On the Purification and Mechanism of Action of 5-Aminoimidazole-4-carboxamide–Ribonucleotide Transformylase from Chicken Liver†

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ABSTRACT: The transformylase from chicken liver catalyzing the formylation of 5-aminoimidazole-4-carboxamide ribonucleotide through the agency of 10-formyltetrahydrofolate has been purified to apparent homogeneity. Inosinicase activity copurifies. This transformylase is not further activated kinetically by the presence of the trifunctional protein in contrast to the glycinamide ribonucleotide transformylase. The enzyme exhibits a >1000-fold preference for the naturally occurring 10-formyltetrahydrofolate cofactor and a sequential reaction pattern. A reinvestigation of the chemical structure of the formylated ribotide product employing ^{13}C and ^{1}H NMR indicated that the imidazole ring remained intact upon formylation, consistent with the originally proposed structure.

5-Amino-4-imidazolecarboxamide–ribonucleotide transformylase (EC 2.1.2.3, AICAR TFase),[1] one of two reduced folate-requiring transformylases involved in purine biosynthesis, catalyzes the formylation of AICAR with 10-formyl-H_4folate to produce FAICAR and H_4folate. The enzyme has been partially purified from chicken liver by Flaks et al. (1957), by Baggott & Krumdieck (1979a) (41- to 158-fold, respectively), and 63-fold from Ehrlich Ascites tumor cells by Geiger & Guglielini (1975). Inosinicase (EC 3.5.4.10, IMP cyclohydrolase) immediately follows AICAR TFase in the purine biosynethic pathway, cyclizing FAICAR to IMP. In all of the above purifications this enzyme was reported to copurify with the transformylase (Baggott & Krumdieck, 1979b), which gave rise to speculation that the two activities were either tightly associated or were on the same protein.

Recently the nature of the chemical reaction catalyzed by these two enzymes has been questioned. The chemical formylation of AICAR was reported to produce a compound other than the expected product (FAICAR). This new compound in which the imidazole residue of FAICAR has undergone hydrolytic cleavage was postulated to readily form IMP in the presence of inosinicase (Baggott & Krumdieck, 1979b).

In this paper we report a new mild purification of AICAR TFase utilizing affinity chromatography which yields a homogeneous protein. The kinetic parameters and stoichiometry of the enzymatic reaction were determined, as well as the stereochemical requirement of the cofactor. Since the other transformylase of purine biosynthesis (GAR TFase) has been found to be involved in a functional enzyme complex (Smith et al., 1980), this possibility also was investigated for AICAR TFase. In addition, we have reexamined the chemical structure proposed for FAICAR and sought evidence for the locus of the inosinicase activity present during the purification.

Experimental Procedures

Materials

5-Aminoimidazole-4-carboxamide-β-1-D-ribofuranosyl 5′-monophosphate (Ba^{2+} salt) (AICAR) was prepared by phosphorylation of the corresponding riboside (Yoshikawa et al., 1967; Murray & Atkinson, 1968). The ribotide was further purified by chromatography on a DEAE-cellulose column (2.5 × 17 cm) using a linear gradient made from 250 mL of H_2O and 250 mL of 3 M ammonium acetate, pH 3.0. AICAR–Sepharose was prepared according to the method of Smith et al. (1980). (±)-L-5,10-Methenyl-H_4folate was prepared by the procedure of Rowe (1968). (±)-L-10-Formyl-H_4folate was prepared by hydrolysis of (±)-L-5,10-methenyl-H_4folate in 50 mM Tris-HCl pH 7.4, 0.1 2-ME for 1 h similar to the method of Rowe (1971). (+)-L-5,10-Methenyl-H_4folate was prepared from folic acid by the method of Blakley (1960), Mathews & Huennekens (1960), and Rowe (1968). (−)-L-5,10-Methenyl-H_4folate was a gift of Dr. John Montgomery, Southern Research Institute. (+)-L-5,10-Methenyl-H_4pteroyltriglutamate was furnished by Gail Wasserman. Purified trifunctional 5,10-methenyl, 5,10-methylene, 10-formyl-H_4folate synthetase (trifunctional protein) was prepared as described by the Smith et al. (1980).

AICAR riboside, tetrahydrofolic acid (H_4folate), folic acid, type II-O ovomucoid, α-1-antitrypsin, aprotinin, pepstatin, protamine sulfate, and bovine serum albumin were purchased from Sigma Chemical Co. Sephadex G-25 (coarse), Sepharose 4B, and QAE-Sephadex A-25 were purchased from Pharmacia Fine Chemicals. Whatman DEAE-cellulose (DE-52) and cellulose CF-1 were purchased from Whatman Ltd., England. Bio-Gel HTP and ammonium persulfate for electrophoresis were purchased from Bio-Rad Laboratories. All other electrophoresis chemicals were purchased from Aldrich Chemical Co. Potassium formate (90% ^{13}C) was purchased from Kor Isotopes. Dimethyl 3,3′-dithiobis(propionimidate) dihydrochloride was purchased from Pierce Chemical Co. All other chemicals were of the highest grade commercially available.

Synthesis of FAICAR. FAICAR was prepared from AICAR by the method of Shaw & Wilson (1962). To a 0.25-g sample of AICAR (Ba^{2+} salt) was added 20 mL of 96% formic acid. After the AICAR dissolved, 10 mL of acetic anhydride

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[1] Abbreviations used: AICAR, 5-aminoimidazole-4-carboxamide-β-1-D-ribofuranosyl 5′-monophosphate; FAICAR, 1′-[(5-formylamino)-4-(aminocarbonyl)-1-imidazolyl]-β-D-ribofuranosyl 5′-monophosphate; IMP, inosine 5′-monophosphate; GAR, glycinamide ribonucleotide; AICA, 5-aminoimidazole-4-carboxamide; H_4folate, tetrahydrofolate; AICAR TFase, AICAR transformylase; trifunctional protein, 5,10-methenyl, 5,10-methylene, 10-formyl-H_4folate synthetase (combined); GAR TFase, GAR transformylase; 2-ME, 2-mercaptoethanol; Me_2SO, dimethyl sulfoxide; $NaDodSO_4$, sodium dodecyl sulfate; BSA, bovine serum albumin; DEAE, diethylaminoethyl; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; LC, liquid chromatography.

