Evidence for the Direct Transfer of the Carboxylate of N^5-Carboxyaminoimidazole Ribonucleotide (N^5-CAIR) To Generate 4-Carboxy-5-aminoimidazole Ribonucleotide Catalyzed by *Escherichia coli* PurE, an N^5-CAIR Mutase†

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ABSTRACT: Formation of 4-carboxy-5-aminoimidazole ribonucleotide (CAIR) in the purine pathway in most prokaryotes requires ATP, HCO_3^-, aminoimidazole ribonucleotide (AIR), and the gene products PurK and PurE. PurK catalyzes the conversion of AIR to N^5-carboxyaminoimidazole ribonucleotide (N^5-CAIR) in a reaction that requires both ATP and HCO_3^-. PurE catalyzes the unusual rearrangement of N^5-CAIR to CAIR. To investigate the mechanism of this rearrangement, [4,7-^{13}C]-N^5-CAIR and [7-^{14}C]-N^5-CAIR were synthesized and separately incubated with PurE in the presence of ATP, aspartate, and 4-(*N*-succinocarboxamide)-5-aminoimidazole ribonucleotide (SAICAR) synthetase (PurC). The SAICAR produced was isolated and analyzed by NMR spectroscopy or scintillation counting, respectively. The PurC trapping of CAIR as SAICAR was required because of the reversibility of the PurE reaction. Results from both experiments reveal that the carboxylate group of the carbamate of N^5-CAIR is transferred directly to generate CAIR without equilibration with CO_2/HCO_3^- in solution. The mechanistic implications of these results relative to the PurE-only (CO_2- and AIR-requiring) AIR carboxylases are discussed.

A new intermediate and two new enzymatic activities have recently been discovered in the purine biosynthetic pathway in prokaryotes (Figure 1) (*1*, *2*). The reaction catalyzed by PurK[1] is the ATP- and HCO_3^--dependent carboxylation of aminoimidazole ribonucleotide (AIR) to N^5-carboxyaminoimidazole ribonucleotide (N^5-CAIR). PurE then catalyzes the unusual rearrangement of N^5-CAIR to 4-carboxy-5-aminoimidazole ribonucleotide (CAIR). The mechanism of conversion of AIR to CAIR appears to be distinct from that observed in some eukaryotic AIR carboxylases, which have no PurK domain and use CO_2 in place of HCO_3^- and ATP (*3*, *4*). PurE could thus represent a unique target for design of antibacterial agents (*5*).

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[1] Abbreviations: CAIR, 4-carboxy-5-aminoimidazole ribonucleotide; AIR, 5-aminoimidazole ribonucleotide; N^5-CAIR, N^5-carboxyaminoimidazole ribonucleotide; PurE, N^5-CAIR mutase or vertebrate AIR carboxylase (EC 4.1.1.21); PurK, N^5-CAIR synthetase; SAICAR, 4-(*N*-succinocarboxamide)-5-aminoimidazole ribonucleotide; PurC, SAICAR synthetase (EC 6.3.2.6); R5P, ribose 5-phosphate; PRPP, 5-phosphoribosyl α-1-pyrophosphate; PRA, 5-phosphoribosylamine (this and all subsequent purine metabolites are β anomers); GAR, glycinamide ribonucleotide; FGAR, formylglycinamide ribonucleotide; FGAM, formylglycinamidine ribonucleotide; LDH, lactate dehydrogenase (EC 1.1.1.27); PK, pyruvate kinase (EC 2.7.1.40); PEP, phosphoenolpyruvate; PEP-C, PEP carboxylase (EC 4.1.1.31); MDH, malate dehydrogenase (EC 1.1.1.37); PRPP-AT, PRPP amidotransferase (PurF, EC 2.4.2.14); GAR syn, GAR synthetase (PurD, EC 6.3.4.13); GAR-TF, GAR transformylase, formate-utilizing form (PurT); FGAR-AT, formylglycinamidine ribonucleotide synthetase (PurL, EC 6.3.5.3); AIR syn, AIR synthetase (PurM, EC 6.3.3.1); TEAB, triethylammonium bicarbonate; TSP, trimethylsilylproprionate; T_1, longitudinal relaxation time; PurKE, fusion of PurK and PurE, found in yeasts and plants.

Two experimental approaches have been used to examine the mechanism of the reversible decarboxylation−recarboxylation catalyzed by PurE to determine if the "CO_2" in the product is derived directly from substrate or from solution. The first one utilizes NMR spectroscopic analysis of the products of the reaction of [4,7-^{13}C]-N^5-CAIR with PurE to determine the fate of the label in the CAIR. The second involves use of [7-^{14}C]-N^5-CAIR and determination of the specific activity of CAIR. To facilitate product analysis and prevent the reverse reaction catalyzed by PurE, CAIR must be converted to 4-(*N*-succinocarboxamide)-5-aminoimidazole ribonucleotide (SAICAR) using SAICAR synthetase (PurC). Results of both experiments show that the carbamate carboxylate from N^5-CAIR is directly transferred to generate CAIR, without equilibration with HCO_3^- and CO_2 in solution. The mechanistic implications of these results will be presented.

