N^5-Carboxyaminoimidazole Ribonucleotide: Evidence for a New Intermediate and Two New Enzymatic Activities in the *de Novo* Purine Biosynthetic Pathway of *Escherichia coli*†

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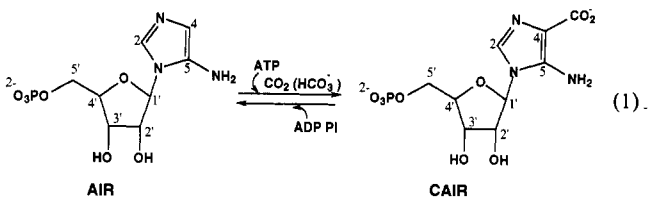
ABSTRACT: Conversion of aminoimidazole ribonucleotide (AIR) to 4-carboxyaminoimidazole ribonucleotide (CAIR) in *Escherichia coli* requires two proteins, PurE and PurK, previously thought to be subunits of a single enzyme, AIR carboxylase. Past studies revealing an ATP requirement for this reaction (Meyer et al., 1992), in conjunction with present studies, reveal that PurE and PurK possess independent catalytic activities. PurK is shown, by NMR spectroscopy, to catalyze the conversion of AIR in the presence of HCO_3^- and ATP to ADP, P_i, and the carbamate of AIR (designated N^5-CAIR). PurE has been shown by NMR spectroscopy and kinetic analysis, to catalyze the reversible conversion of N^5-CAIR and CAIR. N^5-CAIR has a half-life of 0.9 min at pH 7.8 and 30 °C. Thus, two new enzymatic activities and a new intermediate have been discovered in the *de novo* purine biosynthetic pathway of *E. coli*.

Aminoimidazole ribonucleotide (AIR)[1] carboxylase catalyzes the conversion of AIR in the presence of HCO_3^- to carboxyaminoimidazole ribonucleotide (CAIR) (eq 1). Ge-



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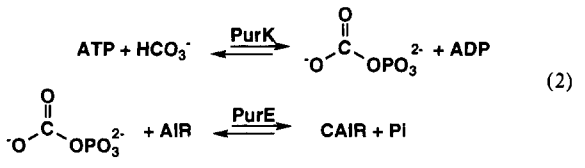
netic studies of Gots et al. (1976) suggested that this enzyme was composed of subunits: one resulting from the *purE* gene and the other from the *purK* gene. Mutants in either *purE* or *purK*, as might be expected, were found to be auxotrophic for purines. Intriguingly, the purine requirement in the *purK*⁻ strain was removed when the bacteria were grown in an atmosphere enriched with CO_2 (Gots et al., 1976). These observations fostered the long-held belief that PurK functions in the AIR carboxylase reaction as a CO_2 carrier. The genes were found by both Smith's (Tiedeman et al., 1989) and Mizobuchi's (Watanabe et al., 1989) laboratories to be components of the same operon. DNA sequencing revealed that PurE is a protein of subunit molecular mass 17 kDa and PurK is a protein of subunit molecular mass 39 kDa. Why a protein of 39 kDa would be required as a CO_2 carrier, when a small molecule such as biotin has been known to function in this capacity, piqued our curiosity.

This function has been partially clarified by our recent studies on PurK and PurE. The overexpressed *purE* and *purK* gene products were purified to homogeneity and their physical and catalytic properties were examined, revealing some surprises (Meyer et al., 1992). Homogeneous PurE is capable of catalyzing the carboxylation of AIR to CAIR at high HCO_3^- concentrations (0.18 M) in the absence of PurK. Furthermore, addition of PurK appeared to have no effect on the activity of PurE. Serendipitously, we made the observation that PurK did possess an activity, hydrolysis of ATP to ADP and P_i. This ATPase activity, however, only occurred in the presence of AIR. Experiments designed to look for the production of CAIR concomitant with this AIR-dependent ATP hydrolysis were all negative. We were subsequently able to show that PurE and PurK are required to catalyze the conversion of AIR to CAIR in the presence of "low" concentrations of HCO_3^- (100 μM) only in the presnce of ATP. Furthermore, every molecule of AIR consumed is accompanied by production of an equivalent amount of ADP and P_i. These studies allowed formulation of the hypothesis described in eq 2. This model