0006-2960/81/0420-0337$01.00/0

was added, and the solution was maintained at 35 °C for 4 h after which time a Bratton–Marshall assay of a 10-μL aliquot indicated no diazotizable amine was present (Flaks & Lukens, 1963). The solution was lyphohilized to dryness and dissolved in 100 mL of H_2O, the pH was adjusted to pH 9 with 3 N NH_4OH, and the solution was applied to a 1 × 10 cm column of QAE-Sephadex A-25. After loading, the column was eluted with a linear gradient made from 150 mL of H_2O and 150 mL of 0.15 M ammonium bicarbonate, pH 9.0, with NH_4OH. The elution profile (absorbance at 270 nm) showed a single major peak. The fractions contained within the peak were pooled, lyophilized to dryness, redissolved in 10 mL of H_2O, and acidified to pH 3 with 1 N HCl to remove any remaining bicarbonate. The pH was then adjusted to pH 9 with concentrated ammonium hydroxide, and the organic phosphate was precipitated with barium acetate by the addition of methanol. The white salt was collected by centrifugation, rinsed with acetone and then ether, and dried in vacuo. A solution of the above barium salt was subjected to the following four tests: (1) acid hydrolysis followed by Bratton–Marshall assay (Flaks et al., 1957); (2) cyclization in base to give the UV spectrum of IMP (Flaks et al., 1957); (3) periodate assay for diols (Dixon & Lipkin, 1954); (4) total phosphate content (Chen et al., 1956). The ratio of ribose/IMP/diazotizable amine/phosphate was 1:1:1:1 within the limits of the methods (±5%).

A sample of FAICAR was further purified by the reverse-phase high-pressure LC technique described under Methods to ensure IMP removal. The extinction coefficient at 248 nm and pH 7.4 (ϵ = 6590 M^{-1}) was calculated by observing the UV spectrum of the purified solution and determining the concentration of FAICAR in solution by use of the periodate assay. Proton and ^{13}C NMR spectra were also obtained for this compound by using a Bruker Instrument WP-200 Fourier transform NMR and are reported under Results.

Synthesis of [^{13}C]Formyl-AICAR. The procedure of Flaks et al. (1957) was modified for synthesis of FAICAR which was 90% ^{13}C enriched in the formyl carbon. AICAR (49 mg of the barium salt) and oven dried potassium formate (90% ^{13}C enriched, 180 mg) were dissolved in 2.5 mL of glacial acetic acid. Acetic anhydride (2.5 mL) was added to the stirred solution which was heated briefly to start the reaction. After 20 min of reaction at room temperature the solution was brought to 50 °C for 70 min. At the end of this time a Bratton–Marshall assay of a 10-μL aliquot indicated that <1% of the original AICAR remained, so the solution was lyophilized to dryness, and the solid was redissolved in 40 mL of H_2O and applied to a 1.1 × 9 cm column of QAE-Sephadex A-25 (bicarbonate form). The column was then eluted with a linear gradient made from 150 mL of H_2O and 150 of 0.2 M ammonium bicarbonate, pH 8.7, with NH_4OH. The elution profile at 248 nm showed two peaks, but only the second gave rise to a ^{13}C signal, although the UV spectra of both compounds were identical. The ^1H NMR of the initial peak material showed no formamide proton and probably resulted from acetylation of AICAR. The proton and ^{13}C NMR spectra for the second peak after isolation by lypohilization are reported under Results.

Methods

Gel Electrophoresis. $NaDodSO_4$ gel electrophoresis was performed on 7.5% gels as described by Weber et al. (1972).

High-Pressure Liquid Chromatography. The identification of synthetic and enzymatically produced nucleotides was accomplished by using either paired-ion reverse-phase or anion-exchange chromatography on an Altex Model 100 high-pressure liquid chromatograph with an Altex 8-μL analytical detector set at 254 nm. Integration of the peaks was accomplished by weighing the peaks from the recorder trace.

Paired-ion reverse-phase chromatography was performed on a Partisil 10-ODS-2 (Whatman) column (4.6 mm × 25 cm) according to the procedure of Rowe et al. (1978). FAICAR was cleanly separated from both AICAR and IMP (retention time = 17.3 min vs. 20.4 min).

Anion-exchange chromatography was performed on a Partisil SAX (Whatman) column (4.6 mm × 25 cm) by a modification of the procedure of Rowe et al. (1978). The column was equilibrated with 10 mM potassium phosphate, pH 2.5, and after sample injection the column was eluted with the same buffer. The retention times (minutes) for AICAR, FAICAR, and IMP are respectively 7.6, 14.0, and 17.2.

Enzyme Assays. All assays were dependent on protein concentration and were initially linear with time. Protein concentration was determined either by assuming an extinction of 1.0 at 280 nm for a 1 mg/mL solution or by a UV biuret technique (Zamenhof, 1957).

AICAR TFase was assayed by following the initial appearance of H_4folate at 298 nm according to the procedure of Black et al. (1978) employing a thermostated Gilford spectrophotometer. The cuvette contained 32.5 μmol of Tris-HCl, pH 7.4, 5 μmol of 2-ME, 25 μmol of KCl, 0.101 μmol of (±)-10-formyl-H_4folate, and enzyme to 0.950 mL under N_2 at 25 °C. The nonenzymatic rate was recorded for 10 min and was subtracted from the initial rate obtained after addition of 0.05 mL of 1.01 mM AICAR (Ba^{2+}). All solutions for the assay were prepared from degassed H_2O and were argon saturated since the blank rate was very sensitive to the amount of oxygen in the solutions. With careful preparation the nonenzymatic rate was <5% of the enzymatic rate.

Inosinicase was assayed by following the appearance of IMP at 248 nm. The cuvette contained 30 μmol of Tris-HCl, pH 7.4, and 0.10 μmol of FAICAR (Ba^{2+}). After a 10-min incubation period the enzyme was added to give a final volume of 1 mL, and the initial rate was recorded as above. GAR TFase was assayed according to the procedure of Caperelli et al. (1980).

