Carboxylases in *de Novo* Purine Biosynthesis. Characterization of the *Gallus gallus* Bifunctional Enzyme†

Steven M. Firestine and V. Jo Davisson*

Department of Medicinal Chemistry and Pharmacognosy, 1333 Robert E. Heine Pharmacy Building, Purdue University, West Lafayette, Indiana 47907-1333

Received May 11, 1994; Revised Manuscript Received July 12, 1994®

ABSTRACT: Two successive steps in *de novo* purine biosynthesis are catalyzed by the enzymes 5-aminoimidazole ribonucleotide (AIR) carboxylase and 4-[(*N*-succinylamino)carbonyl]-5-aminoimidazole ribonucleotide (SAICAR) synthetase. Amino acid sequence alignments of the proteins from various sources suggested that several unusual differences exist within the structure and function of these enzymes. In vertebrates, a bifunctional enzyme (PurCE) catalyzes successive carboxylation and aspartylation steps of AIR to form SAICAR. This is in contrast to the three proteins, PurK, PurE, and PurC, from *Escherichia coli* which have recently been shown to require 2 equiv of ATP for the AIR to SAICAR conversion in the presence of physiological HCO_3^- concentrations (Meyer et al., 1992). A comparative study of these proteins has been initiated using a high-production, heterologous expression system for the *Gallus gallus* AIR carboxylase–SAICAR synthetase and yields purified enzyme following a two-step procedure. Selective assays have been developed for all the enzymatic activities of the bifunctional protein. The *G. gallus* AIR carboxylase has no ATP dependence and displays a K_m for HCO_3^- that is 10-fold lower than that for the related PurE protein from *E. coli,* supporting the hypothesis that the two enzymes require different substrates. No common cofactors or metals are required for catalysis. Each catalytic activity has been shown to be independent by selective inactivation of SAICAR synthetase with the affinity agent 5′-[4-(fluorosulfonyl)benzoyl]-adenosine (FSBA) and inhibition of AIR carboxylase with a tight-binding inhibitor 4-nitro-5-aminoimidazole ribonucleotide (NAIR). The native protein aggregrates, and limited proteolysis indicates that the global structure of the protein involves two independent folding domains, each containing a different catalytic site.

One-carbon metabolism has long been recognized in the *de novo* purine biosynthetic pathway and is often associated with the folate-dependent transformylases. However, a second type of one-carbon metabolism is represented in the pathway, namely, the carboxylation resulting in the formation of 4-carboxy-5-aminoimidazole ribonucleotide (CAIR)[1] catalyzed by the enzyme 5-aminoimidazole ribonucleotide (AIR) carboxylase (see eq 1 in Scheme 1). Carboxylases that utilize aromatic substrates are not modeled by other enzymes in primary metabolism, and the critical catalytic features of the AIR carboxylases are not known. Furthermore, genetic information regarding the interspecies relationship of AIR carboxylases shows that these enzymes represent essential links between purine, CO_2, thiamin, and amino acid metabolism.

Recent investigations of the AIR carboxylase system from *Escherichia coli* have revealed that two distinct enzymatic reactions are required for the carboxylation of AIR. The first enzyme, PurK, catalyzes the N-carboxylation of AIR using ATP and HCO_3^-, to form N^5-carboxyaminoimidazole ribonucleotide (N^5-CAIR) (Mueller et al., 1994). The second reaction is catalyzed by the protein PurE and involves a rearrangement of the carboxyl group from nitrogen to carbon (see eq 2 in Scheme 1). Since the N-carboxylation event is known to occur in the absence of enzymatic catalysis, N^5-CAIR is thought to play the role of a bacterial carboxylation cofactor despite the limited stability of this compound under physiological conditions. Many of the details regarding PurK and PurE function are not clear. However, the critical role of PurK in bacterial physiology has been established since mutations in the *purK* gene result in a dependence on increasing concentrations of exogenous CO_2 (Tiedeman et al., 1989; Watanabe et al., 1989).

Amino acid sequence alignments of the known AIR carboxylases revealed that the bacterial (PurE, PurK), yeast, and fungal (ADE2) proteins all appear to have related functions, while the *Gallus gallus* and *Homo sapiens* enzymes do not have any homology with PurK (Chen et al., 1990; Minet & Lacroute, 1990). In addition, amino acid sequence homologies and results of genetic studies with *G. gallus* cDNA encoding AIR carboxylase implicate another protein function related to PurC or 4-[(succinylamino)carbonyl]-5-aminoimidazole ribonucleotide (SAICAR) synthetase. Biochemical characterization of the *G. gallus* protein is limited to a single *in vitro* study and also indicates a bifunctional protein with

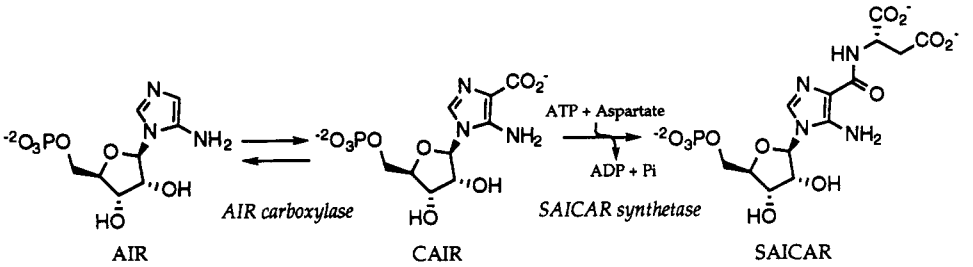
† Financial support was provided by the American Heart Association, Indiana Affiliate, Inc., and the American Cancer Institution (Grant IN-17-31). S.M.F. is a National Institutes of Health predoctoral trainee (GM08298).

® Abstract published in *Advance ACS Abstracts,* September 1, 1994.

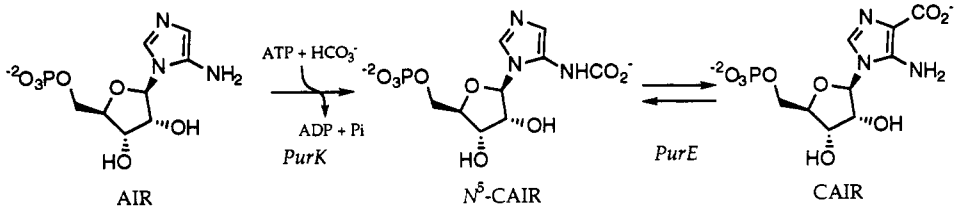
[1] Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; AIR, 5-aminoimidazole ribonucleotide; Amp, ampicillin; ATP, adenosine triphosphate; BES, *N,N*-bis(2-hydroxyethyl)-2-aminoethansulfonic acid; CAIR, 4-carboxy-5-aminoimidazole ribonucleotide; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; DIPEAA, diisopropylethylammonium acetate; EDTA, ethylenediaminetetraacetic acid; FSBA, 5′-[4-(fluorosulfonyl)benzoyl]adenosine; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid); HPLC, high-performance liquid chromatography; ICP, inductively coupled plasma; LB medium, Luria–Bertani medium; M_r, molecular weight; NADH, dihydronicotinamide adenine dinucleotide; NAIR, 4-nitro-5-aminoimidazole ribonucleotide; N^5-CAIR, N^5-carboxyaminoimidazole ribonucleotide; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PEP, phosphoenolpyruvate; SAICAR, 4-[(*N*-succinylamino)carbonyl]-5-aminoimidazole ribonucleotide; SDS, sodium dodecyl sulfate; TEAA, triethylammonium acetate; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; TSP, (trimethylsilyl)propanesulfonic acid.

0006-2960/94/0433-11917$04.50/0

Scheme 1



(1)



(2)

both AIR carboxylase and SAICAR synthetase (eq 1) (Patey & Shaw, 1973). Therefore, the vertebrate form of the enzyme catalyzes succcessive steps in *de novo* purine biosynthesis and has been refered to as PurCE. Specific fusions of *de novo* purine biosynthetic genes in other organisms are known, but their functional significance is not clear (Aimi et al., 1990).

Despite the absence of any homology of the vertebrate proteins with PurK, the *G. gallus* enzyme can functionally complement a *purK* mutant of *E. coli*, and the human cDNA has been shown to complement an *ade2* mutation in *S. cerevisiae* (Chen et al., 1990; Minet & Lacroute, 1990). The absence of a related PurK domain in the vertebrate proteins suggests that the PurCE enzyme contains a catalytic property that serves a PurK function in metabolism. The implications of these results have stimulated our interest in the the structure and mechanism of the AIR carboxylases. We have initiated a comparative biochemical study to obtain a comprehensive view of these related AIR carboxylases. The focus of this paper is the physical and kinetic properties of the bifunctional *G. gallus* protein. To this end, we have expressed the *G. gallus* AIR carboxylase–SAICAR synthetase in *E. coli* and purified the enzyme to apparent homogeneity. The results reported here reveal that the enzyme consists of two independent active sites and that ATP is not required for carboxylation of AIR. The chemical transformations catalyzed by the *G. gallus* and *E. coli* AIR carboxylases are the subject of the following paper in this issue (Firestine et al., 1994).

