gel (Laemmli, 1970) without SDS. One electropherogram was silver-stained, and the other was cut into 3 mm segments which were extracted in 50 mM K−Hepes (pH 7.4) and assayed for AICAR transformylase and IMP cyclohydrolase activities as described above.

Protein Assays. The purified bifunctional enzyme and varying amounts of bovine serum albumin were electrophoresed on an SDS−polyacrylamide gel as described above. The Coomassie Blue stained gel was scanned at 595 nm using a Molecular Dynamics personal densitometer (Molecular Dynamics Inc., Sunnyvale, CA). The protein band densities were quantified using the program ImageQuant, and a plot of the protein band density *versus* amount of bovine serum albumin was used to determine the concentration of the bifunctional enzyme.

Chemical Modifications of Specific Amino Acid Residues with ^{14}C-Labeled Reagents. The conditions used were the same as for unlabeled reagents, except 188 or 375 ng of pure AICAR transformylase−IMP cyclohydrolase was used with 5 mM [^{14}C]phenylglyoxal (24 Ci/mol) or [^{14}C]*N*-ethylmaleimide (7.4 Ci/mol), respectively. After incubation at 37 °C for 9.5 min, an equal volume of 10% (w/v) trichloroacetic acid was added and left on ice for 30 min, and then the precipitated AICAR transformylase−IMP cyclohydrolase was collected by centrifugation (9000*g*, 20 min). The protein pellet was washed with 5% (w/v) trichloroacetic acid 10 times, when soluble radioactivity was minimal. The pellet was then dissolved in 50 μL of 1 M NaOH, neutralized with 50 μL HCl, transferred to a scintillation vial, and counted in

scintill tion cocktail [3.0 g of (2,5-diphenyloxazole−toluene)/Triton X-100 (2:1, v/v)].

Analysis of Titration Data. The second and third acidic groups of FAICAR (S) were titrated with NaOH and the data analyzed as follows:

$$SH_2 \overset{K_{a2}}{\leftrightarrow} SH^- \overset{K_{a3}}{\leftrightarrow} S^{2-} \quad (1)$$

From expressions for these equilibria and conservation of the total concentration of FAICAR, $[S_t]$, the initial total negative charge in the titration mixture, neglecting added NaCl, is:

$$C_i = \frac{K_w}{[H^+_i]} - [H^+_i] + [S_t]\frac{\left(\frac{K_{a2}}{[H^+_i]} + \frac{2K_{a2}K_{a3}}{[H^+_i]^2}\right)}{\left[1 + \left(\frac{K_{a2}}{[H^+_i]}\right)\left(1 + \frac{K_{a3}}{[H^+_i]}\right)\right]} \quad (2)$$

where $[H^+_i]$ is the hydrogen ion concentration at the start of the titration. After addition of b mol of NaOH, an expression for the final total negative charge in the titration mixture, C_f, may be obtained by substituting $[H^+_f]$ for $[H^+_i]$ in eq 2. The increase in total negative charge in the mixture between the initial and final states is equal to the amount of NaOH (b) added:

$$b = C_f - C_i \quad (3)$$

Substitution of the initial and final versions of eq 2 into eq 3 gives an expression for b in terms of initial and final pH, total FAICAR concentration, $[S_t]$, and pK_{a2} and pK_{a3}. Titration data were fitted to eq 3 by nonlinear regression using the program DNRP53 (Duggleby, 1984).

Analysis of Kinetic Data. Experimental data were fitted to the appropriate velocity equation using the program DNRP53 for nonlinear regression analysis (Duggleby, 1984) with all data points given equal weight. It has been assumed that catalysis by IMP cyclohydrolase is much slower than the binding of substrates or inhibitors and that all species of the enzyme are in rapid equilibrium. With this assumption, K_s is the dissociation constant for the substrate, FAICAR (S). Data for the pH dependencies of V_{max} and V_{max}/K_s values of IMP cyclohydrolase were fitted to the equation

$$\log(Y) = \log(Y_m) - \log(1 + [H^+]/K_a) \quad (4)$$

where Y and Y_m represent the values and limiting values, respectively, of V_{max} and V_{max}/K_s (Cleland, 1977). Apparent values for V_{max} and V_{max}/K_s over a pH range from 6.1 to 9.3 were obtained by fitting reaction velocities (v) at 10 FAICAR concentrations to the Michaelis−Menten equation.

Data obtained for competitive inhibition of IMP cyclohydrolase (E) by purine nucleotide derivatives (I) with FAICAR (S) as the varied substrate were fitted to the equation:

$$\frac{v}{V_{max}} = \frac{S}{S + (1 + I/K_i)K_s} \quad (5)$$

where K_s and K_i are dissociation constants for the ES and EI complexes, respectively.