Enzyme Purification. All steps were carried out at 4 °C unless otherwise specified. In several cases it was found to be more convenient to monitor column fractions for enzyme activities other than AICAR TFase. In each case it was determined that AICAR TFase coeluted with the measured enzyme activity.

(*a*) *Extraction.* Chickens obtained from the Poultry Plant, Pennsylvania State University, were killed, and the livers were immediately put on ice. The liver (120 g) was homogenized in a glass–teflon homogenizer with 120 mL of 10 mM potassium phosphate, pH 7.5, 1 mM EDTA, 0.25 M sucrose containing 250 mg of type II-O ovomucoid, 25 mg of α-1-antitrypsin, 2.5 mL of aprotinin, and 15 mg of pepstatin/L. The homogenate was centrifuged at 100000*g* for 90 min, the supernatant then was filtered through glass wool, and the pH was adjusted to pH 7.5 with 3 N NH_4OH after the solution had been made 10 mM in 2-ME.

(*b*) *Protamine Sulfate Precipitation.* To the stirred supernatant was slowly added 30 mL of 2% protamine sulfate in 10 mM potassium phosphate, pH 7.5, and the pH was maintained at pH 7.5 with 3 N NH_4OH. After 20 min, the suspension was centrifuged at 18000*g* for 10 min.

(*c*) *Ammonium Sulfate Fractionation.* The supernatant was brought to 40% saturation by addition of solid $(NH_4)_2SO_4$

while the pH was maintained at pH 7.5 with 3 N NH_4OH. After 50 min the suspension was centrifuged at 18000*g* for 10 min, the pellet was discarded, and the supernatant was brought to 55% saturation as above. The 40–55% pellet then was collected by centrifugation (18000*g*) and dissolved in 50 mL of homogenization buffer which was 10 mM in 2-ME. The redissolved pellet was then dialyzed vs. 10 mM potassium phosphate, pH 7.5 with 10 mM 2-ME.

(*d*) *Bio-Gel HTP Chromatography.* One half of the redissolved pellet solution was applied to a column (6 × 6.5 cm) made from 23 g of Bio-Gel HTP and 23 g of Whatman cellulose CF-1 which had been equilibrated with 10 mM potassium phosphate, pH 7.5 with 10 mM 2-ME. The column was washed with 400 mL of 37.5 mM Tris-HCl, pH 7.5, 10 mM 2-ME, 25% glycerol (v/v), and 10% Me_2SO (v/v), with 500 mL of 75 mM Tris-HCl, pH 7.5, 10 mM 2-ME, 25% glycerol (v/v), and 10% Me_2SO (v/v), and with 800 mL of 22.5 mM potassium phosphate, pH 7.5, 10 mM 2-ME, 25% glycerol (v/v), and 10% Me_2SO (v/v). The protein then was eluted with 75 mM potassium phosphate, pH 7.5, 10 mM 2-ME, 25% glycerol (v/v), and 10% Me_2SO (v/v). The GAR transformylase containing fractions (15 mL each) were combined and dialyzed twice vs. 7.5 mM potassium phosphate, pH 7.5.

(*e*) *AICAR–Sepharose Chromatography.* The dialyzed protein was applied to a column (1.5 × 8.5 cm) of AICAR–Sepharose equilibrated with 7.5 mM potassium phosphate, pH 7.5. The column was washed with 200 mL of 37.5 mM Tris-HCl, pH 7.5, and 250 mL of 75 mM Tris-HCl, pH 7.5, and was eluted with 75 mM Tris-HCl, pH 7.5–75 mM potassium phosphate, pH 7.5. The inosinicase-containing fractions (4 mL each) were pooled and dialyzed vs. 7.5 mM potassium phosphate, pH 7.5.

(*f*) *AICAR–Sepharose Chromatography. AICAR Elution.* The dialyzed protein solution was applied to a column (1.5 × 8.5 cm) of AICAR–Sepharose equilibrated with 7.5 mM potassium phosphate, pH 7.5. The column was washed with 150 mL of 7.5 mM Tris-HCl, pH 7.5, and eluted with 7.5 mM Tris-HCl and 10 mM AICAR (Ba^{2+}) adjusted to pH 7.5 with 1 N HCl. The AICAR TFase containing fractions (4 mL each) were pooled and desalted on a column (2.5 × 42.5 cm) of Sephadex G-25 (coarse) that had been equilibrated with 50 mM Tris-HCl, pH 7.5. The protein solution was then dialyzed vs. 7.5 mM Tris-HCl, pH 7.5.

(*g*) *Concentration.* The entire protein sample was applied to a column (0.5 × 4.5 cm) of AICAR–Sepharose equilibrated with 7.5 mM Tris-HCl, pH 7.5. The column was then eluted with 75 mM Tris-HCl, pH 7.5–75 mM potassium phosphate, pH 7.5. The inosinicase-containing fractions (0.7 mL each) were pooled and dialyzed vs. 7.5 mM potassium phosphate, pH 7.5. After dialysis the enzyme solution was frozen and stored in liquid nitrogen.

Cofactor Stereochemistry and Stoichiometry. The rates of utilization of the 10-formyl-H_4folate diastereomers were determined by observing the rate of production of H_4folate in the AICAR TFase assay. The diastereomeric 10-formyl-H_4folate samples were prepared from (+)-L- and (−)-L-5,10-methenyl-H_4folate by hydrolysis in 50 mM Tris-HCl, pH 7.4 with 0.1 M 2-ME for 1 h under N_2 at 25 °C. The concentration of 10-formyl-H_4folate in the assay was 51.4 μM, and three different concentrations of enzyme were assayed to establish enzyme dependence.

The stoichiometry of the reaction was determined by modification of the standard assay. The cuvettes contained 32.5 μmol of Tris-HCl, pH 7.4, 5 μmol of 2-ME, 25 μmol of KCl, and 0.050 μmol of 10-formyl-H_4folate prepared from (+)-L-5,10-methenyl-H_4folate under N_2. Two cuvettes contained 0.030 μmol and 0.050 μmol of AICAR while a third with no AICAR was run as a control. After a 10-min incubation during which the nonenzymatic rate was 0, enzyme was added to all three cuvettes to bring the volume to 1 mL. The reaction was monitored at 298 nm (formation of H_4folate) until no further change in OD was observed. The protein was then denatured and removed by vortexing the assay solution with chloroform, followed by centrifugation. Aliquots (100 μL) of the aqueous phase were analyzed by high-pressure LC anion-exchange chromatography. The peak area was determined as described above, and the calibration of IMP concentration to peak areas was determined by use of several IMP standards.