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stipulates that the function of PurK is to generate carboxyphosphate, as postulated in the case of biotin-dependent enzymes (Knowles, 1989). This intermediate was proposed to function, either directly or indirectly, to deliver CO_2 to

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[1] Abbreviations: ADP, adenosine diphosphate; AIR, aminoimidazole ribonucleotide; ATP, adenosine triphosphate; BSA, bovine serum albumin; CAIR, 4-carboxyaminoimidazole ribonucleotide; DEAE, diethylaminoethyl; DNA, deoxyribonucleic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FGAM, formylglycinamidine ribonucleotide; FGAR, formylglycinamide ribonucleotide, FGAR-AT, formylglycinamide ribonucleotide amidotranferase; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid); LB medium, Luria–Bertani medium; LDH, lactate dehydrogenase; M9, minimal salts medium; M9CA, minimal salts medium with added casamino acids; M9CAHX, minimal salts medium with added casamino acids and hypoxanthine; MDH, malate dehydrogenase, NADH, β-nicotinamide adenine dinucleotide phosphate, reduced; N^5-CAIR, *N*-carboxyaminoimidazole ribonucleotide; NMR, nuclear magnetic resonance; PEP, phosphoenolpyruvate; PEP-C, phospohenolpyruvate carboxylase; P_i, inorganic phosphate; PK, pyruvate kinase; SAICAR, 5-aminoimidazole-4-(N-succinylcarboxamide)ribonucleotide; TEAB, triethylammonium bicarbonate; Tris, tris(hydroxymethyl)aminomethane.

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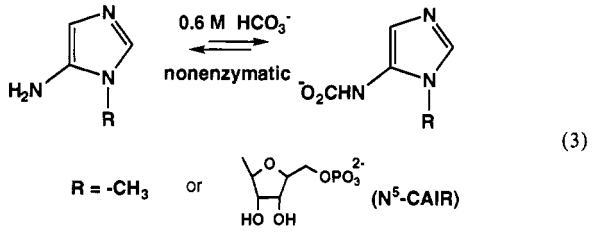
AIR on the PurE protein. The instability of carboxyphosphate, whose half-life is estimated to be less than 70 ms by Sauers et al. (1975), suggested a requisite interaction between PurE and PurK. However, our preliminary studies failed to detect any significant association (Meyer et al., 1992).

Our thinking about the problem was reformulated on reading a paper by Alenin et al. (1987) in which they characterized the nonenzymatic reaction of N^1-alkyl (R)-5-aminoimidazoles [R = ribose 5-phosphate (AIR) or CH_3] with molar concentrations of HCO_3^-. These studies revealed that 5-aminoimidazole derivatives are rapidly and reversibly converted into a new species which is distinct from the N^1-alkyl 4-carboxy-5-aminoimidazoles that form on a much slower time scale. Preliminary ^1H and ^{13}C NMR analysis of the



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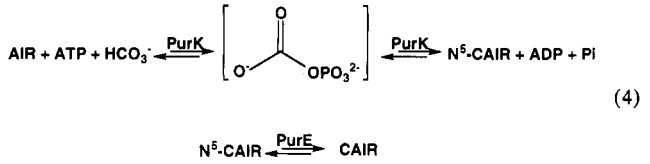
reaction in which R = CH_3 (eq 3) allowed Alenin and coworkers to postulate the formation of the N^5 carbamate. These studies prompted us to reformulate our hypothesis for the mechanism of conversion of AIR to CAIR (eq 4).



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This model predicts that PurE and PurK are not subunits of the same enzyme but that they catalyze two separate steps in the purine biosynthetic pathway. The carbamate product of the PurK-catalyzed reaction between AIR, ATP, and HCO_3^-, N^5-carboxyaminoimidazole ribonucleotide, designated N^5-CAIR, is postulated to be a new intermediate in this pathway. PurE is postulated to catalyze the rearrangement of the N^5-carbamate to CAIR. Evidence is presented in this paper to substantiate this hypothesis.