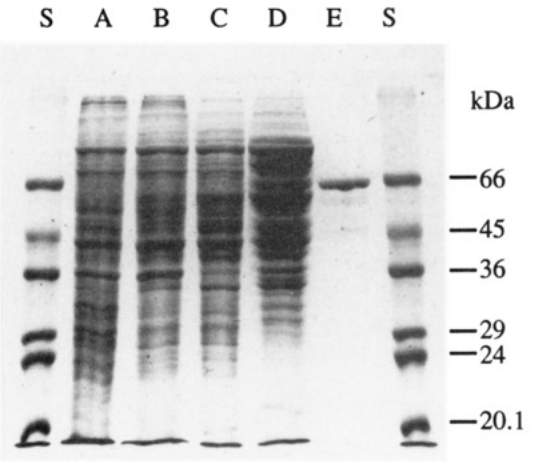


FIGURE 1: SDS−polyacrylamide gel electrophoresis of fractions from the purification of AICAR transformylase−IMP cyclohydrolase. Proteins were stained with Coomassie Blue R250; further details are provided in the Experimental Procedures. S, the standard proteins: bovine albumin, 66.0 kDa; egg albumin, 45.0 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36.0 kDa; carbonic anhydrase, 29.0 kDa; trypsinogen, 24.0 kDa; trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.2 kDa. A, cell-free extract; B, streptomycin sulfate supernatant; C, ammonium sulfate fraction; D, pool from Affi-Gel Blue column; E, pool from AICAR−Sepharose 4B column.

Data for first-order inactivation of IMP cyclohydrolase by chemical modification were fitted to the equation:

$$A = A_0 e^{-kt} \quad (6)$$

where A_0 is the initial enzymic activity, A is the activity at time t, and k is the first-order rate constant for inactivation.

Metabolic Effects of Purine Nucleoside Derivatives. Human CCRF-CEM leukemia cells were grown as described above in two parallel cultures (55 mL) to a density of 7×10^5 cells/mL when a nucleoside (25 μM) was added to one of the cultures. After 4 h exposure to the drug, samples (50 mL) of the control and drug-treated cultures were taken, and metabolites were extracted in ice-cold 0.4 M $HClO_4$ which was then neutralized (Sant *et al.*, 1989). Acid-soluble metabolites were separated by gradient anion exchange HPLC on a Partisil 10-SAX column (25 × 0.46 cm) and quantified using a Spectra-Physics UV2000 ultraviolet detector (Fremont, CA), an LKB Model 2140 Rapid Spectral Detector (Bromma, Sweden), and an LKB Model 1208 Betacord radioactivity monitor (Wallac Oy, Turku, Finland) connected in series as described previously (Sant *et al.*, 1989).

RESULTS

The purification procedure of Allegra *et al.* (1985) for AICAR transformylase−IMP cyclohydrolase using chromatography on Affi-Gel Blue did not yield homogeneous bifunctional enzyme from human MCF-7 breast cancer cells. However, combination of the Affi-Gel Blue column with the AICAR−Sepharose column used by Mueller and Benkovic (1981), with some modification of the elution conditions, did yield apparently pure bifunctional enzyme from human CCRF-CEM leukemia cells (Figure 1E). Comparison of the mobility of the pure human enzyme with that of standard proteins indicated a subunit molecular mass of 62.1 kDa compared with 64.4 kDa for the chicken enzyme, derived

Table 1: Purification of AICAR Transformylase−IMP Cyclohydrolase[a]

fraction	protein concn (mg/mL)	total protein (mg)	AICAR transformylase sp act. (pmol min^{-1} μg^{-1})	AICAR transformylase total act. (nmol/min)	AICAR transformylase purifn factor	IMP cyclohydrolase sp act. (pmol min^{-1} μg^{-1})	IMP cyclohydrolase total act. (nmol/min)	IMP cyclohydrolase purifn factor	ratio of specific activities
cell-free extract	13.4	89.9	1.07	96.2	1.0	37.7	3390	1.0	1:35
streptomycin sulfate supernatant	9.14	56.7	2.25	127	2.1	63.1	3580	1.7	1:28
ammonium sulfate fraction	3.57	17.1	4.77	81.6	4.5	118	2020	3.1	1:25
Affi-Gel Blue column									
predialysis	0.0371	1.10	93.0	102	87	1230	1340	33	1:13
postdialysis	0.0374	1.10	76.8	84.5	72	1710	1880	45	1:22
AICAR−Sepharose 4B									
pool I	0.0386	0.0108	670	7.24	630	29000	313	780	1:44

[a] The bifunctional enzyme was purified from a cell-free extract of human CCRF-CEM leukemia cells (2×10^9 cells/mL) in 4 steps. Enzymic activities presented in this table were determined by radioassay, but the enzyme was quickly located in fractions by the spectrophotometric assay for IMP cyclohydrolase. Further details appear under Experimental Procedures.

from the nucleotide sequence of the cDNA (Ni *et al.*, 1991). The bifunctional enzyme has been purified 780-fold in four steps from a cell-free extract with maintenance of the enzymic activities, AICAR transformylase and IMP cyclohydrolase, in an initial ratio of 1:35 to a ratio of 1:44 for the pure enzyme (Table 1). AICAR transformylase was inhibited by the KCl used for elution of the Affi-Gel Blue column, and there was some loss of this activity in the pure enzyme when compared with that of the original extract. The yield of pure enzyme from the cell-free extract, based upon IMP cyclohydrolase activity, was 9.2%.