Kinetic Parameters. The standard assay was used for determination of the kinetic parameters of AICAR TFase. Data of initial velocities vs. varying AICAR and 10-formyl-H_4folate levels were computer fitted by using Cleland's sequential kinetic program (Cleland, 1967). The 10-formyl-H_4folate was obtained either from (+)-L-5,10-methenyl-H_4folate or (+)-L-5,10-methenyl-H_4pteroyltriglutamate.

Initial velocity data for inosinicase activity at varying concentrations of FAICAR in the normal assay were obtained in order to determine the steady-state kinetic parameters.

Recombination Experiments. A test for the possible association of AICAR TFase and the trifunctional protein was to determine if the specific activity of the pure AICAR TFase increased upon addition of purified trifunctional protein. For this, the standard AICAR TFase assay was modified as follows: into each cuvette was added 32.5 μmol of Tris-HCl, pH 7.4, 5.50 μmol of 2-ME, 25 μmol of KCl, 0.0514 μmol of 10-formyl-H_4folate prepared from (+)-L-methenyl-H_4folate, purified trifunctional protein, and AICAR TFase to 0.950 mL under N_2. After a 20-min incubation period at 25 °C the assay was initiated with 50 μL of 1.01 mM AICAR (Ba^{2+}), and the change in absorbance at 298 nm was recorded. Three different ratios of AICAR TFase to trifunctional protein were used, 10:1, 1:1, 1:10 (mol/mol), respectively, with the concentration of AICAR TFase held constant. As a standard, AICAR TFase was also assayed in the absence of trifunctional protein by the above procedure. For a control an equal amount of BSA by weight was substituted for the trifunctional protein and the assay was repeated as above.

Cross-Linking of AICAR TFase and Trifunctional Protein. An equimolar amount of AICAR TFase and trifunctional protein was dialyzed under N_2 against 50 mM potassium phosphate, pH 7.5, and 25% glycerol (v/v) which had been degassed and N_2 saturated. The solution was made 0.5 mg/mL in protein by dilution with the above buffer and adjusted to pH 8.2 with 1 M potassium hydroxide. Dimethyl 3,3′-dithiobis(propionimidate) dihydrochloride was added as the solid to give a solution of 0.250 mg/mL. The reaction was allowed to proceed for 45 min at room temperature under argon. At the end of this time the pH was adjusted to pH 8.7 with 1 M KOH and the reaction continued for an additional 15 min at room temperature under argon. Solid $NaDodSO_4$ was added to the solution to a concentration of 5 mg/mL, and the protein was denatured by incubation at 37 °C for 2 h.

Two-dimensional $NaDodSO_4$ gel electrophoresis was performed on the denatured protein sample according to the procedure of Coggins et al. (1976). The first dimension was performed on a 5% $NaDodSO_4$ tube gel, and the cross-linker cleavage was accomplished in 0.2 M sodium phosphate, pH 7.2, 0.2% (w/v) $NaDodSO_4$, 10% (v/v) 2-ME in 6 h at room

Table I: Purification of AICAR Transformylase and Inosinicase Activity

fraction	volume (mL)	total protein (mg)	sp act.[a] of AICAR TFase	total act. (units)	purification (yield %)	sp act.[a] of inosinicase	purification (yield %)
homogenate supernatant	125	11.9×10^3	2.07×10^{-3}	24.62	1 (100)	3.31×10^{-2}	1 (100)
protamine sulfate supernatant	160	9.15×10^3	4.21×10^{-3}	38.56	2.0 (157)	4.54×10^{-2}	1.4 (106)
ammonium sulfate, 40-55%	60	2.19×10^3	6.35×10^{-3}	13.91	3.1 (56)	0.105	3.2 (58)
Bio-Gel HTP[b]	500	109	0.110	12.01	53 (98)	0.585	17.7 (16)
AICAR-Sepharose[b]	20	24.5	0.269	6.59	130 (54)	1.55	46.8 (9.6)
AICAR-Sepharose[b] (AICAR elution)	40	5.52	0.618	3.41	299 (28)	4.92	149 (6.9)
concn[b]	2.3	4.23[c]	0.856	3.62	414 (29)	5.09	154 (5.5)

[a] Specific activity is micromole per minute per milligram of protein at 25 °C. [b] Adjusted for 50% use of the $(NH_4)_2SO_4$ fraction. [c] Protein concentration determined by the method of Zamenhof (1957).

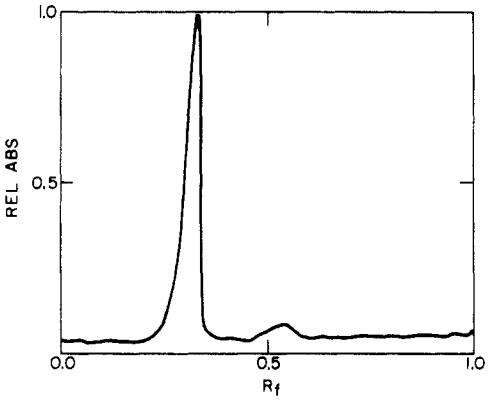


FIGURE 1: $NaDodSO_4$ gel electrophoresis of AICAR fractions obtained from AICAR Sepharose. Electrophoresis was performed according to the method of Weber et al. (1972) on 7.5% polyacrylamide gels.

temperature. The second dimension was carried out on an 11 × 14 × 0.2 cm slab gel with the first dimension tube gel pressed on top.