EXPERIMENTAL PROCEDURES

Materials. Lactate dehydrogenase (LDH, 860 units/mg), pyruvate kinase (PK, 470 units/mg), phosphoenolpyruvate (PEP), PEP carboxylase (PEP-C, 3.6 units/mg), malate dehydrogenase (MDH, 3000 units/mg), and ATP were obtained from Sigma. [2-^{13}C]Gly (99% ^{13}C, lot 83-12204) and [^{13}C]$NaHCO_3$ (99.0% ^{13}C, lot 83-70006) were purchased from Isotec. Dowex 1-X8 was purchased from Bio-Rad. DEAE Sephadex A-25 was obtained from Pharmacia. [^{14}C]$NaHCO_3$ (6.8 mCi/mmol) was purchased from New England Nuclear. S¢intA scintillation fluid was purchased from Packard. 5-Phosphoribosyl-1-pyrophosphate amidotransferase (PRPP-AT, 6.8 units/mg) (*6*), glycinamide ribonucleotide synthetase (GAR syn, 30 units/mg) (*7*), glycin-

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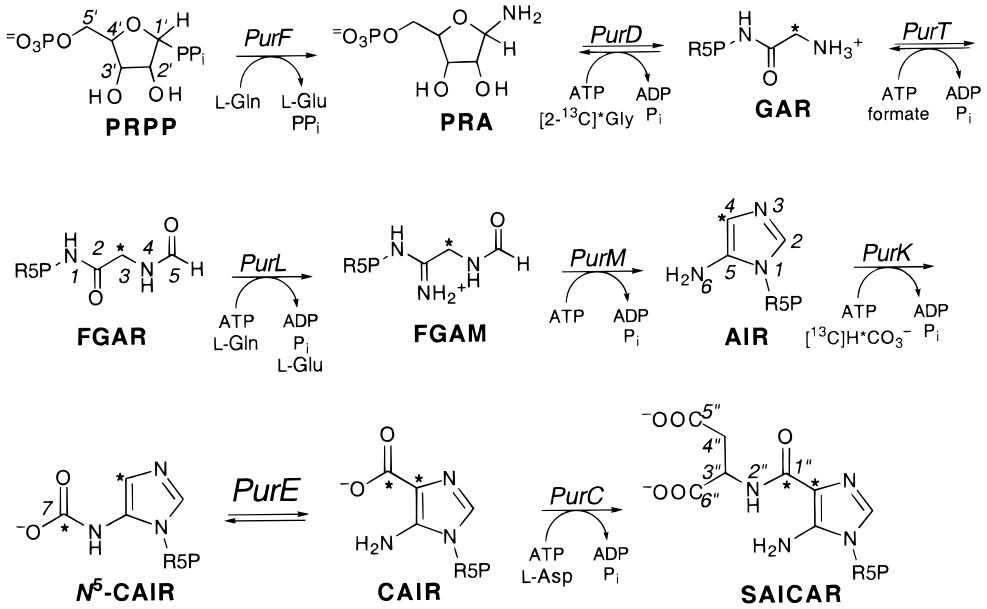

FIGURE 1: Portion of the de novo purine biosynthetic pathway, showing incorporation of label (*) from [2-^{13}C]Gly and [^{13}C]HCO_3^-. R5P is ribose 5-phosphate.

amide ribonucleotide transformylase (GAR-TF or PurT, 14.8 units/mg) (*8*), formylglycinamide ribonucleotide amidotransferase (FGAR-AT, 1.0 unit/mg) (*9*), and AIR synthetase (AIR syn, 1.9 units/mg) (*10*) were isolated as described previously and assayed at 20 °C. The synthesis of AIR, N^5-CAIR, and CAIR, the purification of enzymes, and the assays of PurE, PurK, and PurC were carried out as previously described (*1*, *2*). In all experiments described below, KOH solutions were prepared immediately before use by dissolving solid KOH in distilled water. For all enzymes, a unit is defined as 1 μmol of product formed per minute.

Methods. The orcinol assay for quantitation of reducing pentoses was performed by the procedure described by Dische (*11*). The concentrations of formylglycinamide ribonucleotide (β-FGAR), AIR, and N^5-CAIR were determined using the Bratton−Marshall assay (*12*). The concentration of bicarbonate was determined enzymatically, utilizing PEP-C and MDH (*13*).

Enzymatic Synthesis of [3-^{13}C]FGAR. [3-^{13}C]FGAR was synthesized from PRPP using the first three enzymes in the purine pathway (Figure 1): PRPP-AT (PurF), GAR syn (PurD), and formate-dependent GAR-TF (PurT) (*8*, *14*). In a final volume of 50 mL at 20 °C, the reaction mixture contained 100 mM Tris (pH 7.8), 10 mM $MgCl_2$, 135 mM KCl, 4.86 mM PRPP (243 μmol), 35 mM Gln, 12.1 mM [2-^{13}C]Gly, 2.22 mM ATP, 8.0 mM formate, 20 mM PEP, 37 units of PRPP-AT, 52 units of GAR syn, 700 units of GAR-TF, and 250 units of PK. Prior to enzyme addition, the reaction mixture was adjusted to pH 7.8 and incubated at 20 °C for 5 min. PRPP-AT was added last to initiate the reaction. After 1 h, the reaction mixture was diluted to 500 mL (pH 7.4) with deionized water. The sample was loaded onto a DEAE Sephadex A-25 column (2.5 cm × 10 cm, HCO_3^- form), and the column was developed at 4 °C with a linear gradient from 0 to 500 mM triethylammonium bicarbonate (TEAB, 500 × 500 mL, pH 7.4). Fractions (12 mL) were collected, and 100 μL of every third fraction was analyzed using the orcinol assay. The fractions containing FGAR (fractions 34−44, at 200 mM TEAB) were pooled. To facilitate removal of TEAB, the sample was repeatedly dissolved in water and methanol, and concentrated in vacuo. To prevent anomerization during the concentration process, it was essential that the pH remain slightly basic. This was accomplished through dropwise addition of freshly prepared 1 N KOH. A yield of 50% was determined using the Bratton−Marshall assay: ^{1}H NMR (300 MHz, D_2O, TSP at 0.0 ppm, pH 7.0) δ 8.13 (d, 1, J = 5.7 Hz, formyl), 5.43 (d, 1, J = 5.4 Hz, H1′), 4.20 (m, 1, H2′), 4.12 (m, 2, H3′), 4.06 (m, 1, H4′), 3.97 (m, 2, J = 137.1 Hz, $^{13}CH_2$), 3.89 (m, 2, H5′); ^{13}C NMR (75 MHz, D_2O, MeOH at 50 ppm) δ 56.1 (TEAB), 53.4 (s, C3), 17.3 (TEAB).