Mueller and Benkovic (1981) found that the AICAR transformylase and IMP cyclohydrolase activities from chicken liver copurified to apparent homogeneity, and they concluded that both activities reside on the same polypeptide. These two enzymic activities also copurified from human leukemia cells (Table 1). To confirm that AICAR transformylase and IMP cyclohydrolase activities are carried by a single protein, the purified bifunctional protein was subjected to polyacrylamide gel electrophoresis under nondenaturing conditions. Only one protein band was visible on the silver-stained gel, and peaks for the two enzymic activities, determined from a duplicate track, coincided with this band (data not shown).

The spectrophotometric assay for IMP cyclohydrolase was used to rapidly monitor levels of the bifunctional protein in fractions during the purification but is not sufficiently sensitive for kinetic studies. Mueller and Benkovic (1981) reported a K_s value of IMP cyclohydrolase for FAICAR of less than 1 μM. Using [^{3}H]FAICAR (15 000 Ci/mol) as substrate, a radioassay for IMP cyclohydrolase was developed where the [^{3}H]IMP produced was separated from [^{3}H]-FAICAR by thin-layer chromatography on poly(ethylene imine)−cellulose (Szabados & Christopherson, 1994). A K_s value of 0.87 ± 0.11 μM was determined for FAICAR by this procedure.

To gain insight into the functional groups of purine nucleotide derivatives required for binding at the active site of IMP cyclohydrolase, 19 purine nucleoside 5′-monophosphate derivatives were tested as inhibitors. Assay mixtures, in triplicate, contained 1 μM [^{3}H]FAICAR (15 000 Ci/mol) and 20 μM purine nucleotide derivative. Purine ribotide, 6-chloropurine ribotide, 6-methylmercaptopurine ribotide, 8-azidoadenosine 5′-monophosphate, 8-bromoguanosine 5′-monophosphate, and N^6-methyladenosine 5′-monophosphate did not inhibit IMP cyclohydrolase. N^2-Acetyl-2′-deoxyguanosine 5′-monophosphate, AICAR, AMP, *N*-succino-AMP, IMP, 2′-deoxy-AMP, and GMP decreased IMP cyclohydrolase activity by 10−50%. The potent inhibitors (K_i < 5 μM) identified are listed in Table 2, and their chemical structures are shown in Figure 2. The most potent inhibitor was 2-mercaptoinosine 5′-monophosphate (K_i = 0.094 ± 0.024

Table 2: Inhibition Constants for Potent Inhibitors of IMP Cyclohydrolase[a]

purine nucleotide derivative	dissociation constant [K_s or K_i (μM)]
FAICAR	0.87 ± 0.11
2-mercaptoinosine 5′-monophosphate	0.094 ± 0.024
xanthosine 5′-monophosphate	0.12 ± 0.01
2-fluoroadenine arabinoside 5′-monophosphate	0.16 ± 0.02
6-mercaptopurine riboside 5′-monophosphate	0.20 ± 0.02
adenosine N^1-oxide 5′-monophosphate	0.28 ± 0.03
N^6-(carboxymethyl)adenosine 5′-monophosphate	1.70 ± 0.42

[a] Inhibition patterns in double-reciprocal form were constructed at five FAICAR concentrations (0.1−0.5 μM) and 3 or 4 appropriate inhibitor concentrations using the radioassay for IMP cyclohydrolase and 24.1 pg of pure bifunctional enzyme in assay mixtures of 25 μL. All patterns intersected on the $1/v$ ordinate, indicating competitive inhibition, and data were fitted to eq 5 using the program DNRP53 to obtain values for the inhibition constant (K_i). The dissociation constant (K_s) for FAICAR is included for comparison.