Results

Enzyme Purification. The purification procedure is a modification of the technique used by Caperelli et al. (1980) for the purification of GAR transformylase. The results are outlined in Table I. It was found that the recovery of activity from the last three steps of the purification was very sensitive to the presence of mercaptoethanol. If the buffers used during these steps were 10 mM in mercaptoethanol, the recovery of activity was only one-half to one-third that reported in the table. In contrast, the enzyme was tolerant to 2-ME in the first four steps, and, in fact, 2-ME, along with glycerol and Me_2SO, in the Bio-Gel HTP step seems to be required for the removal of a low (35 000–40 000) molecular weight contaminant which could not easily be separated from AICAR TFase in the subsequent steps. Moreover, dialysis of the purified enzyme with 2-ME-containing buffers seemed to have no effect on its activity.

The ratio of specific activities of inosinicase to AICAR TFase changes from an initial value of ~16 for the first three steps to ~6 for the final three. The transition occurs at the Bio-Gel HTP step, and, since it coincides with an increase in the yield of AICAR TFase, it is apparently due to the removal of a specific transformylase inhibitor. The densitometer scan of a 7.5% $NaDodSO_4$ disc gel is shown in Figure 1. Only a single protein band is present, with the same subunit molecular weight for AICAR TFase as reported by Caperelli et al. (1980).

Cofactor Stereochemistry and Stoichiometry. The AICAR TFase assay with (−)-L-10-formyl-H_4folate derived from (+)-L-5,10-methenyl-H_4folate, the natural stereoisomer, was linear with enzyme concentration, whereas the (+)-L-formyl diastereomer produced from (−)-L-5,10-methenyl-H_4folate gave a lower activity and was linear with respect to enzyme concentration for only ~4 min. During this time, the extent of reaction did not exceed 5%, and afterwards the activity ceased. These results are summarized in Table II. The "burst" phase observed with (+)-L-10-formyl-H_4folate probably is the result of contamination by (−)-L-10-formyl-H_4folate and its rapid consumption by the enzyme. After the burst phase, only (+)-L-10-formyl-H_4folate would be available for use. At the AICAR TFase concentrations employed, utilization of the (+)-L-formyl isomer at a rate 1000-fold less than the naturally occurring diastereomer could have been detected.

Table II: Stereochemical Requirement of Cofactor

relative concn of AICAR TFase	relative sp act. (−)-L-10-formyl-H_4folate	relative sp act. (+)-L-10-formyl-H_4folate (after 20 min)
1	1.00	0.020
2.33	2.24	0.017
16.7	*a*	0.003

[a] Activity too high to be measured.

Table III: Kinetic Parameters for AICAR Transformylase

parameters[a]	10-formyl-H_4folate	10-formyl-H_4pteroyl triglutamate
V_{max} (units/mg)	2.74 ± 0.28	2.40 ± 0.11
K_B(AICAR) (μM)	15.2 ± 3.6	8.4 ± 1.1
K_A(cofactor) (μM)	84 ± 14	0.72 ± 0.19
K_{iA}(cofactor) (μM)	21 ± 11	2.02 ± 0.50

[a] Kinetic parameters are expressed in Cleland's notation.

The stoichiometry of the AICAR TFase reaction was determined by using (−)-L-10-formyl-H_4folate. Since the inosinicase activity in the purified protein is ~6 times the transformylase activity, the ribotide product of the transformylase, FAICAR, would be so rapidly converted to IMP that no significant accumulation of FAICAR would be expected. The ratio of IMP concentration to H_4folate concentration determined as described under Experimental Procedures after quenching of the reaction was found to be 1.1:1 at both AICAR levels.

Kinetics of AICAR Transformylase. Initial velocity studies of AICAR TFase with (−)-L-10-formyl-H_4folate showed a sequential velocity pattern for both the mono- and triglutamate cofactors. The data obtained as described under Experimental Procedures were analyzed by using the Fortran program of

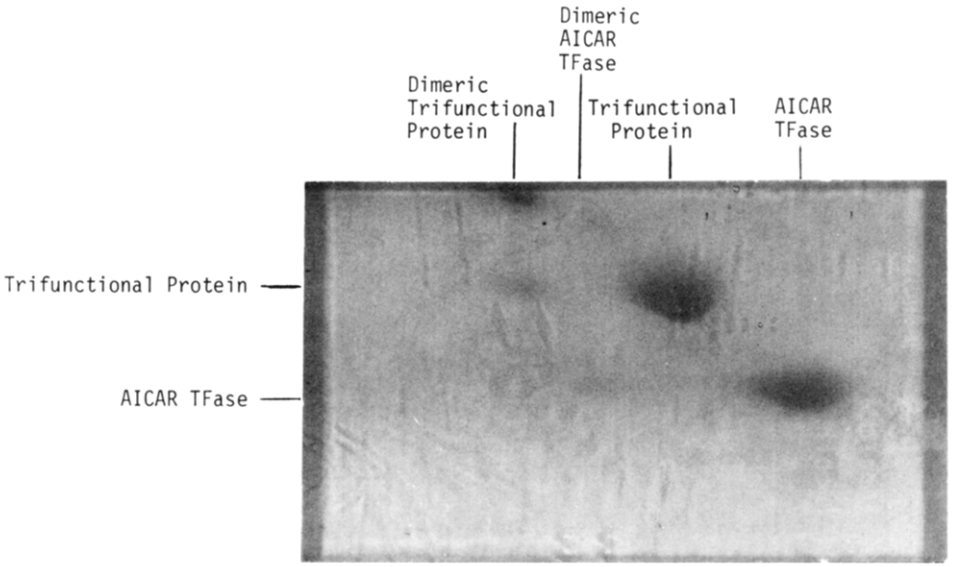


FIGURE 2: Two-dimensional $NaDodSO_4$ gel electrophoresis of cross-linked AICAR TFase and trifunctional protein. The enzymes were cross-linked as described under Experimental Procedures. The first dimension was run in a 7-cm tube gel (5% polyacrylamide), the cross-links then were cleaved with 2-ME, and the second dimension was run by placing the tube gel on top of a 11 × 14 × 0.2 cm slab gel of 7.5% polyacrylamide.

Table IV: Recombination of AICAR TFase with Trifunctional Protein[a]

[AICAR TFase] (nM)	[trifunctional protein] (nM)	relative act.[b]
13.2	0	1
13.2	1.32	1.06
13.2	13.2	1.15
13.2	132	1.26

[a] Assay run as described under Experimental Procedures.
[b] With 0.263 mg of BSA (equivalent to 132 nM trifunctional protein), relative activity is 1.08.