MATERIALS AND METHODS

Materials. Oligonucleotides were synthesized at the Laboratory for Macromolecular Structure of Purdue University and purified by PAGE before use. All solvents, unless otherwise indicated, were HPLC grade. All biochemicals, unless otherwise indicated, were purchased from Sigma. FSBA was dissolved in 50% DMF and used immediately. Dowex AG 50W-X8 was purchased from Sigma and treated with a 5 mM EDTA solution before exchanging to the desired ion. Whatman cellulose thin-layer chromatography plates were purchased from VWR. Triethyl phosphate was purchased from Aldrich and distilled from BaO before use. Phosphorous oxychloride was purchased from Fisher and distilled before use. Diisopropylethylamine was purchased from Aldrich and distilled from $KMnO_4$ and acetic anhydride before use. Pyruvate kinase, lactate dehydrogenase, endoproteinase Glu-C, and NADH were purchased from Boehringer Mannheim. Adenylosuccinate lyase was purchased from Sigma. DEAE-Sepharose FF, SP Sepharose FF, Sephacryl S-200, Sephacryl S-400, and molecular weight standards were purchased from Pharmacia. Molecular weight standards for SDS–PAGE were purchased from Bio-Rad. Immobilon membranes for protein blotting were obtained from Millipore. Protein sequence analyses were conducted at the Laboratory for Macromolecular Structure of Purdue University using Edman degradation chemistry on an ABI 473A sequencer. Analyses using inductively coupled plasma (ICP) emission spectroscopy were performed in the Department of Geology at the University of Michigan. Solutions of CAIR were prepared with 10 mM CHES, pH 9.0 buffer to prevent nonenzymatic decarboxylation. All concentrations, unless otherwise indicated, are the final concentrations in the assays. Analytical and preparative PRP-1 columns were purchased from Hamilton. The preparation and characterization of 4-nitro-5-aminoimidazole ribonucleotide (NAIR) are described elsewhere (Firestine & Davisson, 1993). *E. coli* PurC was a gift from Eric Meyer (MIT).

Ammonium 5-Amino-1-(β-D-ribofuranosyl)imidazole-4-carboxylate 5′-Phosphate (**2**). 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) (1.0 g, 3.87 mmol, dried *in vacuo* over P_2O_5) was prepared by phosphorylation of the corresponding nucleoside by the method of Yoshikowa et al., (1969). Crude AICAR (1.0 g) was dissolved in 7 mL of 6.0 M NaOH, and the solution was refluxed for 5 h. After cooling to room temperature, 50 mL of absolute ethanol was added, with subsequent cooling to −20 °C, and the top layer was decanted leaving a brown syrup. The syrup was repeatedly triturated with 10 mL portions of ethanol and dried *in vacuo* to a gummy solid which was dissolved in 50 mL of water. The pH of the solution was adjusted to the range of 9–10 using 4 N HCl, and a 25 mL portion was diluted to 200 mL with water before applying onto a DEAE-Sepharose column (HCO_3^- form, 2.5 × 22.5 cm). The column was washed at a flow rate of 4 mL min^{-1} with a 240 mL linear gradient from 0 to 250 mM NH_4HCO_3, followed by 120 mL of 250 mM NH_4HCO_3, and finally 120 mL of 500 mM NH_4HCO_3. The resulting fractions (8 mL) were analyzed for absorbance at 265 nm and by TLC (cellulose, 2:1 CH_3CN/100 mM NH_4-

HCO_3). Fractions containing **2** were combined and dried by lyophilization, and the residue was dissolved in 2.5% NH_4OH before drying again by lyophilization (2×) to give a yellow solid (404 mg, 35%). The sodium salt was prepared by dissolving in dilute NH_4OH and passing through a column of AG 50W-X8 (Na^+). TLC (2:1 CH_3CN/100 mM NH_4HCO_3) R_f 0.1; ^1H NMR (500 MHz, D_2O, TSP) δ 7.45 (1H, s, C2), 5.41 (1H, d, J = 5 Hz, C1′), 4.53 (1H, dd, J = 5 Hz, J = 7 Hz, C2′), 4.16 (2H, m, C3′, C4′), 3.94 (2H, m, C5′). FAB MS (–Ve, glycerol) m/z = 338 $[M - 1]^-$.

5-Amino-1-(β-D-ribofuranosyl)imidazole 5′-Phosphate (**3**). *Method A*: *Nonenzymatic Decarboxylation of* **2** *(Bhat et al., 1990).* In a 25 mL flask, **2** (50 mg, 0.132 mmol) was dissolved in 15 mL of 20 mM sodium acetate, pH 4.8 buffer. The reaction was stirred, and argon was bubbled into the solution overnight. The reaction was diluted to 25 mL with distilled water and applied to a DEAE-Sepharose column (2.5 × 22.5 cm) equilibrated with 100 mM triethylammonium acetate (TEAA), pH 7.0. After washing with water, AIR was eluted at a flow rate of 4.0 mL min^{-1} using a 500 mL linear gradient of 0–500 mM TEAA over 2 h. The resulting fractions (8 mL) were analyzed by UV at 260 nm and by HPLC [25 mM diisopropylethylammonium acetate, pH 7.5 (DIPEAA), with a flow rate of 1.0 mL min^{-1} gave a retention time of 4.9 min]. The solid recovered by lyophilization (2×) was dissolved in water and adjusted to pH 7.5. The material was converted to the Na^+ form by passing through a Dowex AG 50W-X8 (Na^+) column. The eluent was dried by lyophilization to a brown solid that was contaminated (30%) with acetate (63 mg).

Method B: *Enzymatic Decarboxylation of* **2**. In a 50 mL Falcon tube, **2** (100 mg, 0.263 mmol) was dissolved in 20 mM Tris-HCl, pH 7.6 buffer containing 0.2 mM EDTA to give a 10 mM solution. *G. gallus* AIR carboxylase (5 μg/mL) was added, and the solution was kept on ice. The reaction was monitored by diluting an aliquot of the reaction mixture into 100 mM Tris-HCl, pH 8.0, and measuring the absorbance at 260 nm using ϵ = 9000 M^{-1} cm^{-1}. After 2 h, the reaction was processed as described above for the nonenzymatic decarboxylation reaction (75 mg, 89%). TLC (7:3 CH_3CN/100 mM NH_4HCO_3) R_f 0.17; IR (KBr) 3411, 1654, 1098, 978, 894 cm^{-1}; ^1H NMR (500 MHz, D_2O, TSP) δ 7.6 (1H, d, J = 1.5 Hz, C2), 6.42 (1H, d, J = 1.0 Hz, C4), 5.66 (1H, d, J = 7.0 Hz, C1′), 4.65 (1H, dd, J = 7.0 Hz, J = 5.5 Hz, C2′), 4.20 (1H, dd, J = 2.5 Hz, J = 5.5 Hz, C3′), 4.25 (1H, m, C4′), 3.92 (2H, m, C5′); ^{13}C NMR (D_2O, TSP) δ 138.5 (C5), 134.9 (C2), 115.2 (C4), 89.7 (C1′), 87.1 (d, $J_{C,P}$ = 8.8 Hz, C4′), 75.7 (C2′), 73.1 (C3′), 66.1 (d, $J_{C,P}$ = 4.0 Hz, C5′).

4-[(N-Succinylamino)carbonyl]-5-aminoimidazole Ribonucleotide (*SAICAR*, **4**). In a 50 mL Falcon tube, **3** (36 mg, 0.122 mmol) was dissolved in 50 mM HEPES, pH 8.0 buffer, containing 200 mM $KHCO_3$, 0.5 mM ATP, 7.5 mM PEP, 80 units of pyruvate kinase, 10 mM aspartic acid, and 25 mM $MgCl_2$. The mixture was placed in a 37 °C bath, and the reaction initiated with 0.23 mg of *G. gallus* AIR carboxylase–SAICAR synthetase. After 4 h, an additional $KHCO_3$ equivalent was added and incubated for 2 h, at which point no change in absorbance at 282 nm was observed. The reaction was diluted to 50 mL and applied to a DEAE-Sepharose column (2.5 × 17 cm, HCO_3^- form). The compound was eluted at a flow rate of 4.0 mL min^{-1} using a 1 L stepwise gradient of 0–400, 400–700, and 700–1000 mM NH_4HCO_3 over 2.5 h, holding for 30 min at the end of each ramp. Fractions were monitored at 260 nm and by the modified Bratton–Marshall procedure (Lukens & Flaks, 1963). Fractions (8 mL) containing SAICAR were pooled and dried twice by lyophilization. Final purification was accomplished by preparative HPLC using 50 mM DIPEAA and 4% CH_3CN at a flow rate of 8.0 mL min^{-1}. After drying by lyophilization, the purified sample was exchanged to the ammonium form using Dowex AG 50W-X8 (NH_4^+) resin (22 mg, 40% yield). TLC (cellulose, 7:3:1 EtOH/H_2O/HOAc) R_f 0.43; ^1H NMR (500 MHz, D_2O referenced to external TSP) δ 7.67 (1H, s, C2), 5.81 (1H, d, J = 7 Hz, C1′), 4.76 (1H, dd, J = 7.0 Hz, J = 5.5 Hz, C2′), 4.72 (1H, dd, J = 4.5 Hz, J = 8.0 Hz, Cα), 4.55 (1H, dd, J = 5.5 Hz, J = 3.0 Hz, C3′), 4.45 (1H, m, C4′), 4.21 (2H, m, C5′), 2.92 (1H, dd, J = 4.5 Hz, J = 16 Hz, Cβ), 2.85 (1H, dd, J = 8.0 Hz, J = 16 Hz, Cβ).