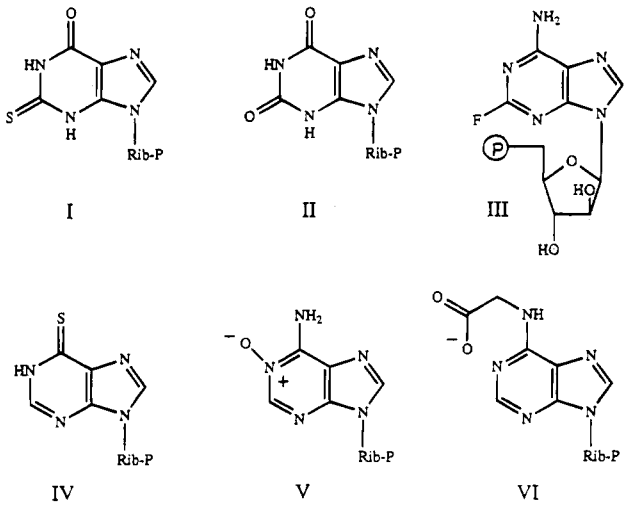


FIGURE 2: Chemical structures of potent inhibitors of IMP cyclohydrolase. **I**, 2-mercaptoinosine 5′-monophosphate; **II**, xanthosine 5′-monophosphate (XMP); **III**, 2-fluoroadenine arabinoside 5′-monophosphate; **IV**, 6-mercaptopurine riboside 5′-monophosphate; **V**, adenosine N^1-oxide 5′-monophosphate; and **VI**, N^6-(carboxymethyl)adenosine 5′-monophosphate.

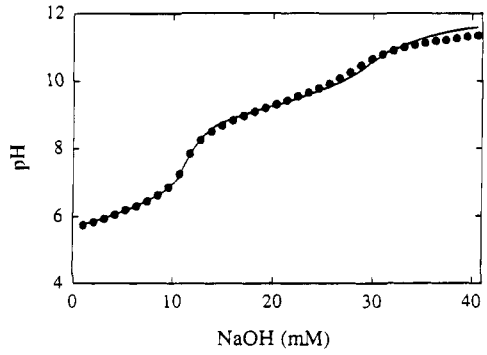


FIGURE 3: Titration of FAICAR with NaOH. Titration mixtures contained, in a total volume of 1.5 mL, 22.8 μmol of FAICAR (disodium salt), 68.6 μmol of HCl, and 806 μmol of NaCl, and NaOH (1.6 M) was added as indicated. The amount of NaOH required to titrate the first phosphate proton of FAICAR has been subtracted before plotting the μmol of NaOH. These data were fitted by nonlinear regression to eq 3 to yield the following values: pK_{a2} = 5.81 ± 0.03, pK_{a3} = 9.41 ± 0.04, and total FAICAR [S_t] = 19.1 ± 0.3 μmol, which were used to generate the theoretical curve through the experimental data.

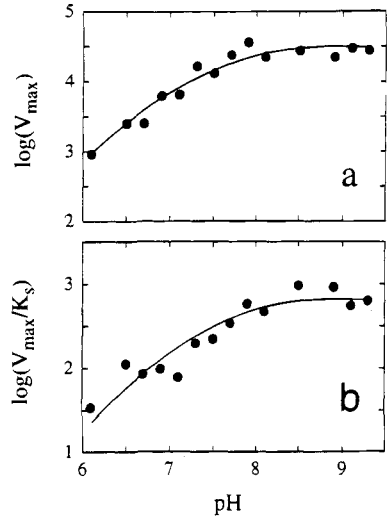


FIGURE 4: Effects of pH upon V_{max} and V_{max}/K_s of IMP cyclohydrolase. The pH values of assay mixtures were maintained with a three-buffer mixture with an ionic strength of 0.1 as described in the Experimental Procedures, and data were fitted to eq 4 to generate the theoretical curves.

μM), and five of these nucleotides have dissociation constants which are less than that of the substrate, FAICAR (K_s = 0.87 ± 0.11 μM).

Information about the functional groups at the active site of IMP cyclohydrolase involved in binding and catalysis was obtained from the pH dependencies of V_{max} and V_{max}/K_s over the pH range 6.1−9.3. The ionization state of the substrate, FAICAR, over this pH range could influence the data obtained, and pK_a values for FAICAR were therefore determined by titration (Figure 3). Data for pH *versus* the amount of 1.6 M NaOH added were fitted to eq 3, and values for pK_{a2} and pK_{a3} of 5.81 ± 0.03 and 9.41 ± 0.04 were determined. Using a three-buffer mixture to maintain constant ionic strength from pH 6.1 to 9.3 (Ellis & Morrison, 1982), the apparent V_{max} is low at pH 6.1 and maximal and constant at alkaline pH (Figure 4a). These data were fitted to eq 4 to give Y_m = (3.12 ± 0.38) × 10^4 pmol min^{-1} μg^{-1} and pK_a = 7.57 ± 0.09, and these parameters were used to draw the theoretical curve through the data of Figure 4a. Values for V_{max}/K_s are also low at acidic pH and are maximal at alkaline pH. These data were also fitted to eq 4 to give Y_m = 691 ± 131 min^{-1} and pK_a = 7.57 ± 0.14.