Enzymatic Synthesis of [4-^{13}C]AIR. In a final volume of 50 mL at 20 °C, the reaction mixture contained 100 mM Tris (pH 7.9), 10 mM $MgCl_2$, 200 mM KCl, 30 mM Gln, 3.16 mM [3-^{13}C]FGAR (159 μmol), 1.8 mM ATP, 10.5 mM PEP, 16 units of FGAR-AT, 15 units of AIR syn, and 200 units of PK. The reaction was allowed to proceed for 20 min. The mixture was then diluted to 500 mL with deionized water at 4 °C and loaded onto a DEAE Sephadex A-25 column (2.5 cm × 10 cm, HCO_3^- form). The column was developed with a linear gradient from 0 to 500 mM TEAB (pH 7.6, 500 × 500 mL). An elution profile (11 mL fractions) was generated by monitoring A_{250} and by the Bratton−Marshall assay. The appropriate fractions containing AIR (fractions 43−50, at 250 mM TEAB) were pooled. The TEAB was removed as described above, maintaining basic pH by addition of 1 N KOH. In the later stages of TEAB removal, the sample was not concentrated completely to dryness and was enzymatically monitored for a decrease in the level of HCO_3^-. The upper limit for the [^{12}C]HCO_3^- concentration ranged from 12 to 73 μmol. An average yield (from eight preparations) for this reaction was 30%: ^{1}H NMR of [4-^{13}C]AIR (D_2O, pH 8.0, TSP at 0.0 ppm) δ 7.9 (d, J = 7.6 Hz, H2), 6.5 (d, J = 195 Hz, H4), 5.75 (d, J = 4.9, H1′), 4.65 (t, H2′), 4.53 (t, H3′), 4.42 (m, H4′), 3.97 (m, H5′); ^{13}C NMR (75 MHz, D_2O, MeOH at 50 ppm) δ 113.6 (s, C4).

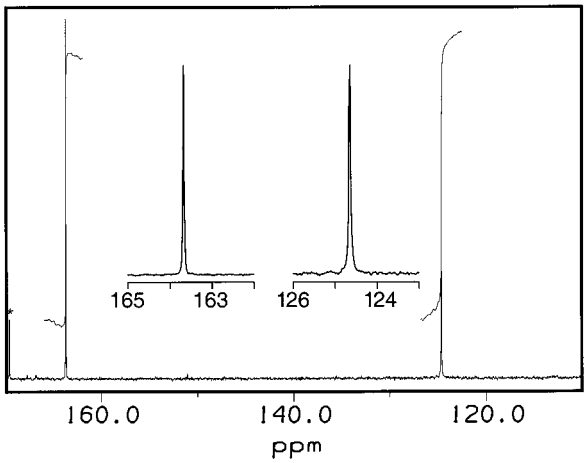

FIGURE 2: Downfield region of the proton-decoupled ^{13}C NMR (75 MHz) spectrum of [4,7-^{13}C]-N^5-CAIR used as a substrate for PurE. This spectrum was collected with an acquisition delay of 60 s ($5 \times T_1$). The (*) peak is bicarbonate. In the inset is shown an expanded view of the C7 (left) and C4 (right) resonances.

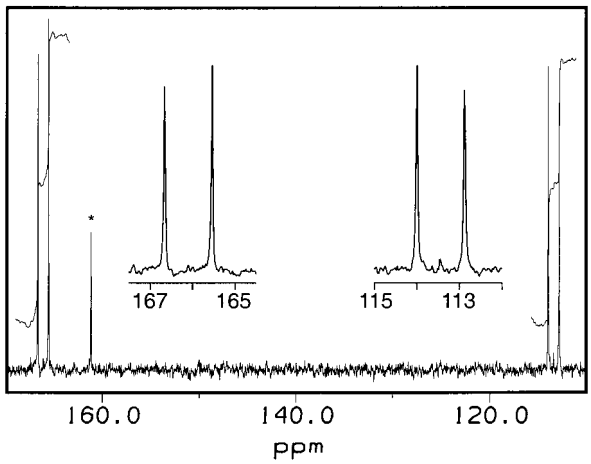

FIGURE 3: Downfield region of the proton-decoupled ^{13}C NMR (75 MHz) spectrum of [4,1″-^{13}C]SAICAR recovered following reaction of [4,7-^{13}C]-N^5-CAIR with PurE and PurC. This spectrum was collected with an acquisition delay of 23 s ($>5 \times T_1$). The (*) peak is bicarbonate. In the inset is shown an expanded view of the C1″ (left) and C4 (right) resonances, each exhibiting a J_{C-C} of 84.2 Hz.

Enzymatic Synthesis and Isolation of [4,7-^{13}C]-N^5-CAIR. In a final volume of 10 mL, a 15 mL Falcon tube contained 200 mM Tris (pH 8.0), 12 mM $MgCl_2$, 60 mM KCl, 4.5 mM ATP, 10 mM PEP, 50 μmol of [4-^{13}C]AIR, 156 units of PurK, and 200 units of PK. The solution was equilibrated for 5 min at 20 °C. Solid [^{13}C]$NaHCO_3$ was added to the reaction mixture to produce a final concentration of 120 mM. An excess of labeled bicarbonate was added to dilute the unlabeled bicarbonate (upper limit of 10% ^{12}C) originating from the AIR isolation. The reaction was initiated by the addition of PurK. After 50 s at 20 °C, the reaction was quenched with 1 mL of 10 M KOH (20 °C).