General Recombinant DNA. Restriction digests were performed with plasmid DNA at 0.1 μg μL^{-1}. All DNA fragments for cloning purposes were purified by agarose gel electrophoresis and extracted using the Gene Clean protocol (Bio 101). Ligation reactions were performed with 0.1 μg of the appropriate vector DNA in 10 μL volumes and 1.5 units of T4 DNA ligase. Ligation mixtures (5 μL) were used to transform *E. coli* XL-1 Blue (Stratagene) or DH5α (Yale Genetic Stocks), and the desired recombinant plasmids were selected by restriction digestion. All other recombinant DNA methods were based upon standard protocols (Sambrook et al., 1989).

Construction of Plasmid Vector pD5-pur. Two plasmid clones containing the cDNA encoding the *G. gallus purCE* gene, pZD1 and pZD4 (Chen et al., 1990), were used as a source of different portions of the coding DNA. Briefly, the 693 bp fragment from *Eco*RI–*Spe*I digestion of pZD1 and the 881 bp fragment from *Nde*I–*Eco*RI digestion of pZD4 were ligated with the *Nde*I–*Spe*I 2.9 kb vector fragment of pDL-Nde (Davisson et al., 1989). This vector is a constitutive *E. coli* expression vector derived from Bluescript KS (Stratagene). After isolation of the recombinant plasmid pD2-*pur*, the 3′-nontranslated region was removed by digestion with *Apa*LI–*Spe*I. A synthetic linker with the appropriate restriction overhangs was ligated with the digested vector to replace this region. The sequence of the linker for the sense strand was 5′-TGCACCTTGTAAGGATCCA-3′ and for the antisense strand was 5′-CTAGTGGATCCTTACAAGG-3′. After annealing, the linker was phosphorylated with polynucleotide kinase prior to ligation with pD2-*pur* to arrive at the recombinant plasmid pD3-*pur*. The entire coding sequence for the *G. gallus* AIR carboxylase–SAICAR synthetase was isolated as an *Nde*I–*Bam*HI restriction fragment and cloned into the same sites in the T7 promoter vector pET 3a (Novagen) to provide the desired expression vector pD5-*pur*.

Purification of AIR Carboxylase–SAICAR Synthetase. All cultures were grown at 37 °C with shaking at 200 rpm. *E. coli* BL21 (DE3) pLysS cells were freshly transformed with the plasmid pD5-*pur* and selected on LB plates with Amp (100 μg mL^{-1}) and chloramphenicol (20 μg mL^{-1}). Single colonies were used to inoculate 3 mL aliquots of LB/Amp (100 μg mL^{-1}) and grown for 8–12 h before use as a 4% inoculum in 25 mL of LB/Amp (100 μg mL^{-1}) media. These cultures were grown for 6–8 h and used to inoculate 1 L portions of fresh medium lacking Amp. Cultures were grown to a cell density of OD_{550} = 0.9, and then IPTG was added to a final concentration of 1 mM. After an additional 4–5 h, the cells were harvested by centrifugation, and the cell pastes were stored at –80 °C.

All steps in the protein isolation were carried out at 4 °C. *E. coli* BL21 (DE3) pLysS/pD5-*pur* cells (approximately 3.5 g L^{-1} of culture) were thawed and resuspended in 50 mM

Tris-HCl and 1 mM EDTA, pH 8.0. The suspension was sonicated in a 20 s on, 10 s off cycle for a total of 3 min. The debris was removed by centrifugation for 15 min at 17000*g*. The supernatant was made 1% (w/v) in streptomycin sulfate, and the resulting cloudy solution was allowed to stand for 10 min. After centrifugation at 17000*g* for 10 min, the supernatant was diluted to 50 mL with water and applied to a SP Sepharose FF column (2.5 × 6 cm) equilibrated with 50 mM Tris-HCl and 1 mM EDTA, pH 8.0, at a flow rate of 1.8 mL min^{-1}. The column was washed with the same buffer before eluting the protein with a linear gradient of 100 mL of 50 mM Tris-HCl and 1 mM EDTA, pH 8.0, and 100 mL of 200 mM Tris-HCl, 200 mM KCl, and 1 mM EDTA, pH 8.0. The fractions containing PurCE enzymatic activities were concentrated using a PM10 membrane. The concentrated protein was applied to a S-200 gel filtration column (2.5 × 89 cm) which had been equilibrated with 50 mM Tris-HCl, 100 mM KCl, and 1 mM EDTA, pH 8.0 buffer. The protein was eluted using the same buffer at a flow rate of 1.8 mL min^{-1}. The protein that eluted was concentrated using a PM10 membrane and stored in 0.5 mL aliquots at 4 °C.

Removal of Metals from Protein. Metal-free enzyme (2 mL) was prepared by equilibrium dialysis against 4 L of 50 mM Tris-HCl, pH 8.0, and 1 mM EDTA, with 12 mequiv of Chelex (Na^+) resin. After 4 h, the sample was dialyzed against fresh buffer without EDTA.

Enzymatic Assays. Assay A: SAICAR Synthetase Assay with NADH (Meyer et al., 1992). In a 1 mL cuvette, 50 mM HEPES, pH 7.8 buffer, containing 25 mM $MgCl_2$ and 1.6 μg of enzyme was mixed and allowed to stand for 5 min at 37 °C. To the cuvette were added 0.48 mM AIR, 50 mM $KHCO_3$, 2.0 mM PEP, 4.0 units of pyruvate kinase, and 17 units of lactate dehydrogenase. The conversion of AIR to CAIR was monitored at 260 nm using $\Delta\epsilon$ = 9000 M^{-1} cm^{-1}. The assay was initiated by sequential addition of 1 mM ATP, 2 mM NADH, and 10 mM aspartic acid, and the change in absorbance at 340 nm was monitored using ϵ = 6200 M^{-1} cm^{-1}.

Assay B: SAICAR Synthetase Assay without NADH (Casey & Lowenstein, 1987). The assay was performed as above except that lactate dehydrogenase and NADH were eliminated. The reaction was initiated with 10 mM aspartic acid and monitored at 282 nm using $\Delta\epsilon$ = 8480 M^{-1} cm^{-1}.

Assay C: CAIR Decarboxylation Assay (Meyer et al., 1992). In a 1 mL total volume, 50 mM Tris-HCl and 0.5 mM EDTA, pH 8.0, and 150 μM CAIR were incubated at 30 °C. The reaction was initiated by the addition of enzyme (23–35 ng) while monitoring the decrease at 260 nm using $\Delta\epsilon$ = 9000 M^{-1} cm^{-1}.

Assay D: AIR Carboxylation Assay at 260 nm (Meyer et al., 1992). In a 1 mL total volume, 50 mM Tris-HCl and 0.5 mM EDTA, pH 8.0, AIR (typically 0.3 mM), and 200 mM $KHCO_3$ were incubated at 30 °C. The reaction was initiated by the addition of enzyme (23–60 ng), and the increase in absorbance at 260 nm was monitored.

Assay E: Coupled Assay for Carboxylation of AIR. In a 1 mL total volume, 50 mM HEPES, pH 8.0, 0.05 μg of enzyme, and 25 mM $MgCl_2$ were preincubated for 5 min at room temperature. To the solution were added 100 mM $KHCO_3$, 2.0 mM PEP, 1.0 mM ATP, 4.0 units of pyruvate kinase, 10 mM aspartic acid, and 0.24 unit of *E. coli* SAICAR synthetase. The reaction at 37 °C was initiated by the addition of 0.5 mM AIR and monitored at 282 nm using $\Delta\epsilon$ = 8480 M^{-1} cm^{-1}.

Effect of Mg^{2+} on CAIR Decarboxylation. The effect of Mg^{2+} on the rate of decarboxylation was determined using Assay C with the exception that 50 mM HEPES, pH 8.0, was used as the buffer and $MgCl_2$ (0–50 mM) was included in the solution.

CO_2-Dependent Carboxylation Assays. In a total volume of 1 mL within stopper-fitted cuvettes were combined 200 mM Tris-HCl, pH 8.0, 0.55 mM AIR, 0.23 μg of *G. gallus* AIR carboxylase, and 0–25 mM $MgCl_2$. The reaction was cooled to 10 °C and initiated by addition of a CO_2 solution to a final concentration of 20 mM, and the change in absorbance at 260 nm was recorded. UV–vis difference spectra of the CO_2-dependent carboxylation reactions revealed that the product formed had a spectrum identical to CAIR and was also a substrate for SAICAR synthetase. The CO_2 solutions were prepared by continuous delivery of CO_2(g) into 100 mL of water at 4 °C, and the concentration was estimated from solubility constants (0.2871 g/100 g of water) (Dean, 1985).