Table 3: Inactivation of IMP Cyclohydrolase by Reagents Specific for Particular Amino Acid Residues[a]

amino acid residue	pH	preincubation conditions	k (min^{-1})	k/k_0
arginine	8.0	control	0.0356	
		phenylglyoxal	0.166	4.66
		phenylglyoxal + FAICAR	0.0267	0.75
lysine	8.2	control	0.0576	
		citraconic anhydride	0.115	2.00
tyrosine	7.4	control	0.0469	
		N-acetylimidazole	0.0808	1.72
cysteine	7.0	control	0.0116	
		N-ethylmaleimide	0.156	13.4
		N-ethylmaleimide + FAICAR	0.0135	1.16
histidine	6.0	control	0.0504	
		diethyl pyrocarbonate	0.148	2.94
		diethyl pyrocarbonate + FAICAR	2.22	44.0

[a] Pure bifunctional enzyme (2.90 ng) was preincubated at the indicated pH with the modification reagent (5 mM) in the absence and presence of FAICAR (10 μM, 5000 Ci/mol), and 4 samples (5 μL) were taken at 3 min intervals from 30 s for radioassay of IMP cyclohydrolase. A third, control preincubation indicated the stability of the enzyme of that pH. Data for IMP cyclohydrolase activity as a function of time were fitted to eq 6 for exponential decay with the program DNRP53 to obtain first-order rate constants (k). The ratio k/k_0 indicates the rate of decay relative to the control.

To determine which types of amino acid residues are essential for IMP cyclohydrolase activity and whether they are at the active site, the pure enzyme was incubated with a variety of reagents which chemically modify particular amino acids (Table 3; Lundblad & Noyes, 1984). IMP cyclohydrolase was incubated at a pH suitable for the particular modification but without the modification reagent, and the first-order rate constant (k_0) was determined for decay of enzymic activity. First-order rate constants (k) were also determined for enzyme incubated with the modification reagent, and if significant inactivation occurred relative to the control ($k/k_0 > 2$), then enzyme was also incubated with the reagent plus [^{3}H]FAICAR (10 μM, 5000 Ci/mol). Protection of the enzyme by the substrate against inactivation by the reagent would provide evidence that the amino acid susceptible to modification was at the active site or essential for maintaining the active conformation of the enzyme (Table 3). IMP cyclohydrolase was inactivated by phenylglyoxal, and FAICAR protected against this inactivation, providing evidence for an arginine residue at the active site. *N*-Ethylmaleimide rapidly inactivated the enzyme, and again FAICAR protected the enzyme, consistent with a cysteine residue at the active site. Diethyl pyrocarbonate slowly inactivated the enzyme, but the presence of FAICAR stimulated the inactivation 15-fold. These data provide evidence for a histidine residue essential to catalytic activity which is not at the active site and may become more exposed due to a conformational change induced by the binding of FAICAR. To determine how many arginine and cysteine residues were modified, purified AICAR transformylase−IMP cyclohydrolase was incubated with [^{14}C]phenylglyoxal and [^{14}C]*N*-ethylmaleimide, precipitated, and washed extensively prior to determination of the molar ratio of reagent/enzyme subunit, assuming that 2 mol of phenylglyoxal or 1 mol of *N*-ethylmaleimide modifies an arginine or cysteine residue, respectively, per mole of enzyme subunit. [^{14}C]-Phenylglyoxal (5 mM, 24 Ci/mol) modified 12.4 ± 0.4 (*n*

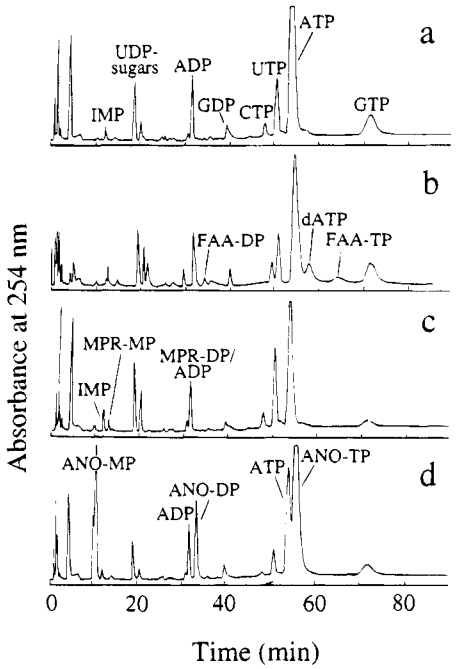


FIGURE 5: Metabolic effects of purine nucleoside derivatives upon human CCRF-CEM leukemia cells. The drug (25 μM) was added to leukemia cells at a density of 7×10^5 cells/mL and after 4 h, samples (50 mL) were taken and cell extracts prepared as described in the Experimental Procedures. Metabolites were separated by gradient anion exchange HPLC and quantified by ultraviolet absorbance. (a) Control, (b) 2-fluoroadenine arabinoside, (c) 6-mercaptopurine riboside, and (d) adenosine N^1-oxide.