MATERIALS AND METHODS

Materials. Bovine serum albumin (fraction V, BSA), lactate dehydrogenase (LDH, 860 units/mg), pyruvate kinase (PK, 470 units/mg), phosphoenolpyruvate (PEP), phosphoenolpyruvate carboxylase (PEP-C, 3.6 units/mg), malate dehydrogenase (MDH, 3000 units/mg) and adenosine triphosphate (ATP) were obtained from Sigma Chemical Co. Filters of 0.45 μm and Centricon ultrafiltration units were purchased from Millipore Corp. DEAE A-25 Sephadex was obtained from Pharmacia. [1-^{14}C]Aspartate (220 mCi/mmol), $KH^{13}CO_3$ (WCS 171, batch 8, 90 atom %) and $H_2{}^{18}O$ (WOW 420, batch 3, 95 atom %) were purchased from Amersham. [γ-*amido*-^{15}N]-L-glutamine (lot CH-211, 99% ^{15}N) was from Cambridge Isotope Laboratories. Isotope ratio mass spectrometry was performed by Geochron Laboratories, Cambridge, MA. β-Formylglycinamide ribonucleotide (β-FGAR) was synthesized as described by Mueller (1993), and was typically a 40:60 ratio of α:β-anomers. All reported concentrations are for β-FGAR. Formylglycinamide ribonucleotide amidotransferase (FGAR-AT) and AIR synthetase were purified using the techniques described by Schendel et al. (1989) and Schrimsher et al. (1986), respectively. The syntheses of AIR and CAIR, the purification of enzymes PurE, PurK, and PurC [5-aminoimidazole-4-(*N*-succinylcarboxamide)ribonucleotide (SAICAR) synthetase), and the assays for these enzymes were accomplished as previously described (Meyer et al., 1992). *Escherichia coli* TX209 (*purK*$^-$) was a gift from John Smith, R & D Systems Inc., Minneapolis, MN. Restriction enzymes and DNA molecular mass markers were purchased from New England Biolabs.

General Methods. Protein assays were done according to the method of Lowry et al. (1951) using BSA (ϵ_{279} = 0.667 mL mg^{-1} cm^{-1}) as a standard. Phosphate assays were accomplished using the method of Ames and Dubin (1960) with ATP as a standard (ϵ_{260} = 15.4 mM^{-1} cm^{-1}). Orcinol assays for quantitation of reducing pentose sugars were conducted using the method of Dische (1962) with ATP as a standard. Bratton–Marshall assays were carried out as described (Schrimsher et al., 1986). All standard molecular biological methods were carried out according to Sambrook et al. (1989). Fixed-wavelength UV/visible assays were carried out using a Cary 210 spectrophotometer. Assays in which multiple wavelengths were monitored were done on a Hewlett-Packard 8452A diode array spectrophotometer. NMR spectra were recorded on either a Varian VXR-500 NMR or a Varian Unity-300 NMR spectrometer. ^{13}C NMR spectra were collected using broad-band proton decoupling. All chemical shifts are reported in parts per million relative to the methyl peak of 1,1,1-trimethylsilylpropanesulfonic acid set at 0.0 ppm as an internal standard, unless otherwise noted. Plasmid DNA was isolated using a Magic Miniprep kit from Promega. DNA was purified from agarose gels using U.S. Bioclean, from U.S. Biochemical Corp.

Determination of the Bicarbonate Concentration. Assays contained 50 mM HEPES (pH 7.7), 2 mM PEP, 2 mM $MgCl_2$, 0.2 mM NADH, 0.25 unit of PEP-C, and 5 units of MDH in a final volume of 400 μL. Reaction mixtures, without enzymes, were degassed by bubbling argon through the reaction mixture for 30 min and then preincubated at 37 °C for 2 min just before use. Addition of PEP-C and MDH resulted in rapid consumption of ~15% of the NADH due to contaminating bicarbonate in solution. Once a background rate was established, a small volume (typically 1–5 μL) of the sample to be analyzed for bicarbonate was added and the ΔA_{340} was recorded. Bicarbonate concentration was calculated using ϵ_{340} = 6220 M^{-1} cm^{-1}.