Is CAIR Released from the Enzyme? In a 1 mL cuvette, 50 mM HEPES, pH 8.0, 50 mM $KHCO_3$, 1.6 μg of *G. gallus* AIR carboxylase–SAICAR synthetase, 2.0 mM PEP, 1.0 mM ATP, 4.0 units of pyruvate kinase, 10 mM aspartic acid, 25 mM $MgCl_2$, and *E. coli* SAICAR synthetase (0 or 0.24 unit, 7.7 μg) were added and incubated at 37 °C. The reaction was initiated by the addition of 0.32 mM AIR and the increase at 282 nm monitored using $\Delta\epsilon$ = 8480 M^{-1} cm^{-1}.

Determination of SAICAR Extinction Coefficient (Lukens & Flaks, 1963). The concentration of a stock SAICAR solution was determined by cleavage of SAICAR to AICAR and fumarate by adenylosuccinate lyase. In a 1 mL total volume, 10 μL of a SAICAR solution was added to 20 mM HEPES, 100 mM KCl, pH 7.8, and 0.1 unit of adenylosuccinate lyase. The cleavage of SAICAR to AICAR and fumarate was monitored at 268 nm, and the concentration of SAICAR was estimated using ϵ = 12 700 M^{-1} cm^{-1}. This extinction coefficient was calculated from spectra of known concentrations of AICAR (ϵ_{268} = 12 348 M^{-1} cm^{-1}) and fumarate (ϵ_{268} = 388 M^{-1} cm^{-1}) under conditions of the assay. The extinction coefficient for SAICAR was determined by placing known concentrations of SAICAR into 50 mM HEPES, pH 8.0, and recording the absorbance spectra. These data gave extinction coefficients for SAICAR of ϵ_{268} = 14 829 M^{-1} cm^{-1} and ϵ_{282} = 9227 M^{-1} cm^{-1}.

Effect of Added Cofactors. To a 1 mL cuvette containing 50 mM Tris-HCl, pH 8.0, 120 μM AIR, and 400 mM $KHCO_3$ were added varying amounts of biotin (0–5 μM) and ATP (0–5 μM). The reaction was initiated by addition of 5 × 10^{-3} unit (0.02 μg, 0.4 pmol) of *G. gallus* AIR carboxylase and monitored at 260 nm. The reactions in the presence of thiamin (0–5 μM) were carried out using the same conditions.

Determination of [AIR]/[CAIR] at Equilibrium in the Presence and Absence of Mg^{2+}. Method A: HPLC Determination. In 1 mL Eppendorf tubes, 0.5 mM CAIR was added to 50 mM HEPES, pH 8.0, containing $MgCl_2$ (0–50 mM) and 200 mM $KHCO_3$. The tube was sealed with parafilm and placed into a 37 °C bath. After 1 and 2 days, 50 μL aliquots were analyzed by HPLC in 25 mM DIPEAA, pH 7.0. Under these conditions with a flow rate of 1.0 mL min^{-1} and monitoring at 260 nm, AIR had a retention time of 4.9 min and CAIR had a retention time of 14.0 min. The ratio of the areas for these two compounds was corrected for differences in extinction coefficients at 260 nm and used to determine the ratio of AIR/CAIR at equilibrium in the presence and absence of Mg^{2+}.

Method B: UV Determination. In a 1 mL cuvette, 92 μg of *G. gallus* AIR carboxylase was added to 50 mM HEPES, pH 8.0, containing 25 mM $MgCl_2$. To the cuvette were added

81–812 μM AIR and 50 mM $KHCO_3$. The reaction was monitored at 260 nm until no further change was observed. The amount of CAIR was calculated using $\Delta\epsilon$ = 9000 M^{-1} cm^{-1}, and the amount of AIR was obtained by subtracting the above number from the starting concentration of AIR.

Stoichiometery. In a 1 mL cuvette, 50 mM HEPES, pH 8.0, 1.0 mM ATP, 25 mM $MgCl_2$, 2.0 mM PEP, 43 units of lactate dehydrogenase, 10 units of pyruvate kinase, 100 μM NADH, 23 μg of *G. gallus* AIR carboxylase–SAICAR synthetase, and 50 μM CAIR were combined and incubated at 37 °C. The absorbance at 340 nm was monitored until a constant absorbance value was obtained before final addition of 10 mM aspartic acid. After 15 min, the absorbance at 340 nm was used to determine the consumption of ATP. The concentration of SAICAR produced was determined using Assay B with 50 μM CAIR. The absorbance difference at 282 nm was determined, and the amount of SAICAR produced was calculated using $\Delta\epsilon$ = 7700 M^{-1} cm^{-1}, obtained by subtracting the extinction coefficient for CAIR (ϵ_{282} = 1500 M^{-1} cm^{-1}) from the extinction coefficient for SAICAR (ϵ_{282} = 9220 M^{-1} cm^{-1}).

Steady-State Kinetic Analysis. Kinetic parameters for CAIR, AIR, and $KHCO_3$ for *G. gallus* AIR carboxylase were determined under the following conditions. For CAIR, Assay C was conducted using 0.023 μg of enzyme with CAIR varied from 10 to 100 μM. Assay D was used for AIR with 0.058 μg of enzyme, and substrate concentrations varied from 25 to 200 μM while $KHCO_3$ was held constant at 100 mM. For $KHCO_3$, Assay E was conducted using 0.058 μg of enzyme with 10–200 mM $KHCO_3$ and 0.34 mM AIR. Kinetic parameters for CAIR, ATP, and aspartic acid for *G. gallus* SAICAR synthetase were determined as follows. CAIR was generated *in situ* from AIR and $KHCO_3$ as described in Assay B, and all parameters were determined using 1.6 μg of enzyme. CAIR (2.9–40 μM) was generated from AIR (20–140 μM) with constant 50 mM $KHCO_3$, while ATP and aspartic acid were held constant at 1 and 10 mM, respectively. ATP concentrations were varied from 5 to 100 μM in the presence of 37 μM CAIR and 10 mM aspartic acid. Aspartic acid concentrations were varied from 0.5 to 10 mM with 35 μM CAIR and 1 mM ATP. The Michaelis constants for both *G. gallus* AIR carboxylase and SAICAR synthetase were estimated by fitting the velocity data as a function of substrate concentration to $v = VA/(K_A + A)$ as executed by the program Enzyme Kinetics (Trinity Software).

Determination of Native and Subunit Molecular Weight for AIR Carboxylase–SAICAR Synthetase. The subunit molecular weight was determined by SDS–PAGE (12%) according to the procedure of Laemmli (1970). Molecular mass standards included phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). The native molecular mass was estimated by size exclusion chromatography using Sephacryl S-400 equilibrated with 50 mM Tris-HCl, 100 mM KCl, and 1 mM EDTA, pH 8.0. The column (2.5 × 80 cm) was calibrated using thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), and albumin (67 kDa), and the void volume (164 mL) was determined using Blue dextran.

Preparation of Protein for N-Terminal Sequencing. *G. gallus* AIR carboxylase–SAICAR synthetase (0.1 mg) was exchanged into 50 mM NH_4HCO_3 using a NAP-10 column (Pharmacia). The protein fractions were concentrated to dryness and redissolved in 0.1% TFA. A sample (350 pmol) of this solution was subjected to Edman degradation on an ABI 473A automated sequencer. The yield was 50% for the first cycle, and the average repetitive yields for 24 cycles were 85–90%.

Limited Digestion of AIR Carboxylase. In a final volume of 300 μL, 0.1 μg of endoproteinase Glu-C was added to 50 mM NH_4HCO_3 containing 1.2 mg of *G. gallus* AIR carboxylase–SAICAR synthetase. The reaction was incubated at 37 °C, and samples were removed and diluted with 10% SDS electrophoresis sample buffer and heated at 98 °C for 15 min. Samples were frozen at –20 °C before analysis by SDS–PAGE.

Proteolytic Fragment Isolation. Samples from a 20% SDS–PAGE were electroblotted onto a PVDF membrane (Immobilon) using the Mini-Trans Blot apparatus from Bio-Rad according to the manufacture's protocols. The electroelution was run at constant voltage (70 V) for 1 h using 25 mM Tris, 192 mM glycine, and 20% methanol, pH 8.3. The PVDF membrane was washed with water (3×) and stained with Coomassie blue. The two major protein fragments were cut from the membrane using a razor blade and subjected to Edman degradation sequence analysis.

Affinity Labeling and Inhibition. 5'-[4-(Fluorosulfonyl)-benzoyl]adenosine (FSBA). In an Eppendorf tube, FSBA (0–2.5 mM, 1.5% DMF) was added to 50 mM BES, pH 7.1, 25 mM $MgCl_2$, ATP (0 or 200 μM), and 49 nM of *G. gallus* AIR carboxylase–SAICAR synthetase to a total volume of 1.0 mL. The tubes were kept on ice, and at appropriate time points, 100 μL aliquots were assayed for SAICAR synthetase activity according to Assay B with the exception that 50 mM BES, pH 7.1 buffer was used. AIR carboxylase activity was determined using Assay C with 10 μL of the reaction solution.