= 3) residues/subunit of AICAR transformylase−IMP cyclohydrolase, and the presence of FAICAR (10 μM) decreased the ratio to 10.5 ± 0.7 (n = 3) residues/subunit. [^{14}C]*N*-Ethylmaleimide (5 mM, 7.4 Ci/mol) modified 10.7 ± 0.5 (n = 3) residues/subunit of AICAR transformylase−IMP cyclohydrolase, and the presence of FAICAR (10 μM) decreased the ratio to 9.0 ± 1.0 (n = 3) residues/subunit.

The purine nucleotide derivatives **I**−**VI** (Figure 2) would not readily enter human cells because of the 5′-phosphate group. Therefore, the equivalent nucleosides were obtained, or prepared enzymically from the nucleotides (**III**, **VI**). IC_{50} values were determined for the six nucleosides. **I**, **II**, and **VI** did not inhibit cellular growth up to concentrations of at least 40 μM while IC_{50} values were obtained for **III**, 0.20 μM; **IV**, 7.5 μM; and **V**, 1.1 μM. The metabolic effects of these six purine nucleoside derivatives were determined with human CCRF-CEM leukemia cells growing in culture, as described in the Experimental Procedures, using techniques established in our laboratory (Sant *et al.*, 1992). Cells were exposed to drug (25 μM) for 4 h with an untreated control culture grown in parallel, and perchloric acid extracts were prepared, neutralized, and analyzed by gradient anion exchange HPLC. Nucleoside mono-, di-, and triphosphates in cell extracts were detected by their absorbance at 254 nm. Comparison of drug-treated elution profiles with the control profile showed that **I**, **II**, and **VI** appeared to have no effect while 2-fluoroadenine arabinoside (**III**), 6-mercaptopurine riboside (**IV**), and adenosine N^1-oxide (**V**) induced significant effects (Figure 5). Phosphorylated forms of these three purine nucleoside derivatives were identified as new peaks in the mono-, di-, or triphosphate regions of the ultraviolet elution profiles. Use of an on-line ultraviolet diode array detector showed that these phosphorylated drugs had wavelengths of maximal absorbance (λ_{max} values) which corresponded with the parent nucleoside: For 2-fluoroadenine arabinoside, λ_{max} = 259 nm; 6-mercaptopurine riboside, λ_{max} = 320 nm; and adenosine N^1-oxide, λ_{max} = 231 nm. Cellular concentrations of these phosphorylated drugs were determined by comparison with standards and are presented in Table 4. If IMP cyclohydrolase were inhibited in growing leukemia cells by high concentrations of the monophosphate derivatives of these drugs (6-mercaptopurine 5′-monophosphate and adenosine N^1-oxide 5′-monophosphate, Table 4), a significant accumulation of FAICAR and perhaps AICAR should be found in cell extracts. FAICAR and AICAR elute just before IMP within the first 12 min of the elution profiles of Figure 5. No accumulations of these purine precursors were seen in any of the six nucleoside-treated cultures, even when cells were grown in the presence of [^{14}C]bicarbonate (150 μM, 55.8 Ci/mol) or [^{32}P]orthophosphate (1.1 μM, 9.13 × 10^6 Ci/mol) to radiolabel these intermediates (data not shown; Brooke *et al.*, 1990).

Table 4: Cellular Concentrations of Phosphorylated Forms of Purine Nucleoside Derivatives[a]

purine nucleoside derivative	cellular concentration (μM)		
	monophosphate	diphosphate	triphosphate
2-fluoroadenine arabinoside	*b*	84	140
6-mercaptopurine riboside	690	64	*b*
adenosine N^1-oxide	2800	1100	7800

[a] Human CCRF-CEM leukemia cells were exposed to the six purine nucleosides (25 μM, 4 h) derived from the nucleotides of Figure 2. Samples (50 mL) of these cultures were taken, and extracts were analyzed by HPLC as shown in Figure 5. Peaks for phosphorylated forms of the nucleosides, observed for three of the drugs, were integrated and expressed as cellular concentrations by comparison with appropriate standards and using a cellular volume of 0.42 pL (R. Z. Shi and R. I. Christopherson, unpublished experiments). [b] Not detectable (see Figure 5).

DISCUSSION

The availability of pure, human AICAR transformylase−IMP cyclohydrolase has enabled a detailed study of the catalytic properties of the latter enzymic activity. Six potent inhibitors of IMP cyclohydrolase have been discovered (Figure 2, Table 2) which provide valuable information about the structural requirements for binding at this active site. The basic purine heterocyclic structure is required with a ribose or arabinose 5′-monophosphate group. An electronegative substituent, such as sulfur, oxygen, or fluorine, in the 2-position on the purine ring with an oxygen or amino group in the 6-position (Figure 2, **I**, **II**, or **III**) gave the most potent inhibition (Table 2). All inhibitors (**I**−**VI**) have electronegative substituents in the 6-position and adenosine N^1-oxide 5′-monophosphate (**V**) has a charge separation at the 1-position (N^+-O^-) which could be similar to an intermediate along the reaction pathway FAICAR → IMP. XMP (**II**) is a naturally-occurring intermediate in the reaction sequence IMP → XMP → GMP, and the K_i value obtained of 120 nM (Table 2) suggests that this inhibition could have regulatory significance in growing cells, perhaps when L-glutamine levels limit the conversion of XMP → GMP. Two of the inhibitors, **III** as the nucleotide and **IV** as the nucleobase, are in clinical use as anticancer drugs (Cheson, 1992; Stet *et al.*, 1991), but none of the six nucleoside