The reaction mixture was then diluted with 300 mL of 10 mM KOH and loaded onto a Dowex 1-X8 column (7 mL, $^-$OH form). The column was developed with a linear gradient from 0 to 300 mM KCl (250 × 250 mL in 50 mM TEA, 4.5 mL fractions). The TEA solution (pH 11.4) was prepared and used immediately without adjusting the pH. An elution profile was generated by monitoring A_{240} and A_{250} and for a positive response in the Bratton−Marshall assay. When applicable, 0.5 mL from every fifth fraction was assayed for radioactivity by scintillation counting. In a typical reaction, AIR eluted in fractions 47−53, while [4,7-^{13}C]-N^5-CAIR eluted in fractions 60−66. To maintain alkaline conditions, 300 μL of 2 N KOH was added to the pooled fractions, and the sample was concentrated in vacuo (50% yield). The presence of high concentrations of salt prevented rechromatographing either N^5-CAIR or AIR on an anion exchange column. The N^5-CAIR was typically stored as a solution (pH >12) at −80 °C. The purity of the [4,7-^{13}C]-N^5-CAIR sample was determined by NMR spectroscopy: ^1H NMR (300 MHz, TSP, D_2O, pD 14) δ 7.93 (d, 1, J = 8.5 Hz, C2H), 6.84 (d, 1, J = 191.6 Hz, C4H), 5.49 (d, 1, J = 4.92 Hz, H1′), 4.4 (m, 1, H2′), 4.3 (m, 1, H3′), 4.21 (m, 1, H4′), 3.91 (m, 2, H5′); ^{13}C NMR (75 MHz, MeOH at 50 ppm, D_2O, pD 14) δ 169.6 (CO_3^{2-}), 163.8 (s, C7), 124.6 (s, C4) (Figure 2).

Methanol Extraction of N^5-CAIR. The KCl and $KHCO_3$ in the N^5-CAIR sample can be removed by methanol extraction. The N^5-CAIR and salt solution was evaporated to dryness in a round-bottom flask and kept at 4 °C. The resulting solid was triturated with 4 mL of HPLC grade methanol at 4 °C. The salts were then allowed to settle, and the sample was centrifuged in 1.5 mL Eppendorf tubes in a microcentrifuge for 20 s. The supernatant was then transferred into a round-bottom flask. The methanol extraction of the salts was repeated four times. To ensure basic conditions, 25 μL of 1 N KOH was added to the MeOH/N^5-CAIR mixture. The combined methanol fractions were pooled, and the solvent was then removed in vacuo. A 10% loss in the amount of N^5-CAIR occurred during the desalting process based on the Bratton−Marshall assay. The concentrations of salts were greatly reduced (final concentrations of $KHCO_3^-$ were 50−100 mM).

Enzymatic Conversion of [4,7-^{13}C]-N^5-CAIR to [4,1″-^{13}C]-SAICAR. In a final volume of 5 mL at 20 °C, the reaction mixture contained 200 mM Tris (pH 8), 10 mM KCl, 10 mM $MgCl_2$, 14 mM Asp, 1.62 mM ATP, 4.0 mM PEP, 250 units of PK, 132 units of PurC, 11.3 units of PurE (0.07 μmol), and 0.92 mM [4,7-^{13}C]-N^5-CAIR. A sample of the reaction mixture without enzyme (45 μL) was mixed with N^5-CAIR (5 μL) to determine the final reaction pH with pH paper. The buffer was then adjusted accordingly so that the final reaction mixture had a pH of 8. The reaction was initiated by addition of N^5-CAIR and was quenched after 141 s with 1 mL of 13 N KOH. The mixture was diluted with 300 mL of 10 mM KOH at 4 °C and then loaded onto a Dowex 1-X8 column (8 mL, OH^- form). The column was developed with a linear gradient from 0 to 500 mM KCl (300 × 300 mL, 50 mM TEA, pH 11, 2.75 mL fractions). An elution profile was generated by monitoring A_{268} and A_{260}. Fractions (79−96) containing [4,1″-^{13}C]SAICAR and minimal ATP were pooled. The sample was concentrated in vacuo (yield of 60%): ^1H NMR (300 MHz, 0.8 mM, TSP, D_2O, pD 6) δ 7.56 (d, 1, $J_{C,H}$ = 10.5 Hz, C2H), 5.72 (d, 1, J = 6.5 Hz, H1′) (the other resonances were obscured by ATP and TEAB); ^{13}C NMR (75 MHz, MeOH at 50 ppm, D_2O, pD 14) δ 166.26 (d, $J_{C,H}$ = 84.19 Hz, C1″), 161.2 (CO_3^{2-}), 113.54 (d, J = 84.19 Hz, C4) (Figure 3).

^{13}C T_1 Relaxation Determinations. Using the inversion−recovery method and a Varian 300 MHz NMR spectrometer equipped with a broad-band probe operating at 75.429 MHz,

individual resonance intensities for N^5-CAIR and SAICAR were fit to eq 1 by an exponential least-squares analysis to determine their longitudinal relaxation values (T_1) (*15*). The equation is appropriate for a 90° pulse width, which was determined in a separate experiment.

$$M_z = M_o(1 - e^{-d_2/T_1}) \quad (1)$$

In this expression, M_z is the steady state z magnetization, M_o is the equilibrium magnetization, T_1 is the longest longitudinal relaxation value, and d_2 is the interval between pulses.