Cleland (1967). The kinetic parameters obtained from the program are listed in Table III.

Recombination. It has been shown that the other transformylase in the purine biosynthetic pathway, GAR TFase, requires the presence of the trifunctional 5,10-methenyl, 5,10-methylene, 10-formyl-H_4folate synthetase in order to be catalytically active (Smith et al., 1980). One demonstration of this was the activation of partially purified GAR TFase by recombination with purified trifunctional protein. This same type of experiment was done with AICAR TFase and trifunctional protein, and the results are summarized in Table IV.

There is some activation of AICAR TFase by the trifunctional protein in excess of the nonspecific activation by BSA, but with a 10:1 molar ratio of the trifunctional protein to AICAR TFase the trifunctional protein specific activation is only ~18%. In contrast, for a 1:1 molar ratio of partially purified GAR TFase to trifunctional protein the trifunctional protein specific activation is about 4–5-fold, so it appears that the catalytic activity of AICAR TFase is only weakly influenced by the presence of trifunctional protein, and the trifunctional protein is not required for AICAR TFase to function.

Cross-Linking. The two-dimensional $NaDodSO_4$ gel procedure described under Experimental Procedures was used by Smith et al. (1980) to demonstrate the physical association between GAR TFase and the trifunctional protein. The results of attempts to cross-link AICAR TFase with trifunctional protein are shown in Figure 2. The major spots on the slab gel represent AICAR TFase and trifunctional subunits. The minor, off-diagonal spots represent dimeric trifunctional protein and AICAR TFase. No AICAR TFase–trifunctional cross-links were observed.

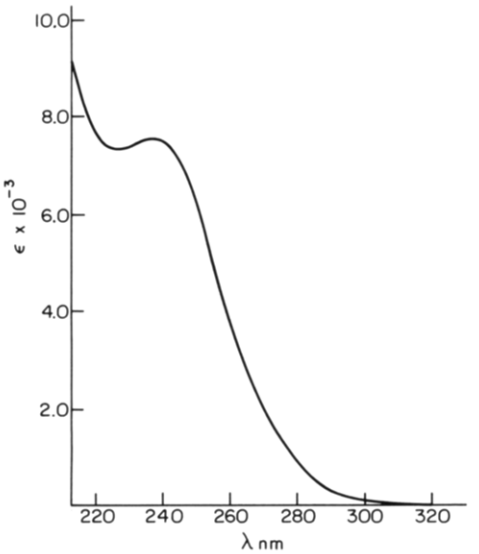
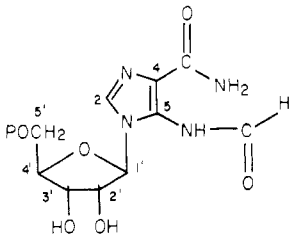


FIGURE 3: UV spectrum of FAICAR in 50 mM Tris-HCl, pH 7.4, ϵ is given in L/mol·cm.

Verification of the Structure of FAICAR. Several published syntheses of FAICAR (Flaks et al., 1957; Muramatsu et al., 1965; Shaw & Wilson, 1962) were tried and gave products with the identical UV spectrum shown in Figure 3, which is substantially different from the spectrum for FAICAR described by Flaks et al. (1957) with λ_{max} of 270 nm and an extinction coefficient of 11 300 M^{-1}. However, the other properties attributed to FAICAR are identical with those for the product of our AICAR formylation. The phosphate/ribose ratio is 1:1, acid hydrolysis liberates 1 mol of diazotizable amine, and base treatment produces 1 mol of IMP/mol of formylation product.

The ^{13}C resonances of the formylation product are summarized in Table V referenced to structure **1**. Assignment of the ribose resonances was based on the published spectra of several nucleotides (Dorman & Roberts, 1970). The assignment of the C-2 resonance on the imidazole ring was based on the published ^{13}C spectra of purine nucleotides (Dorman & Roberts, 1970) and purine nucleosides (Jones et al., 1970),



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the ^{13}C spectrum of AICA (Sadtler 1631C; *Sadtler Standard Carbon-13 NMR Spectra*, 1978), and our ^{13}C spectrum of AICAR riboside (unpublished results). In addition, the fully coupled ^{13}C NMR spectrum of the AICAR formylation product showed that only three nonribose resonances were split by hydrogen nuclei. Two of these resonances were in the carbonyl region of 160–180 ppm (Levy & Nelson, 1972) while the third was at 134.9 ppm with J = 215.3 Hz.

The three resonances in the carbonyl region were assigned by utilizing the product obtained from formylation of AICAR with ^{13}C-enriched formate. Only two ^{13}C resonances were readily observed for this compound, with chemical shifts of 167.6 and 164.5 ppm. The two were also the carbonyl resonances split in the fully coupled ^{13}C NMR spectrum, with J = 205.9 and 205.7 Hz, respectively. The chemical shifts and two peaks in the fully decoupled ^{13}C NMR spectrum are consistent with an N-substituted formamide; e.g., *N*-methylformamide has chemical shifts of 166.3 and 163.2 ppm depending on conformation (Sadtler 2993C). The other resonance in the carbonyl region did not correspond to a formate salt contaminant (172.3 ppm, Sadlter 3045C) and so must be the carbonyl carbon of the carboxamide.

The final two resonances were assigned to positions C-4 and C-5 of the imidazole ring, even though they differ from the chemical shifts seen for similar carbons on other nucleotides (Dorman & Roberts, 1970) and on AICAR riboside (117.0 and 149.6 ppm, respectively, unpublished results). The rationale for this difference will be discussed later.