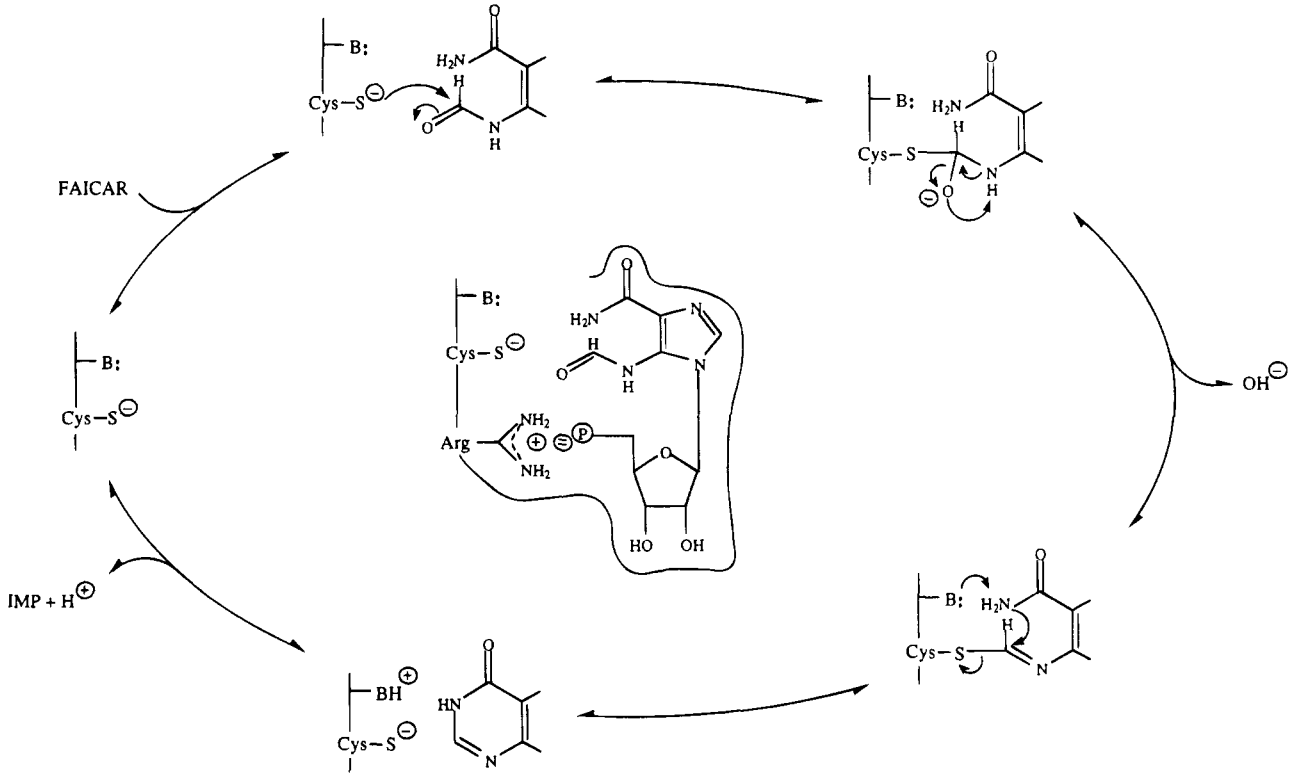


FIGURE 6: Proposed active site of IMP cyclohydrolase and mechanism of catalysis.

derivatives induced inhibition of IMP cyclohydrolase in growing leukemia cells. However, it is of interest to note that 2-fluoroadenine arabinoside induced a 3-fold accumulation of dATP (Figure 5b) consistent with inhibition of DNA synthesis by the triphosphate derivative of the drug. Multiple sites of inhibition induced by 6-mercaptopurine in the *de novo* purine pathway have been proposed (Weber, 1983), but the similarity of Figure 5c to Figure 5a suggests that misincorporation of mercaptonucleotides into DNA might be the primary mechanism of toxicity. These six purine analogs are inhibitors of IMP cyclohydrolase *in vitro* (Figure 2), but may not inhibit the conversion of FAICAR → IMP in growing leukemia cells because (1) some of the nucleosides were not taken up by cells and/or were not subsequently phosphorylated; (2) the active site of IMP cyclohydrolase may not be directly accessible to inhibitors in growing cells due to formation of a multienzyme complex for purine nucleotide biosynthesis (Rowe *et al.*, 1978); or (3) the specific enzymic activity of IMP cyclohydrolase is 44-fold higher than that of AICAR transformylase (Table 1), and more potent inhibitors (K_i < 94 nM, Table 2) would be required to block the pathway. The much higher specific enzymic activity of IMP cyclohydrolase could account for the absence of FAICAR in growing leukemia cells.