Enzymatic Synthesis of [7-^{14}C]-N^5-CAIR with [^{14}C]HCO_3^- by PurK. In a final volume of 5 mL at 20 °C, a 15 mL Falcon tube contained 200 mM Tris (pH 8.0), 6 mM $MgCl_2$, 20 mM KCl, 5 mM ATP, 10 mM PEP, 1.35 μmol of AIR, 92 units of PurK, and 125 units of PK. In the hood, a granule of [^{14}C]$NaHCO_3$ was transferred with a glass microcapillary into the reaction mixture. The reaction was initiated with PurK and allowed to proceed for 30 s at 20 °C. The reaction was quenched with 1 mL of 10 M KOH. The isolation procedure previously described for [4,7-^{13}C]-N^5-CAIR with anion exchange chromatography and methanol extraction was used to purify [7-^{14}C]-N^5-CAIR. To prevent the loss of the label during scintillation counting, [7-^{14}C]-N^5-CAIR was diluted into 1 mL of 140 mM KOH before addition of the scintillation fluid. The N^5-CAIR was quantitated using the Bratton−Marshall assay. Typical specific activities were 1.1−1.67 × 10^6 cpm/μmol. With a successful protocol for isolation of [4,7-^{13}C]-N^5-CAIR in hand, an NMR spectrum was not recorded for the ^{14}C compound.

Synthesis of [1″-^{14}C]SAICAR from [7-^{14}C]-N^5-CAIR. In a final volume of 5 mL at 20 °C, the reagents were added in the following order: 200 mM Tris-HCl (final pH of 8, from an initial pH of 7.3), 20 mM KCl, 5.2 mM $MgCl_2$, 1 mM ATP, 3 mM PEP, 7 mM Asp, 11.6 units of PurE, 123 units of PurC, 250 units of PK, and 0.27 mM [^{14}C]-N^5-CAIR (1.17 × 10^6 cpm/μmol, impurities being 5.0 mM HCO_3^- and 260 mM KCl). As described above, the effect of N^5-CAIR on the final pH was determined prior to the actual experiment. The reaction was quenched after 35 s with 1 mL of 13.5 M KOH. The mixture was diluted to 300 mL with 10 mM KOH at 4 °C and loaded onto a Dowex 1-X8 column (7 mL, OH^- form). The compounds were eluted with a linear gradient from 0 to 500 mM KCl (320 × 320 mL, 50 mM TEA, pH 11.4). An elution profile was generated by monitoring A_{268} and A_{260} and by scintillation counting of every fifth fraction (0.5 mL). On the basis of relative specific activities, the [^{14}C]SAICAR was pooled and then rechromatographed on a DEAE Sephadex A-25 column (6 mL, HCO_3^- form) in an effort to remove ATP. SAICAR was eluted with a linear gradient from 0 to 750 mM TEAB (pH 7.5, 200 × 200 mL), pooled, and then concentrated in vacuo. The specific activity was determined from the UV spectrum of SAICAR in 100 mM Tris-HCl (pH 8.0) and by scintillation counting.

Preparation of the Escherichia coli PurE R46K Mutant. Using standard molecular biology techniques (*16*), the PurE/PurK coding region was excised from the pJS355 plasmid (*1*) and ligated into the *Eco*RI−*Hin*dIII sites of pUC118. Single-stranded DNA was produced using R408 helper phage (*40*) and an *E. coli* CJ236 (*dut ung*) host (*16*). Using the Kunkel mutagenesis procedure (*17*), the *Eco*RI site was replaced with a new, unique *Nde*I site at the initiator Met codon. The *Nde*I−*Hin*dIII fragment from this construct was placed into the T7 overexpression vector pET23a to generate the plasmid pNC2, which was used as a wild-type template. Mutagenesis was performed using the oligonucleotide 5′-CGAAGCTGAAAAGCTTATCGGGGGT**TTT**GTGAGC-AGAAACCA, which in addition to the PurE R46K mutant (bold) encodes a silent mutation introducing a second *Hin*dIII site (underlined) to aid in mutant screening. The resulting construct pCKO2 was transformed into BL21(DE3) cells, which were grown at 37 °C in LB medium with 0.1 g/L ampicillin to an A_{600} of ≈2, at which point the cells were harvested by centrifugation and frozen in liquid nitrogen. Mutant PurE was purified to >95% homogeneity (17 mg of enzyme per gram of wet cell weight) as previously described (*1*). The conversion of N^5-CAIR → CAIR was measured at either 23 or 37 °C, using excess PurK, ATP, PEP, and PK to generate a saturating, steady-state level of N^5-CAIR (*18*). PurC, Asp, NADH, and LDH were also present in excess to convert the PurE product CAIR to SAICAR, with concomitant ADP formation and NADH oxidation, which was monitored at 340 nm (*1*).

Can R46K PurE Use CO_2 and AIR as a Substrate? A 55 mM solution of CO_2 was prepared in 50 mM potassium acetate at pH 5.4 and 4 °C. This solution was added to a solution of 100 mM Tris and 0.15 mM AIR to give a final CO_2 concentration of 22 mM at pH 8.1. The reaction was started by addition of 2.6 × 10^{-3} unit of PurE or R46K PurE and the formation of CAIR monitored at 282 nm (*4*).