A summary of the ^{1}H NMR spectrum of the AICAR formylation product is included in Table VI. The chemical shifts for the ribose protons were assigned by comparison with the ^{1}H NMR spectrum of inosine (Sadlter, 27584M; *Sadtler Standard NMR Spectra*, 1980) and by analysis of the splitting pattern. The ^{1}H NMR spectrum of the ^{13}C-enriched formylation product showed that only the peaks at 8.18 and 7.98 ppm were split by the ^{13}C nucleus. In accord with the ^{13}C spectrum, these two resonances are quite close to the observed value of 8.10 ppm for the formyl proton of *N*-methylformamide (Sadtler 9350M) and thus arise from two different conformations of the formamide with respect to the rest of the ribotide. Additional evidence that these two resonances resulted from restricted rotation was their coalescence at ~60 °C to a single signal. The final resonance was assigned to the C-2 imidazole proton and is consistent with the observed chemical shift of the C-8 proton of inosine (Sadtler, 27594M), adenosine (Sadtler, 27540M), zanthosine 5′-monophosphate (Aldrich 125A; Pouchert & Campbell, 1974), and guanosine 5′-monophosphate (Aldrich, 126A; Pouchert & Campbell, 1974).

K_m Determination of Inosinicase. The difference in extinction coefficient between IMP and FAICAR at 248 nm and pH 7.4 was calculated to be 5710 M^{-1} based on the published value for the extinction coefficient for IMP (Burton, 1974) and our FAICAR value permitting the monitoring of IMP concentration. Analysis of initial velocity data revealed that the value of K_m for FAICAR was <1 μM, too low to be measured accurately with our present technique. A minor (5%) IMP contaminant in the FAICAR was found to have no effect on the inosinicase assay.

Table V: ^{13}C NMR of FAICAR

assignment	ppm[a]
O=CH	167.6, 164.5
O=CNH_2	165.4
C-5/C-4	127.4/125.4
C-2	134.9
C-1′	87.8
C-2′	69.8
C-3′	74.6
C-4′	84.0
C-5′	63.4

[a] In D_2O, pH 7, relative to Me_4Si. Dioxane was used as an internal reference, and the chemical shifts were converted to the Me_4Si scale by using the dioxane-Me_4Si shift difference of 66.5 ppm.

Table VI: ^{1}H NMR of FAICAR

assignment	ppm[a]	integration
O=CH	8.18 (s), 7.98 (s)	1.13
H-2	7.93 (s)	0.81
1′	5.41 (d)	1.00
2′	4.29 (t)	0.96
3′	4.18 (t)	1.29
4′	4.05 (m)	1.21
5′	3.80 (m)	2.22

[a] In D_2O, pH 7, relative to Me_4Si. Dioxane was used as an internal reference, and chemical shifts were converted to the Me_4Si scale by using the dioxane-Me_4Si shift difference of 3.51 ppm.

Discussion

The purification of AICAR TFase to apparent homogeniety was successful largely due to the use of an AICAR affinity chromatography step with a specific AICAR elution. The results of this purification indicate that both AICAR TFase and inosinicase reside on the same peptide since, with the exception of one step, they copurify during the isolation procedure. More substantial evidence is that the purified AICAR TFase, which is a single peptide by $NaDodSO_4$ gel electrophoresis, contains a significant amount of inosinicase activity.

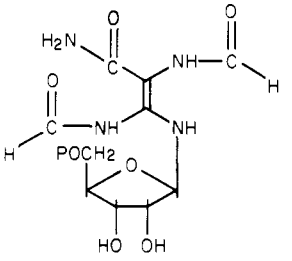
The presence of these sequential activities of purine biosynthesis on the same protein leads to speculation about their interaction. One possibility is that FAICAR produced by the transformylase "channels" to the inosinicase active site without being released into solution. Another possibility is that only a single active site is present on the protein and FAICAR is only a short-lived intermediate. The tight coupling of the two activities would eliminate the need for the hydrolytically labile FAICAR to be released into solution, where cleavage of the formamide would represent a "futile cycle" of the reduced folate cofactor.

The trifunctional 5,10-methenyl, 5,10-methylene, 10-formyl-H_4folate synthetase prepares reduced folate cofactors which are utilized by two transformylases in the purine biosynthetic pathway, GAR TFase and AICAR TFase. Previous experiments have revealed, through kinetic analysis and chemical cross-linking of proteins, that GAR TFase and the trifunctional protein form a functional protein complex (Smith et al, 1980). The recombination and cross-linking experiments presented here do not indicate that a similar type of protein complex is formed between AICAR TFase and the trifunctional protein. The small degree of trifunctional protein specific activation of the AICAR TFase may be an indication that 10-formyl-H_4folate produced by the trifunctional protein is "channeled" directly to the active site of the transformylase,

but this type of interaction would be apparent only when both enzymes were functioning and would not have been seen in this study.

Characterization of AICAR TFase with respect to its steady-state kinetic parameters reveals a sequential reaction pattern and no evidence for a formyl transfer from cofactor to enzyme followed by cofactor dissociation. Thus, both transformylases do not use a classical "ping-pong" mechanism encountered in other acyl transfer processes (Caperelli et al., 1980). Increasing the folate glutamyl tail to three residues does not alter the general sequential pattern, but it does result in a 100-fold decrease in K_A for the cofactor from 84 to 0.72 μM. In addition, the assignment of kinetic parameters is reasonable only if an ordered sequence with initial binding by the H_4folate cofactor is presumed since the value of K_{ia}—the dissociation constant for the first species from the enzyme—decreases with changing cofactor. This trend can be rationalized in terms of an increased affinity for the enzyme caused by the triglutamate residue, but not by AICAR initially binding in which case K_{ia} should remain constant. Baggott & Krumdieck (1979a), who were working with a less purified enzyme, reported K_m's for mono- and triglutamate cofactors of 674 μM and 1.65 μM, respectively. They also reported that the apparent K_m for AICAR was not strongly affected by folate cofactor concentration. The AICAR TFase unlike GAR TFase exhibits a >1000-fold preference for the naturally occurring H_4folate cofactor. As expected there is a 1:1 stoichiometry with respect to 10-formyl-H_4folate utilization and IMP formation.