4-Nitro-5-aminoimidazole Ribonucleotide (NAIR). In a 1 mL cuvette, *G. gallus* AIR carboxylase–SAICAR synthetase (0.5 nM) was incubated with NAIR (0–10 μM) in 50 mM Tris-HCl, pH 8.0 buffer at 37 °C for 10 min. Residual *G. gallus* AIR carboxylase activity was determined by addition of 150 μM CAIR to the incubation mixture and monitoring the reaction as stated for Assay C. The nonenzymatic rate of CAIR decomposition (1.04×10^{-3} μmol min^{-1}) under conditions of the assay was subtracted from all measured rates. SAICAR synthetase activity was determined by preincubating 5.0 nM enzyme with NAIR (0–100 μM) in 50 mM HEPES, pH 8.0, in a 1 mL cuvette at 37 °C for 10 min and analyzed as in Assay B except that AIR and $KHCO_3$ were omitted and the reactions were initiated with CAIR.

RESULTS

Heterologous Expression of G. gallus PurCE. The original cloning strategy for the *G. gallus purCE* cDNA involved genetic complementation in *E. coli* of a *purK* deficiency (Chen et al., 1990). Although expression of the gene was demonstrated, the levels of protein were not suitable for isolation and characterization. Our efforts began with the original plasmid vectors and used a simple reconstruction of the *G. gallus* cDNA coding region in a constitutive expression vector (Davisson et al., 1989). Plasmid pD3-*pur* confers pur^+ phenotypes to the *E. coli* strains that are deficient in PurK (TX209), PurE (TX257), PurK–PurE (NK6051), or PurC (NK6056). Optimal cloning of the coding DNA into a T7 RNA polymerase vector followed by expression in *E. coli* BL21(DE3)pLysS provided one major induced protein of apparent molecular mass 46 kDa, consistent with the calculated molecular weight deduced from the gene sequence. Although the original reported vectors for the high-level expression failed in a similar DNA construct (Chen et al., 1990), we suggest that our success is attributable to the cassette subcloning of only the coding region of the cDNA.

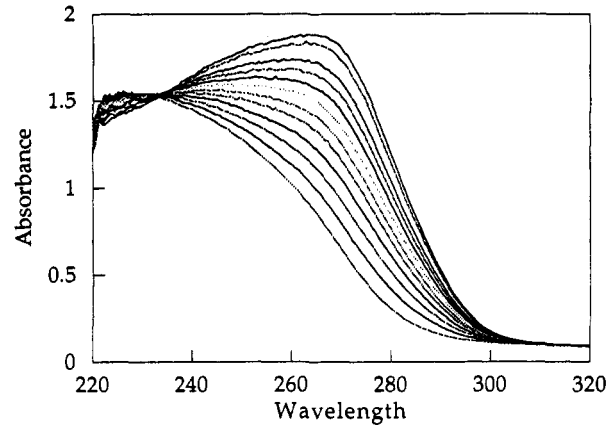


FIGURE 1: UV–vis spectra over time for the reaction of AIR to SAICAR using Assay E. Each line represents a 0.2-min increment.

AIR Carboxylase Assays. The amino acid sequence alignments of the related purine biosynthetic genes, and previous analysis of the enzyme isolated from liver tissue, suggested *G. gallus* AIR carboxylase and SAICAR synthetase exist as a bifunctional protein that catalyzes successive steps in the *de novo* purine pathway. The need for suitable assays for both the purification and kinetic analyses of these two enzyme activities required the adaptation and refinement of available systems. Routine analyses of AIR carboxylase were based upon the conversion of CAIR to AIR in the nonphysiological direction. This assay was simplified by a favorable equilibrium which lies toward AIR (Meyer et al., 1992).

Direct observation of the enzyme-catalyzed reaction for AIR to CAIR was more problematic and required high levels of $KHCO_3$. A second coupled enzyme assay was made possible by use of *E. coli* SAICAR synthetase purified from a recombinant overexpression strain (Meyer et al., 1992). The overall spectral changes for the conversion of AIR to SAICAR (shown in Figure 1) allowed for continuous monitoring at 282 nm where the absorbance of AIR and CAIR is negligible. By including appropriate amounts of the monofunctional *E. coli* SAICAR synthetase, the specific activity of the *G. gallus* AIR carboxylase was found to increase from 7 to 40 units mg^{-1}. Since the assay monitors the production of SAICAR, this >5-fold stimulation indicated that CAIR is released in the steady-state turnover of AIR carboxylase, and the free pool is the substrate for both the *E. coli* and *G. gallus* SAICAR synthetases.

Metal Effects on AIR/CAIR Equilibrium. Several critical details of the bifunctional enzyme were characterized in the process of developing the coupled AIR carboxylase–SAICAR synthetase assay. Since SAICAR synthetase was found to require Mg^{2+}, the effects of the metal on the AIR carboxylase reaction were studied. The specific activity for the decarboxylase reaction decreased as a function of total Mg^{2+} concentration, resulting in a 10-fold reduction at 25 mM. In contrast, the specific activity for the carboxylation reaction was increased only 1.5-fold by the presence of Mg^{2+} under the same conditions.

In order to further demonstrate the basis for the differential effects of $MgCl_2$ upon the AIR carboxylase activity, a CO_2-dependent assay was employed (Firestine et al., 1994). Under these conditions, the time required to attain CO_2/HCO_3^- equilibrium is prolonged (Cooper et al., 1968). The AIR/CAIR equilibrium characteristics of the *G. gallus* AIR carboxylase reaction can be observed by monitoring the reaction progress over an extended time period at 10 °C as shown in Figure 2. In the absence of $MgCl_2$, the CAIR concentration increased over the first 8 min and then slowly

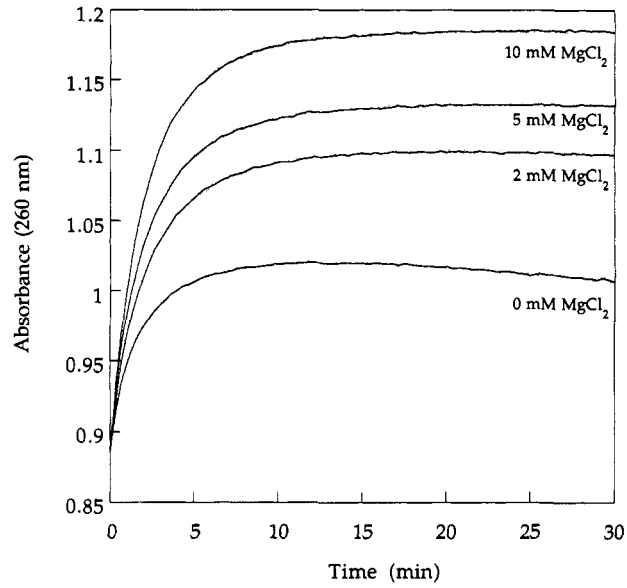


FIGURE 2: Effect of $MgCl_2$ on the progress of CAIR formation by *G. gallus* AIR carboyxlase in the presence of 20 mM CO_2 at 10 °C.

decreased over the next 20 min. The subsequent decrease in absorbance at longer times is attributed to the changes in the concentrations of CO_2 and HCO_3^- (eq 3) over the period of the experiment, which subsequently affects the AIR/CAIR equilibrium.

$$HCO_3^- \rightleftharpoons CO_2 + AIR \underset{\text{AIR carboxylase}}{\rightleftharpoons} CAIR \rightleftharpoons CAIR \cdot Mg^{+2}$$
$$\downarrow$$
$$CO_2(g) \qquad (3)$$

In the presence of $MgCl_2$, a very different time course for the AIR/CAIR equilibrium was observed (Figure 2). The rate and extent of AIR conversion in these carboxylations were modified by inclusion of metal, while the initial velocities for carboxylation were not affected. Up to 25% of the AIR was converted to CAIR in the incubation with 10 mM $MgCl_2$. As described in eq 3, the net effect is a slower rate of AIR/CAIR equilibrium attributed to formation of a CAIR–Mg^{2+} complex which is not a substrate for the *G. gallus* AIR carboxylase.

SAICAR Synthetase Assays. Assays for SAICAR synthetase activity were complicated by the AIR carboxylase-catalyzed reaction for CAIR to AIR which was faster than the ATP- and aspartate-dependent conversion of CAIR to SAICAR. To overcome this problem, AIR, $KHCO_3$, and AIR carboxylase–SAICAR synthetase were incubated in the presence of the $MgCl_2$ for a time period suitable to achieve CAIR/AIR equilibrium. The final nucleotide concentrations were verified by UV absorbance of the solutions as well as by HPLC measurements, and the equilibrium ratio of AIR/CAIR with 25 mM $MgCl_2$ was shown to be 2.0. The synthetase reaction was then initiated by addition of ATP and aspartic acid. Direct observation of SAICAR production was accomplished at 282 nm as described above (see Figure 1), and the effective extinction coefficient was determined by a comparison to the rates of ATP consumption. This correlation resulted in an $\Delta\epsilon_{282} = 8480\ M^{-1}\ cm^{-1}$. To verify this result, a purified sample of SAICAR was used to determine the extinction coefficient. By subtracting the extinction coefficient of AIR ($\epsilon_{282} = 620\ M^{-1}\ cm^{-1}$) from that of SAICAR ($\epsilon_{282} = 9227\ M^{-1}\ cm^{-1}$), a value of $\Delta\epsilon_{282} = 8600\ M^{-1}\ cm^{-1}$ was determined.