RESULTS

Synthesis of Labeled [4-^{13}C]AIR (Figure 1). Our previous studies have shown that PurE catalyzes the chemically unprecedented conversion of N^5-CAIR to CAIR. As an initial step in the investigation of the mechanism of this reaction, we determined the source of the carboxylate of CAIR. Two approaches to this problem, both involving specific isotopic labeling, were undertaken. One involved ^{13}C labeling of N^5-CAIR and NMR spectroscopy, and the second involved ^{14}C labeling and scintillation counting. Both approaches were hindered by the chemical instability of N^5-CAIR at pH <12, the ubiquitous presence of bicarbonate, and the difficulties associated with the quantitation of amounts of each nucleotide.

The availability of all of the enzymes in the purine pathway allowed the biosynthetic preparation of isotopically labeled AIR. The first three enzymes in the purine pathway (PRPP-AT, GAR syn, and GAR-TF) were used to incorporate [2-^{13}C]Gly into [3-^{13}C]FGAR in 50% yield (Figure 1). The product was characterized by ^{1}H and ^{13}C NMR spectroscopy. [3-^{13}C]FGAR was then converted to [4-^{13}C]AIR using the next two enzymes in this pathway (FGAR-AT and AIR syn, Figure 1). The resulting [4-^{13}C]AIR was purified using anion exchange chromatography, giving a 30% yield of the desired product. The ^{1}H and ^{13}C NMR spectra were used to establish the position of the ^{13}C label at C4 of AIR as well as its purity. The incubation time in the second series of enzymatic transformations was crucial, as longer reaction times resulted in formation of a compound which elutes after AIR during its purification. The compound had an UV spectrum and an absorption spectrum after reaction with the Bratton−Marshall reagent similar to those of CAIR (λ_{max} =

520 nm, while AIR is at 502 nm). The identity of this species has not been determined.

Enzymatic Synthesis of ^{13}C- or ^{14}C-Labeled N^5-CAIR. Utilizing [^{13}C]- or [^{14}C]bicarbonate and PurK, isotopic label was incorporated into the carbamate of N^5-CAIR. The amount of PurK (156 units) and the 50 s incubation time were chosen to maximize formation of N^5-CAIR and minimize its decomposition to AIR ($k = 0.23$ min^{-1}, $t_{1/2} =$ 3 min, pH 8, 20 °C). Our previous studies showed that alkaline conditions (pH 12) *must* be maintained during purification and storage of N^5-CAIR to reduce the extent of its decarboxylation ($k = 0.00144$ min^{-1}, $t_{1/2} = 8$ h, pH 12, 20 °C; E. Meyer, unpublished observations). For its isolation, the reaction mixture was loaded onto a Dowex 1-X8 exchange column and then eluted with a KCl gradient in 50 mM TEA. Under these conditions, the bicarbonate utilized during its synthesis elutes prior to N^5-CAIR. Even with many precautions, however, some contaminating bicarbonate is always present in the product at the end of the purification due to rapid dissolution of CO_2 in basic solutions. After the fractions containing N^5-CAIR were identified, they were pooled and concentrated in vacuo and extracted with methanol to reduce the levels of the contaminating salts KCl and $KHCO_3$. Overall yields for N^5-CAIR were typically 30%. The low yields in part result from its decomposition to AIR. The purity of the sample was established with ^{1}H NMR spectroscopy (Figure 2), since the aromatic and C1 protons of AIR and CAIR have chemical shifts different from those of N^5-CAIR (*2*, *19*).

[7-^{14}C]-N^5-CAIR was synthesized by a similar procedure. Its specific activity ($1-1.6 \times 10^6$ cpm/μmol) was determined with the Bratton−Marshall assay to determine the nucleotide concentration in conjunction with scintillation counting. Quantitation with the Bratton−Marshall assay is problematic since the first step in this procedure decarboxylates N^5-CAIR to AIR before diazotization (*20*). Thus, contaminating AIR causes the concentration of N^5-CAIR to be overestimated, leading to an underestimate of its specific activity. Careful attention to pH during the isolation of N^5-CAIR can reduce the level of contaminating AIR to less than 5%, as judged by NMR methods.

Integration Parameters for ^{13}C Spectra of [4,7-^{13}C]-N^5-CAIR and [4,1″-^{13}C]SAICAR. Meaningful integration of ^{13}C spectra, necessary to establish the extent of transfer of the C7 carboxylate of [4,7-^{13}C]-N^5-CAIR to [4,6-^{13}C]CAIR, requires determination of the longitudinal relaxation time (T_1) of each of these carbons. Accurate integration requires an acquisition delay that is 3−5-fold greater than the longest T_1 (*21*). Therefore, using the inversion−recovery method, the T_1 values of C7 and C4 of N^5-CAIR and C1″ and C4 of SAICAR were determined and found to be 12.6, 0.5, 3.2, and 4.6 s, respectively. For integration of N^5-CAIR spectra, an acquisition delay of 60 s was used, while for SAICAR spectra, a delay of 23 s was used.

Enzyme-Catalyzed Conversion of [4,7-^{13}C]-N^5-CAIR to [4,1″-^{13}C]SAICAR. To address the question of whether the "CO_2" from the carbamate of N^5-CAIR is transferred directly to C4 to produce [4,6-^{13}C]CAIR, without exchanging with the CO_2 in solution, [4,7- ^{13}C]-N^5-CAIR was incubated with PurE. The reaction was carried out in the presence of PurC, ATP, and Asp to trap the CAIR as SAICAR (Figure 1). This is an essential procedure since the PurE-catalyzed reaction is reversible. With 4.6 μmol of [4,7-^{13}C]-N^5-CAIR and 11.3 units of PurE, the conversion was complete after 24 s, limiting the amount of nonenzymatic decarboxylation of N^5-CAIR to 13%.