The product of the chemical and enzymatic formylation of AICAR has been established to be FAICAR (**1**). Difficulty was encountered in the previous characterization of this compound (Baggott & Krumdieck, 1979b) by ^{1}H NMR due to the fact that for purine or purine-like compounds, imidazole and formyl protons may have quite similar chemical shifts. The only way to unequivocally distinguish between these two possibilities is by ^{13}C NMR. The UV spectrum of this compound is not what was reported in the literature (Flaks et al., 1957), nor is it what one would expect from its structure. An analysis using space-filling models confirms the suggestion of Baggott & Krumdieck (1979b) that the carboxamide and formamide moieties of FAICAR are unable to be coplanar with the imidazole ring due to steric hindrance, thus forcing them out of resonance. However, their hypothesis that the imidazole ring of FAICAR suffers hydrolytic cleavage to give *trans*-α,β-diformamide-β-(5′-phosphoribosylamino)acrylamide



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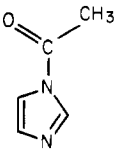
(**2**) is clearly untenable. The presence of an H-2 and a C-2 at the expected resonance for the imidazole methine and the unambiguous enrichment of a single carbonyl with restricted rotation upon formylation of AICAR with ^{13}C formate accounting for the two formamide ^{13}C resonances are only consistent with structure **1**. Owing to the loss of exocyclic resonance, the UV spectrum would be expected to resemble that of imidazol-1-yl methyl ketone (**3**) in which the imidazole ring is intact and the only exocyclic conjugation is through



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a carbonyl attached to a nitrogen of the imidazole. The UV spectrum of this compound (Sadtler, 19137UV; *Sadtler Standard UV Spectra*, 1980) closely resembles that which we find for FAICAR, including the shoulder at 240 nm, indicating that cleavage of the imidazole ring is not necessary to obtain the UV spectrum of the AICAR formylation product.

The decrease in resonance by steric crowding is probably also responsible for the anomalous ^{13}C NMR resonances at C-4 and C-5 of FAICAR. The decreasing resonance of the carboxamide would cause an increase in electron density at the C-5 carbon and an upfield shift in its signal. The formylation of the amine of AICAR, plus its decreased resonance with the imidazole ring would lead to a decrease in electron density at the C-4 carbon and a downfield shift in its ^{13}C NMR signal. The net result of this would be that the difference in chemical shifts between the C-4 and C-5 positions would be much less for FAICAR than for AICAR. In fact, the observed chemical shifts for these two carbons are virtually the same in FAICAR and approach the value for the C-4 and C-5 carbons of imidazole (Levy & Nelson, 1972).

One remaining question is the nature of the species which gives rise to the UV spectrum originally reported. One possibility, since the spectrum so closely resembles AICAR, is that the sample being prepared under acidic conditions lost either its formyl or ribose group by acid hydrolysis to give either AICAR or the free base, which has a UV spectrum similar to AICAR (Baggott & Krumdieck, 1979b).

Acknowledgments

We thank Alan Freyer for his technical assistance with the NMR and Dr. G. K. Smith, Dr. R. F. Dietrich, and Dr. J. F. Marlier for their advice and discussion.

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Earthworm Bioluminescence: Characterization of High Specific Activity *Diplocardia longa* Luciferase and the Reaction It Catalyzes†

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ABSTRACT: *Diplocardia longa* luciferase purified by an improved procedure differs from that first described by Bellisario et al. [Bellisario, R., Spencer, T. E., & Cormier, M. J. (1972) *Biochemistry 11*, 2256-2266] in having much higher specific activity (40×) and firmly bound, EPR-silent copper. Improved assay conditions suggest that this protein acts as a catalyst in a bioluminescent reaction involving the degradation of 3-(isovalerylamino)-1-hydroxypropane hydroperoxide. This substrate is formed spontaneously on the addition of hydrogen peroxide to *D. longa* luciferin (3-(isovalerylamino)propanal). The quantum yield of the bioluminescence for this substrate is 3%. Detailed physical and chemical analyses of high specific activity *D. longa* luciferase indicate that it is a large (300 000 daltons), asymmetric (f/f_0 = 1.63, with 0.4 g/g hydration), multisubunit enzyme. It contains carbohydrate (6%), lipid (2%), and copper (up to 4 mol/300 000 daltons). The amino acid composition is unusual with 11% by weight of the residues being either proline or hydroxyproline.

In the bioluminescence of the earthworm *Diplocardia longa*, hydrogen peroxide and 3-(isovalerylamino)propanal (earthworm luciferin) react in the presence of the protein *D. longa* luciferase with emission of blue-green light (Bellisario & Cormier, 1971; Bellisario et al., 1972; Ohtsuka et al., 1976; Mulkerrin & Wampler, 1978). Previous work on this protein (Bellisario & Cormier, 1971; Bellisario et al., 1972) has shown that it is large (300 000 daltons), is asymmetric (f/f_0 = 2.1; with no hydration and $\bar{v}$ = 0.73) and is composed of multiple subunits. Analysis of the purified protein also revealed a reproducible titer of bound, EPR-detectable copper (Bellisario & Cormier, 1971), but further study (Bellisario et al., 1972) showed that 90% of this copper could be removed with no loss of activity. A second important observation, reported by Bellisario et al. (1972), was the lack of a catalytic role for the protein under their assay conditions. In contrast, the data reported here show that *D. longa* luciferase can be prepared with much higher specific activity and that this protein does indeed contain firmly bound, but EPR-silent, copper. In addition, by alteration of the assay conditions to protect the protein from the denaturing action of hydrogen peroxide, turnover of the enzyme can be demonstrated. However, since the physical data for protein prepared according to this new procedure (Rudie, 1977; Mulkerrin & Wampler, 1978) are nearly identical with those reported for the previous, low specific activity protein (Bellisario et al., 1972), we conclude that the differences between the two preparations are explained by a difference in the fraction of active enzyme.

The physical data reported here extend the physical description of the protein to include the carbohydrate, lipid, and amino acid compositions, and a more accurate evaluation of the physical parameters. Interestingly, amino acid analysis reveals that luciferase contains 5.8% by weight of hydroxyproline.

A reinvestigation of the in vitro reaction shows that the true substrate of the bioluminescence is a luciferin peroxide adduct. The stoichiometry and quantum yield data suggest that luciferase catalyzes the luminescent degradation of 3-(isovalerylamino)-1-hydroxypropane hydroperoxide. These data also indicate that the earthworm bioluminescence reaction is quite similar to the copper-catalyzed chemiluminescence which

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0006-2960/81/0420-0344$01.00/0