The effects of pH upon V_{max} and V_{max}/K_s for IMP cyclohydrolase (Figure 4) are consistent with the ionization of a single amino acid residue of the enzyme which must be unprotonated for the binding of substrate (V_{max}/K_s, Figure 4b, $pK_a = 7.57 \pm 0.14$) and for catalysis to occur (V_{max}, Figure 4a, $pK_a = 7.57 \pm 0.09$). The identical pK_a values obtained for both data sets and the upward slope of 1.0 for Figure 4 strongly suggest that the binding of FAICAR does not affect the ionization of this residue. The pK_a values obtained for FAICAR ($pK_{a2} = 5.81 \pm 0.03$, $pK_{a3} = 9.41 \pm 0.04$, Figure 3) indicate that the ionization state of the substrate would not significantly influence the pH profiles obtained for V_{max} and V_{max}/K_s (Figure 4). The value for pK_{a2} of 5.81 would represent the second ionization of the 5′-phosphate group while the pK_{a3} of 9.41 represents dissociation of the N=C(H)N= proton of the imidazole ring of FAICAR. Thus, within the range of pH values used (Figure 4), the dianion of FAICAR would predominate over the monoanion by 5-fold at pH 6.5 and 15-fold at pH 7.

The identity of the amino acid residue with a pK_a of 7.57, which is required in the basic form for catalysis, might be determined by chemical modification of IMP cyclohydrolase with reagents specific for particular amino acids. Strong evidence for the presence of arginine and cysteine residues at the active site was obtained from these experiments (Table 3). The presence of FAICAR (10 μM) reduced the ratio of residues modified/subunit of AICAR transformylase−IMP cyclohydrolase by 1.9 arginines and 1.7 cysteines, consistent with the presence of these residues at the active site or being required to maintain the active conformation of IMP cyclohydrolase. An arginine side chain would be too basic to have a pK_a of 7.57, but cysteine, in an appropriate microenvironment at the active site, could be required in the basic (CH_2S^-) form for catalysis. An arginine residue with a positive charge could interact with the negatively-charged 5′-phosphate of the substrate, FAICAR. The binding of FAICAR may induce a conformational change in the enzyme, indicated by a 15-fold increase in reactivity of a remote histidine residue. IMP cyclohydrolase was not inhibited by a variety of metal chelators, suggesting that an enzyme-bound metal atom was not involved in catalysis.

The data presented in this paper provide information about the catalytic mechanism of IMP cyclohydrolase. Purine nucleoside derivatives, lacking the 5′-phosphate group, do not inhibit the enzyme, and an electrostatic interaction may occur between this negatively-charged phosphate and a

positively-charged arginine residue of the active site (Figure 6). All inhibitors (Figure 2) have electronegative substituents in the 6-position, and a complementary pocket may be found at the active site. We propose that a cysteine residue at the active site has a pK_a of 7.57 and that this cysteine must be in the thiolate (S^-) form for productive binding of FAICAR as shown for the catalytic cycle of Figure 6. The initial step of catalysis could be nucleophilic attack by the thiolate anion on the 5-formamido carbon of FAICAR with formation of a tetrahedral, oxyanion transition state, a mechanism which bears some resemblance to a reversal of the mechanism for a thiol protease (Walsh, 1979). However, there is currently no experimental data for such a covalent transition state. Ni *et al.* (1991) have aligned the amino acid sequences for AICAR transformylase−IMP cyclohydrolase from chicken, *Escherichia coli,* and *Bacillus subtilis*, which contain 9, 7, and 1 cysteine residues, respectively. The single cysteine of the *B. subtilis* enzyme was aligned with a cysteine of the *E. coli* sequence and was 14 residues in the N-terminal direction from cysteine 288 of the chicken sequence. A minor realignment of these three sequences would maintain the approximately 36% sequence similarity between the avian and bacterial enzymes but would conserve a cysteine residue required for the catalytic mechanism of IMP cyclohydrolase. The alignment published by Ni *et al.* (1991) showed 6 arginine residues conserved between the three species; chemical modification of the human bifunctional enzyme (Table 3) indicates that an arginine residue(s) is essential for the catalytic activity of IMP cyclohydrolase. Modification experiments with [^{14}C]phenylglyoxal and [^{14}C]*N*-ethylmaleimide suggest that the human enzyme contains more arginine and cysteine residues than the enzymes discussed above.

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