Table 1: Purification of *G. gallus* AIR Carboxylase–SAICAR Synthetase[a]

step	volume (mL)	protein (mg)	Assay C[b]	total units	Assay E[c]	total units	Assay B[d]	total units
crude	25	141	71	10053	13	1791	2.3	324
streptomycin	27	104	76	7862	14	1414	2.5	261
SP Sepharose	8	40	157	6280	23	920	4.4	176
S-200	9	21	222	4662	36	756	7.2	151

[a] 1 L of cells. [b] Decarboxylation of CAIR to AIR. [c] Carboxylation of AIR to CAIR. [d] Production of SAICAR.

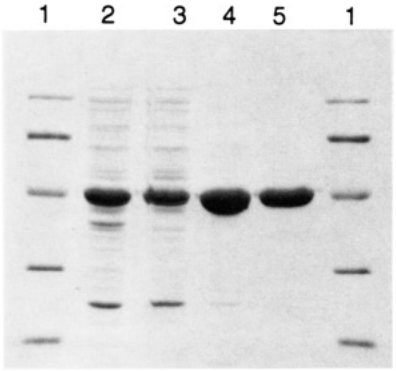


FIGURE 3: SDS–PAGE (12%) analysis of the purification of *G. gallus* AIR carboxylase–SAICAR synthetase. Molecular weight standards as stated in the Materials and Methods (lanes 1); crude cellular extract (lane 2); 1% streptomycin sulfate (lane 3); SP-Sepharose (lane 4); S-200 (lane 5).

Enzyme Purification and Chacterization. The high-level expression system developed for the *G. gallus* enzyme reduced the purification process to a two-column protocol based upon earlier efforts and is summarized in Table 1 and Figure 3. Three catalytic properties indicated a 3-fold purification of the protein to apparent homogeneity. The observed subunit mass of the enzyme was 46 kDa by SDS–PAGE, which is consistent with the calculated molecular weight of 47 245 deduced from the coding cDNA. A native molecular mass was estimated by size exclusion chromatography to be ≥330 kDa, consistent with at least a heptamer of subunits. The UV–vis spectrum of the purified protein showed no unusual features, with a maximum centered at 280 nm.

In contrast to the earlier reports of the *G. gallus* AIR carboxylase–SAICAR synthetase, the stability of the catalytic activities did not require thiol reagents (Patey & Shaw, 1973). Protein isolated from the S-200 chromatography was stable at 4 °C for at least 6 months. Longer term storage (even with the addition of glycerol) and prolonged dialysis (>12 h) consistently resulted in loss of approximately 50% of the catalytic activity. Unlike an earlier report (Patey & Shaw, 1973), no unusual changes in electrophoretic mobility were observed upon addition of thiol reagents to the recombinant protein.

The protein was subjected to amino acid sequence analysis, and the first 21 residues were verified as the following: Pro-Ala-Ala-Ser-Glu-Leu-Lys-Leu-Gly-Lys-Lys-Val-Asn-Glu-Gly-Lys-Thr-Lys-Glu-Val-Tyr. The Met and Ala residues at positions 1 and 2 were apparently removed in a post-translational process since these amino acids were included in the coding region of the DNA.

Cofactors. The enzyme was investigated to determine if addition of various cofactors could enhance the specific activity of *G. gallus* AIR carboxylase. Addition of ATP, biotin, and thiamin up to a concentration 10 000-fold greater than that of *G. gallus* AIR carboxylase resulted in no change in specific activity. Addition of various metals, Zn^{2+}, Co^{2+}, Cu^{2+}, and Mn^{2+}, resulted in precipitation of CAIR–metal complexes from solution. Treatment of the protein with a 10^4-fold excess of *o*-phenanthroline for 60 min resulted in no change in specific activity, indicating the absence of accessible, enzyme-bound metals required for catalysis.

Table 2: Metal Analysis of AIR Carboxylase–SAICAR Synthetase

sample[a]	protein (μM)	metals (μM)	% act.
1	48.5	Mg^{2+} (28.6), Ca^{2+} (121), Zn^{2+} (4.9)	100[b]
2	44.5	Mg^{2+} (5), Ca^{2+} (15)	70[b]
3	44.0	Mg^{2+} (3.9), Ca^{2+} (18)	90[b]/100[c]

[a] Sample 1: dialyzed against 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 6 g of Chelex, for 1 h. Sample 2: dialyzed as for sample 1 but for 30 h. Sample 3: dialyzed against 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 6 g of Chelex, for 4 h, and then changed to 50 mM Tris-HCl, pH 8.0, and 6 g of Chelex for 4 h. [b] Determined using Assay C. [c] Determined using Assay D.

Table 3: Steady-State Kinetic Parameters for AIR Carboxylase–SAICAR Synthetase

enzyme	substrate	K_m	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1} min^{-1})
AIR carboxylase	AIR	76 μM	2400	3×10^7
AIR carboxylase	$KHCO_3$ (CO_2)	23 mM (<0.8 mM)	1900	7×10^4
AIR carboxylase	CAIR	20 μM	10800	5×10^8
SAICAR synthetase	CAIR	3 μM	240	8×10^7
SAICAR synthetase	ATP	10 μM	192	2×10^7
SAICAR synthetase	aspartate	1.4 mM	211	2×10^5

Protein dialyzed against EDTA and Chelex was found to retain 90% of the catalytic activity. The metal content of the protein samples was assessed by ICP spectrometry. As shown in Table 2, the only significant metals found in the protein samples were Ca^{2+} and Mg^{2+}, and their quantities have no correlation with the carboxylase/decarboxylase activities. These metals bound with high affinity to the protein; however, they are not essential for catalysis. In contrast, low-affinity magnesium binding sites required for full SAICAR synthetase activity are suggested by the positive effect of preincubation.

Stoichiometry. One critical issue for the *G. gallus* AIR carboxylase–SAICAR synthetase is the overall stoichiometery of substrates and products. This is especially important in consideration of the recent discovery that the corresponding system in *E. coli* uses 2 mol of ATP for every mole of AIR converted to SAICAR (Meyer et al., 1992). In the presence of 49.9 ± 0.31 nmol of AIR/CAIR, 50 mM $KHCO_3$, and the *G. gallus* enzyme, a total of 48.8 ± 0.33 nmol of ATP was consumed and 53.6 ± 1.2 nmol of SAICAR was produced. Therefore, the total consumption of AIR in the presence of excess $KHCO_3$ revealed a 1:1 molar ratio with ATP, and SAICAR. ATP was required only for the aspartylation of CAIR and establishes the lack of a PurK function within the *G. gallus* AIR carboxylase protein.

Steady-State Kinetics. The kinetic constants for all of the catalytic activities of the *G. gallus* AIR carboxylase–SAICAR synthetase were determined and are summarized in Table 3. These data establish that the carboxylase and synthetase properties of the protein can be treated as separate catalytic units. The k_{cat}/K_m values for the carboxylase and synthetase reactions indicate efficient utilization of the substrates with the exception of $KHCO_3$. Although the kinetic data are

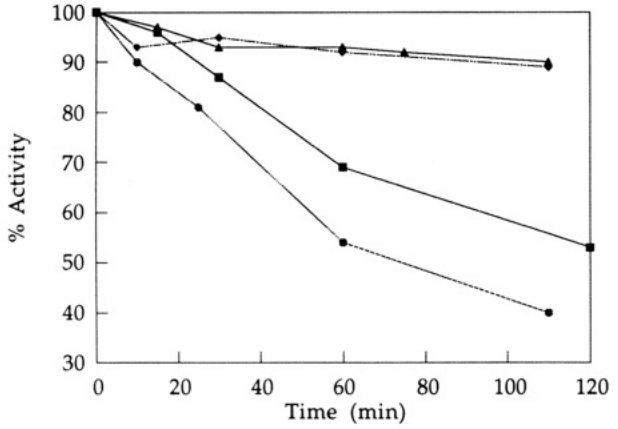


FIGURE 4: Inactivation of *G. gallus* AIR carboxylase–SAICAR synthetase by FSBA. (■) Activity of SAICAR synthetase (Assay B) after preincubation with 0.5 mM FSBA. (●) Activity of SAICAR synthetase (Assay B) after preincubation with 2.5 mM FSBA. (♦) Activity of SAICAR synthetase (Assay B) after preincubation with 0.5 mM FSBA and 200 μM ATP. (▲) Activity of AIR carboxylase (Assay C) after preincubation with 2.5 mM FSBA. All assays were compared to the appropiate control which lacked FSBA.

reproducible, the *G. gallus* carboxylase appears to use $KHCO_3$ at low efficiency and suggests that HCO_3^- is not the physiological substrate. Despite this ambiguity, the *G. gallus* carboxylase does accelerate the carboxylation of AIR by a factor of $\geq 10^6$ when compared to the rate of the chemical carboxylation (7×10^{-4} min^{-1}) (Meyer et al., 1992; Mueller et al., 1994). Another interesting feature is the 10-fold greater k_{cat}/K_m for the single substrate decarboxylase activity. This suggests that the kinetic measurements of the *G. gallus* enzyme may not reflect the true substrate requirements for the carboxylation reaction.