Table 1: ^{13}C NMR Integration of Starting Material ([4,7-^{13}C]-N^5-CAIR) and Product ([4,1″-^{13}C]SAICAR)[a]

[4,7-^{13}C]-N^5-CAIR	C4	C7	[4,1″-^{13}C]SAICAR	C4	C1″
expt 1 (Figure 2)	100	98	expt 1 (Figure 3)	99	100
expt 2	100	97.2	expt 2	100	98.6

[a] Values given are relative integral areas in each of the four experiments.

The SAICAR generated was purified by anion exchange chromatography and analyzed by ^{13}C NMR methods. The integration of this material relative to starting material allowed quantitation of the amount of ^{13}C transferred. If ^{12}C from CO_2 in solution was uniquely incorporated into SAICAR during this conversion, then the ^{13}C resonance of the C4 of SAICAR would appear as a singlet at 113.5 ppm. On the other hand, if ^{13}C from the carbamate is transferred to C4, both carbons would appear as doublets with a J_{C-C} of 84.2 Hz. By integration of the relative intensities of the observed resonances, the amount of direct transfer can be quantitated. From two separate reactions, the integration analyses revealed 97% retention of the label during this enzymatic process (Table 1 and Figure 3). This result establishes that the 4-carboxylate group of CAIR originates from N^5-CAIR. Within the limits of detection, no exchange with [^{12}C]HCO_3^-/CO_2 from solution was detected during the PurE-catalyzed reaction.

Quantitation of the [1″-^{14}C]SAICAR. Our initial efforts to address the question of "CO_2" transfer catalyzed by PurE focused on ^{14}C-labeled N^5-CAIR. Problems with the determination of accurate specific activities of the starting material and product, however, suggested that the ^{13}C method described above would be more quantitative. The radiolabel method, as briefly described, gives results in qualitative agreement with the NMR experiments. The conversion of N^5-CAIR to SAICAR was carried out by incubating 1.35 μmol of [7-^{14}C]-N^5-CAIR with PurE (11.6 units) for 7 s, minimizing its nonenzymatic decomposition to AIR (5%). The [^{14}C]SAICAR was isolated by anion exchange chromatography, although separation from ATP present in the reaction mixture was incomplete. The results of two separate experiments revealed that approximately 50% of the label appeared to be lost during the PurE-catalyzed reaction, and as a result, the specific activity of SAICAR was half of that of N^5-CAIR. Errors in this experiment are associated with an inability to obtain an accurate determination of the specific activity of the starting material due to its decomposition and the presence of salts which preclude its purification to homogeneity. The specific activity of SAICAR was also difficult to establish due to its comigration with ATP from the reaction mixture in several chromatographic systems and the difficulty of determining the extinction coefficient of SAICAR (*1*). Given these problems, and that the quantities of CO_2/HCO_3^- in solution during these experiments would be expected to dilute by a factor of ~100 any [^{14}C]CO_2 that escaped from the active site during the reaction, these results suggest quantitative transfer of the carboxylate by PurE.

Mutagenesis of a Partially Conserved Lysine. As noted in the introductory section, vertebrate organisms appear to

utilize CO_2, AIR, and PurE to make CAIR, while plants and microbes appear to use HCO_3^-, ATP, AIR, PurK, and PurE to make the same product. We considered the possibility that a conserved lysine might be a site of transient carbamate formation in the CO_2-dependent PurE reaction and may be a key determinant of CO_2 specificity. An alignment of the protein sequences of four vertebrate AIR carboxylases and the PurE from *Archaeoglobus fulgidus* [an archaeon that grows in an abyssal vent environment (*22*) where concentrations of CO_2 are very high] reveals a single conserved lysine. A comparison with the 19 sequences from all PurKE requiring AIR carboxylases reveals that they have no conserved lysines in the PurE domain and an arginine (R46) is found in place of the conserved lysine of the vertebrate PurEs. To test the hypothesis that K46 might function as a CO_2 carrier and remove the PurK requirement for CAIR formation altogether, the *E. coli* PurE R46 was changed to a lysine by site-directed mutagenesis. The mutant PurE still required N^5-CAIR as a substrate and had a turnover number for the production of CAIR of 0.6 s^{-1} (per monomer; 2 units/mg), or ~5% of that of the wild-type enzyme. No CAIR was produced when AIR was incubated with CO_2 and R46K-PurE at 10 °C. This result suggests that in neither class of PurE is covalent catalysis by lysine likely involved, although the complementary experiment in which the vertebrate PurE has its lysine mutated to an arginine is required.

DISCUSSION

Our results suggest that the carbon of the N^5-CAIR carbamate is transferred directly to the C4 of the same molecule of N^5-CAIR from which it was generated and does not exchange with CO_2 in the media during the PurE-catalyzed reaction. The use of N^5-CAIR to deliver a one-carbon equivalent is unusual, but remarkably similar to the well-characterized, though chemically enigmatic, carboxybiotin cofactor that is also involved in one-carbon transfers (*23*). Both PurK and biotin carboxylase systems use HCO_3^- and ATP, and presumably make carboxyphosphate to generate a carbamate from non-nucleophilic nitrogens. Both enzymes are structurally homologous (*24*; J. B. Thoden, T. J. Kappock, J. Stubbe, and H. M. Holden, unpublished observations) members of the ATP grasp superfamily (*25*). They are further structurally homologous to two domains of carbamoyl phosphate synthetase, one of which is involved in the generation of carboxyphosphate, which in the presence of ammonia generates carbamate (*26*). The differences in the chemical reactivities of the amide of biotin, the amino group of AIR, and NH_3 toward carboxyphosphate are reflected in the relative stabilities of their carboxylated products [half-lives of 30 min (*23*), 30 s (*19*), and 20 ms (*27*), respectively]. The similarities in the three available enzyme structures should soon allow elucidation of the basis for their differential reactivities with the putative carboxyphosphate.