One problem with the steady-state data presented above is that, at the bicarbonate levels used in these studies, a third nucleotide exists in solution with AIR and CAIR. A recent account outlines the reaction between AIR and HCO_3^- which results in carboxylation on the exocyclic amine nitrogen to form N^5-CAIR (Mueller et al., 1994). This intermediate has a short lifetime under the conditions of our measurements ($t_{1/2}$ 0.7 min) but could represent up to 16% of the total nucleotide pool. The binding of N^5-CAIR to the *G. gallus* AIR carboxylase may lead to overestimates of the K_m value for AIR; the interaction of the enzyme with this nucleotide is addressed in the following paper (Firestine et al., 1994).

Affinity Labeling and Inactivation. Although the lack of steady-state shuttling of CAIR to *G. gallus* SAICAR synthetase suggests that the two enzymes are independent, a second approach toward analysis of the possible interdependence of the two catalytic sites of the protein was pursued. The selective inactivation of either the AIR carboxylase or the SAICAR synthetase was attempted using agents directed to each active site. As shown in Figure 4, incubation of the enzyme with an ATP affinity analog FSBA resulted in the loss of SAICAR synthetase activity. This inactivation was dependent upon both the time of incubation and FSBA concentration. Inclusion of saturating concentrations of the substrate ATP protected against the loss of SAICAR synthetase activity, indicating that the reagent was competing for the ATP binding site. Most importantly, 90% of the AIR carboxylase activity (measured as CAIR decarboxylase) remained. The small (10%) loss of AIR carboxylase activity could be due to nonspecific binding of the FSBA; however, differences in sensitivity to FSBA suggest no interdependence of the two catalytic activities.

A parallel experiment was pursued to corroborate the selective inactivation data with FSBA. The goal was to identify whether inhibition of AIR carboxylase activity affected the turnover of SAICAR synthetase. In related studies, our efforts have led to the discovery of a slow, tight-binding inhibitor of AIR carboxylase. This compound, NAIR, is the 4-nitro analog of the metabolite CAIR (Firestine & Davisson, 1993). Incubation of the *G. gallus* bifunctional enzyme for 10 min at 37 °C with varied molar ratios of NAIR prior to addition of substrate resulted in inhibition of the AIR carboxylase. The degree of AIR carboxylase inhibition was within the limits of detection at the highest concentration used for NAIR (Table IV). Despite the complete inhibition by tight binding of NAIR in the AIR carboxylase active site, SAICAR synthetase activity was not significantly altered. Together, the data show that these agents selectively inhibit or inactivate the catalytic properties of the protein, and under these conditions there is no interdependence of the two functions in *G. gallus* AIR carboxylase–SAICAR synthetase.

Table 4: Effect of NAIR on *G. gallus* AIR Carboxylase–SAICAR Synthetase

NAIR protein	% act. of *G. gallus* AIR carboxylase[a]	% act. of *G. gallus* SAICAR synthetase[b]
20000	0	92 ± 9.0
2000	1.6 ± 1.0	100 ± 8.0
200	4.0 ± 2.0	100 ± 7.0
20	33 ± 3.5	91 ± 8.0

[a] Determined using Assay C. [b] Determined using Assay B.

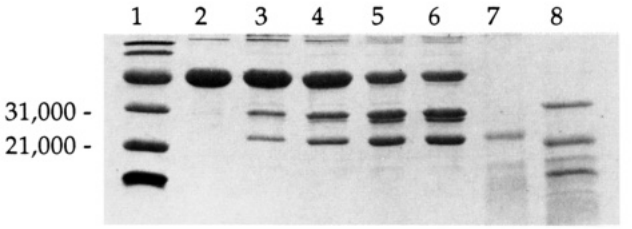


FIGURE 5: SDS–PAGE (20%) analysis of limited digests of *G. gallus* AIR carboxylase–SAICAR synthetase by endoproteinase Glu-C. Molecular weight standards as stated in the Materials and Methods (lane 1); undigested protein (lane 2); digestion at 4 h (lane 3); digestion at 8 h (lane 4); digestion at 16 h (lane 5); digestion at 24 h (lane 6); digestion at 48 h (lane 7); low molecular weight standards: carbonic anhydrase 31 000; soybean trypsin inhibitor 20 400, 19 700; lysozyme 14 400 (lane 8).

Limited Proteolysis. A previous study using deletion mutagenesis and genetic complementation of *E. coli* suggested that the *G. gallus* AIR carboxylase–SAICAR synthetase protein had independent folded domains (Chen et al., 1990). As shown above, our efforts have substantiated that the protein contains distinct catalytic active sites. This model suggests that each of the catalytic sites resides in a distinct folding domain bearing functional homology to related proteins from other organisms. To establish a direct experimental basis for this model, *G. gallus* AIR carboxylase–SAICAR synthetase was subjected to limited digestion with several proteases. In Figure 5 are presented the results for digestion of the protein with endoproteinase Glu-C. This enzyme was chosen because amino acid sequence alignments revealed a glutamic acid-rich region spanning the two putative domains which functionally relate to SAICAR synthetase and AIR carboxylase. The endoproteinase Glu-C cleavage gave two predominant fragments of molecular mass 27 and 20 kDa, which is consistent with the expected molecular weights for the corresponding monofunctional *E. coli* proteins (PurC and PurE). The 20 kDa fragment was particularly stable to the protease treatment, remaining intact for up to 24 h. Both fragments were isolated by electroblotting onto PVDF membranes and analyzed for their amino acid sequence. Figure 6 summarizes the sequence

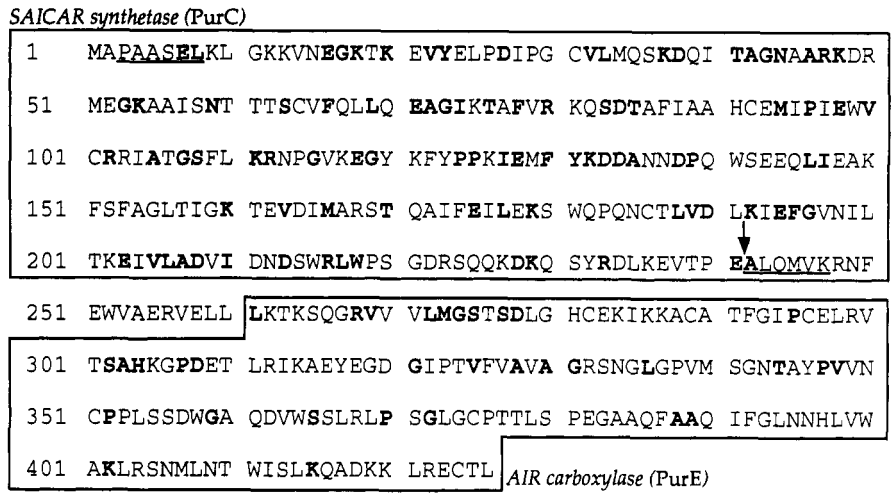


FIGURE 6: Cleavage site of endoproteinase Glu-C. Amino acids in bold indicate residues which have the highest sequence homology to other AIR carboxylases and SAICAR synthetases (Chen et al., 1990). Amino acids underlined indicate sequences determined from fragments isolated by SDS–PAGE followed by electroblotting to the PVDF membrane. The arrow indicates the site of cleavage by endoproteinase Glu-C. Boxed regions outline the two domains predicted by amino acid sequence alignments.

analyses for these protein fragments and shows that the major internal protease cleavage site was at Glu-241. This position is one of the three glutamic acids that reside near the proposed interdomain region, which, upon cleavage, provides the 20-kDa protein constituted by amino acids Glu-241 to Leu-426. Sequence analysis of the first eight amino acids in the higher molecular weight fragment gave P-A-A-S-E-L, indicating that the 27-kDa protein was Pro-3 to Pro-240. These results are consistent with a model for which the native structure has a solvent-exposed region between two folded domains. Each domain is partially protected from extensive proteolytic degradation.

DISCUSSION

Despite their synonymous function in *de novo* purine biosynthesis, the AIR carboxylase genes characterized to date exhibit limited amino acid sequence homologies. In *E. coli*, a second protein, PurK, has been identified as an AIR-, ATP-, and HCO_3^--dependent carboxylase that forms a chemically unstable nucleotide intermediate N^5-CAIR (Mueller et al., 1994). PurE catalyzes the final carbon–carbon bond-forming reaction utilizing N^5-CAIR, and this protein bears sequence homology to the C-terminal region of the *G. gallus* AIR carboxylase–SAICAR synthetase (PurCE). The heterologous expression and purification of the *G. gallus* protein have allowed for the direct comparison of the catalytic functions of the bacterial and vertebrate enzyme systems. In contrast to the *E. coli* PurK/PurE system, the stoichiometry of the overall reactions catalyzed by the *G. gallus* enzyme demonstrates that ATP is not utilized for carboxylation of AIR, and the ATPase activity analogous to the *E. coli* PurK (Meyer et al., 1992) was absent. These results are consistent with the lack of amino acid sequence homology between the *G. gallus* and PurK protein but do not explain the ability of the *G. gallus* gene to complement an *E. coli* strain with a *purK*$^-$ genotype (Chen et al., 1990).

Effect of Metals. The bifunctional nature of the *G. gallus* protein presented unique problems for the development of selective assays. Analysis of the synthetase activity proved especially difficult since the enzyme-catalyzed decarboxylation of CAIR occurs with higher turnover. However, metal effects on the enzyme-catalyzed equilibrium between AIR and CAIR were used for the synthetase assays by preincubation of the enzyme-nucleotide mixtures followed by reaction initiation with aspartic acid.