The mechanism by which a "CO_2" equivalent is transferred has been the subject of a longstanding debate in the biotin case (*28*−*33*). A concerted mechanism in which decarboxylation of biotin and deprotonation of substrate accompany the formation of the new carbon−carbon bond has been proposed and eliminated (*28*−*31*). Stepwise enol (enolate) formation, followed by nucleophilic attack on the carbamate of biotin, or enolate attack on CO_2 generated from decarboxylation of carboxybiotin, has been proposed and modeled (*23*). What is most curious is that this chemically enigmatic mechanism has now reappeared in the purine pathway.

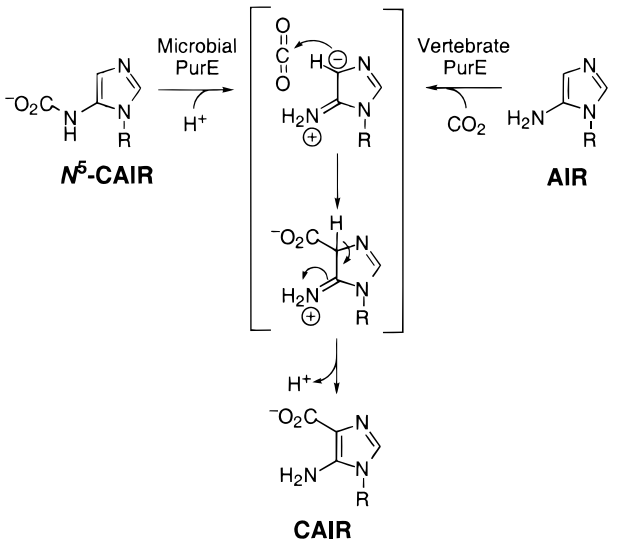

FIGURE 4: Common intermediate proposed for the PurE reaction in vertebrates and microbes, which have divergent substrate requirements.

The same mechanistic options can be considered in the PurE case as well. The concerted mechanism in the PurE case is unrealistic, as the reaction is intramolecular and would involve a four-membered cyclic transition state, rather than the six-membered cyclic transition state in biotin. A recent mechanism for PurE, based on CO_2 addition to N^5-CAIR, is ruled out by the present experiments (*34*). The mechanism thus favored would be enzyme-mediated decarboxylation generating CO_2 and AIR, which then could react to generate product (Figure 4). This mechanism is essentially that proposed for the vertebrate PurE systems which appear to have no PurK counterpart. This mechanism is unappealing, as the cell has just expended an ATP molecule to carboxylate HCO_3^- instead of using CO_2 directly. However, if the concentrations of CO_2 are low in the PurKE-requiring organisms, this approach might provide a vehicle for harnessing "CO_2" equivalents and delivering them to the site where chemistry must occur. In contrast to HCO_3^-, it is not readily apparent how an enzyme would bind CO_2, which has no charge and no net dipole. It is thus interesting to note that one microbe (*A. fulgidus*) known to lack a PurK equivalent only grows in a CO_2-enriched environment.

An alternative mechanism involving direct nucleophilic attack on the carbon of the carbamate of N^5-CAIR requires that PurE bind both AIR and N^5-CAIR simultaneously. This mechanism would regenerate AIR and form CAIR. While not particularly appealing, this mechanism at present cannot be ruled out. It is very difficult to remove all of the AIR from N^5-CAIR due to its rapid decomposition.

One final mechanistic option that could be incorporated into any of the above schemes involves covalent catalysis using an active site lysine. Carbamates of lysine have been detected in ribulose bisphosphate carboxylase (*35*), phosphotriesterase (*36*), and urease (*37*), although none of these carbamates plays a direct role in catalysis as would be the case for PurE. As noted above, in the 23 sequences from organisms that use PurK/PurE for carboxylation of AIR, there

are no conserved lysines. Exchange of an arginine for a lysine residue, which is found at one position in every member of the CO_2-utilizing subfamily of PurE enzymes, did not alter the N^5-CAIR substrate specificity of *E. coli* PurE. While this experiment is technically difficult, due to the nonenzymatic reaction of AIR with CO_2 and the rapid hydration of CO_2 to bicarbonate, CO_2 does not appear to compete effectively with N^5-CAIR as a substrate for the mutant protein. Thus, the covalent catalysis option seems to be unreasonable.

The favored mechanism is thus decarboxylation of N^5-CAIR in the active site to form AIR and carbon dioxide creating a high, local concentration of CO_2 in the active site, which can then react with the C4 of AIR (Figure 4). As we have previously shown, the C4 of AIR is rapidly protonated as shown by the exchange of the C4 proton at pH 7.2 ($k_{obs} = 0.09$ min^{-1}) (*38*). Thus nucleophilic attack at C4 could occur on an electrophilic carbon dioxide. The feasibility of this mechanism is also apparent from model studies of AIR with CO_2 in solution (*2*, *39*). NMR analysis of this reaction, at high bicarbonate concentrations, shows that N^5-CAIR forms very rapidly (the kinetic product), while CAIR forms at a much slower rate (the thermodynamic product). The extent of the reaction is dependent on the concentration of bicarbonate or CO_2. The recent crystal structures of PurK (J. B. Thoden, T. J. Kappock, J. Stubbe, and H. M. Holden, unpublished observations) and PurE (I. I. Mathews, T. J. Kappock, J. Stubbe, and S. E. Ealick, unpublished observations) should allow formulation of a more detailed mechanistic model for this intriguing AIR carboxylase reaction.

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