The divalent metal cation effect on the *G. gallus* AIR carboxylase is significant to the AIR and CAIR equilibrium. Previous model studies have established a series of CAIR–transition metal complexes which are stablized toward buffer-catalyzed decarboxylation (Litchfield & Shaw, 1971). In addition, CAIR–metal complexes have been suggested to be poor substrates or inhibitors of the AIR carboxylase (Chipperfield et al., 1988). The experimental conditions of the previous model studies failed to measure a stable CAIR–Mg^{2+} complex, but an effect on the extent of AIR carboxylase reaction has been mentioned (Patey & Shaw, 1973). Our results in the CO_2 addition reactions indicate that free CAIR concentrations must be reduced when $MgCl_2$ is present, since only the rate of the CAIR to AIR reaction (Figure 2) was affected. These results are consistent with the steady-state turnover of the *G. gallus* AIR carboxylase, which showed a 10-fold reduction for CAIR decarboxylation in the presence of 25 mM $MgCl_2$ with only minor effects on the rate of AIR carboxylation. Also consistent with this postulate are the steady-state kinetic parameters for the *G. gallus* SAICAR synthetase which implicate a CAIR–Mg^{2+} complex as the substrate. Together, the CAIR-stabilizing effect by divalent metal cations and the substrate binding properties of the SAICAR synthetase may conspire to affect the rate of the CAIR decarboxylation and its biosynthetic transformation to SAICAR.

Comparison with Other Carboxylases. Steady-state kinetic analyses of all the catalytic properties of *G. gallus* AIR carboxylase–SAICAR synthetase were possible and are consistent with those for the corresponding *E. coli* enzymes. A significant property of the *G. gallus* AIR carboxylase is the catalytic efficiencies (k_{cat}/K_m) for the nucleotide substrates which are near diffusion control. When compared to the nonenzymatic reactions, the enzyme is responsible for accelerating carboxylation and decarboxylation by factors of $\geq 10^6$ and $\geq 10^7$, respectively. One significant difference between the *G. gallus* and bacterial forms of the enzyme that exists involves the HCO_3^--dependent carboxylation of AIR. In *E. coli*, the two-protein PurK/PurE carboxylase system has been shown to function at low HCO_3^- concentrations only in the presence of ATP. In the absence of ATP, the PurE-catalyzed carboxylation requires a chemical reaction between AIR and HCO_3^- before turnover to CAIR. The *G. gallus* AIR carboxylase, which has no ATP dependence, displays a K_m for HCO_3^- that is nonphysiological but distinctly lower

than that for the PurE-catalyzed carboxylation, supporting the hypothesis that different substrates are required. Evidence is provided in the following paper that CO_2 is the actual substrate for the *G. gallus* enzyme, making estimates of K_m less accurate but more consistent with the probable physiological role (Firestine et al., 1994).

Previous data regarding the biochemical properties of the *G. gallus* AIR carboxylase–SAICAR synthetase are limited. The recombinant protein has an estimated native M_r, that suggests it exists at least as a heptamer of bifunctional subunits. Similar aggregate properties have been observed for the proteins isolated from liver tissue and the related enzymes from *E. coli* (Patey & Shaw, 1973; Meyer et al., 1992). Despite the potential mechanistic corollary to other carboxylases, there have been no previous reports of a cofactor dependence for AIR carboxylases. Our preliminary studies have failed to identify any metal or cofactor requirements for the protein-dependent carboxylation. The involvement of biotin as a cofactor has been ruled out since ATP is not required for the carboxylation reaction, and a systematic search for metal cofactors by chemical and spectroscopic methods has proven negative. Since their substrates are aromatic materials, AIR carboxylases catalyze a unique metabolic transformation and obviously have evolved important functions for CO_2 fixation. Therefore, an understanding of the essential protein structural features involved in the efficient carboxylation of their respective nucleotide substrates is a subject deserving further experimental attention.

Global Structure and Function. The original cloning and amino acid sequence alignments of the AIR carboxylases and SAICAR synthetases suggested that the protein consisted of two independent domains (Chen et al., 1990). A genetic analysis of the bifunctional enzyme revealed that deletion of 106 amino acids from the C-terminal region encompassing the putative AIR carboxylase-domain gave a partially functional protein which complemented an *E. coli* strain deficient in SAICAR synthetase, but not the AIR carboxylase deficient strains. Conversely, a similar complementation analysis showed that deletion of amino acids 17 and 18 affected the SAICAR synthetase function but not the function of the AIR carboxylase. The amino acid sequence alignments implicate a region of approximately 20 residues in the protein presumed to connect two functional domains. Limited proteolysis of the purified protein now indicates that this interdomain segment is exposed in the native protein and that the predicted folded domains are resistant to further digestion despite the presence of 23 additional sites. These corroborative data implicate a global protein structure for the vertebrate AIR carboxylase–SAICAR synthetase having two independent folding domains each responsible for different catalytic functions.

Fusion of catalytic proteins involved in sequential steps in a critical metabolic pathway offers potential for cooperative or conditional domain interactions. A relationship of this type seemed especially attractive for *G. gallus* AIR carboxylase since AIR has a limited half-life under physiological conditions, and the carboxylation reaction has an equilibrium that favors substrate. The fusion of AIR carboxylase and SAICAR synthetase suggested an evolutionary pathway designed to remove the ATP requirement for carboxylation and render the process irreversible via direct transfer of the intermediate CAIR to the SAICAR synthetase catalytic site. Useful assays for the *G. gallus* enzyme required addition of exogenous SAICAR synthetase as a coupling enzyme to maintain optimal turnover of CAIR, indicating that a significant portion of the CAIR is released from the AIR carboxylase active site. Selective, active site-directed inactivations with tight-binding inhibitors or affinity analogs also demonstrate that the carboxylase and synthetase activities are not interdependent. Although the shuttle postulate is attractive, the current data regarding the global structure and independent catalytic properties of the *G. gallus* enzyme do not support an obligatory shuttling event under *in vitro* steady-state conditions. There are few documented cases of biochemical shuttling events, and they are difficult to detect *in vitro* (Dunn et al., 1990; Anderson et al., 1991). Shuttling in *de novo* purine biosynthesis could be dependent upon other protein–protein interactions not addressed in these studies.

ACKNOWLEDGMENT

We would like to thank Howard Zalkin, Jack Dixon, Erik Meyer, and JoAnne Stubbe for helpful discussion in the early stages of this work.

REFERENCES

Aimi, J., Qiu, H., Williams, J., Zalkin, H., & Dixon, J. (1990) *Nucleic Acids Res. 18*, 6665–6672.
Anderson, K. A., Miles, E. W., & Johnson, K. A. (1991) *J. Biol. Chem. 266*, 8020–8033.
Casey, P. J., & Lowenstein, J. M. (1987) *Biochem. Pharmacol. 36*, 705–709.
Chen, Z., Dixon, J. E., & Zalkin, H. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3097–3101.
Chipperfield, J. R., Humble, R. W., Iveson, G. K. K., Mackenzie, G., & Shaw, G. (1988) *Nucleosides Nucleotides 7*, 571–576.
Cooper, T. G., Tchen, T. T., Wood, H. G., & Benedict, C. R. (1968) *J. Biol. Chem. 243*, 3857–3863.
Davisson, V. J., Sirawaraporn, W., & Santi, D. V. (1989) *J. Biol. Chem 264*, 9145–9148.
Dean, J. A. (1985) *Lange's Handbook of Chemistry*, McGraw-Hill.
Dunn, M. F., Aguilar, V., Brzović, P., Drewe, W. F., Jr., Houben, K. F., Leja, C. A., & Roy, M. (1990) *Biochemistry 29*, 8598–8607.
Firestine, S. M., & Davisson, V. J. (1993) *J. Med. Chem. 36*, 3484–3486.
Firestine, S. M., Poon, S.-W., Mueller, E. J., Stubbe, J., & Davisson, V. J. (1994) *Biochemistry* (following paper in this issue).
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Litchfield, G. J., & Shaw, G. (1971) *J. Chem. Soc. (B)*, 1474–1484.
Lukens, L. N., & Flaks, J. G. (1963) *Methods Enzymol. 6*, 671–702.
Meyer, E., Leonard, N. J., Bhah, B., Stubbe, J., & Smith, J. M. (1992) *Biochemistry 31*, 5022–5032.
Minet, M., & Lacroute, F. (1990) *Curr. Genet. 18*, 287–291.
Mueller, E. J., Meyer, E., Rudolph, J., Davisson, V. J., & Stubbe, J. (1994) *Biochemistry 33*, 2269–2278.
Patey, C. A. H., & Shaw, G. (1973) *Biochem. J. 135*, 543–545.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Vols. 1 and 2, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Tiedeman, A. A., Keyhani, J., Kamholz, J., Daum, III, H. A., Gotts, J. S., & Smith, J. M. (1989) *J. Bacteriol. 171*, 205–212.
Watanabe, W., Sampei, G.-I., Aiba, A., & Mizobuchi, K. (1989) *J. Bacteriol. 171*, 198–204.
Yoshikawa, M., Kato, T., & Takanishi, T. (1969) *Bull. Chem. Soc. Jpn. 42*, 3505–3508.