Nonenzymatic Conversion of AIR to N^5-CAIR Monitored by NMR Spectroscopy. A solution, 600 μL, containing AIR (29 mM) and 200 mM potassium phosphate in D_2O (pD 6.0) was placed in an NMR tube. After an initial spectrum was recorded, solid $KHCO_3$ (56.1 mg, 0.63 mmol) was added to give a final volume of 700 μL and the NMR tube was sealed using a flame. The reaction was initiated by inverting the tube and mixing the solid and liquid phases. ^{1}H NMR spectra were recorded at various intervals from 1 min to 600 h. Each spectrum was integrated to determine the amount of each species formed at a given time. At the end of the experiment the tube was opened and the pD was determined to be 8.0.

Enzymatic Synthesis of [amidino-^{15}N]-β-FGAM. The reaction mixture contained 40 mM Tris-HCl (pH 7.5), 20 mM ATP, 8.8 mM [γ-*amido*-^{15}N]glutamine, 80 mM KCl, 30 mM $MgCl_2$, 11 mM PEP, 1.87 mM β-FGAR, 20 units of PK, and 5 units of FGAR-AT in a final volume of 4 mL. The mixture containing everything except the enzymes was preincubated at 37 °C for 10 min, and the reaction was initiated

by addition of FGAR-AT. After 25 min, the mixture was diluted to 100 mL with water at 4 °C and applied to a DEAE A-25 column (2.5 × 10 cm, HCO_3^- form). The column was washed with 100 mL of water and the product was eluted with a 500 × 500 mL linear gradient from 0 to 400 mM triethylammonium bicarbonate (TEAB) pH 7.6. β-FGAM eluted at 75 mM TEAB, determined using the orcinol assay. Fractions containing product were concentrated *in vacuo* and assayed by Bratton–Marshall end point assay, which indicated the recovery of 5.2 μmol β-FGAM (70% yield).

Enzymatic Synthesis of [5-amino-^{15}N]-β-AIR. The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 20 mM ATP, 400 mM KCl, 3 mM $MgCl_2$, 14 mM PEP, 1.7 mM [*amidino*-^{15}N]-β-FGAM, 10 units of PK, and 3 units of AIR synthetase in a final volume of 2 mL. The mixture without enzymes was preincubated for 10 min at 37 °C and the reaction was then initiated by addition of PK and AIR synthetase. The reaction was allowed to proceed for 30 min, after which time the mixture was diluted to 100 mL with cold water and loaded onto a DEAE A-25 column (2.5 × 10 cm, HCO_3^- form). The column was developed with a 500 × 500 mL gradient of 0–500 mM TEAB, pH 7.6. Fractions were monitored using A_{250} and the Bratton–Marshall and orcinol assays. AIR eluted at 200 mM TEAB and the appropriate fractions were pooled and concentrated *in vacuo* to give 1.7 μmol of AIR (51% yield). ^{13}C NMR (125 MHz, D_2O, $^{13}CH_3OH$ standard δ = 48 ppm) 135.1 (d, J = 12 Hz, C5), 131.5 (s, C2), 111.6 (s, C4), 86.4 (s, C1′), 83.8 (d, J = 8.4 Hz, C4′), 72.5 (s, C3′), 69.9 (s, C2′), 62.8 (d, J = 3.8 Hz, C5′), and a small amount of HCO_3^- (δ 167.5, s). Assignments are based on the work of Schendel and Stubbe (1986) and Alenin et al. (1987). The ^{13}C–^{15}N coupling observed at the C5 resonance is in the range expected (Levy & Lichter, 1979).

^{13}C NMR Study of [5-amino-^{15}N,carbamoyl-^{13}C]-N^5-CAIR. [*5-amino*-^{15}N]AIR (3.8 μmol) and $NaH^{13}CO_3$ (375 μmol) were combined in 600 μL of D_2O (final pD 7.4) and incubated on ice for 30 min. The sample was then adjusted to pD 12.5 using 1 M KOD, and $^{13}CH_3OH$ was added as a standard (δ = 48 ppm). The final volume was 1.2 mL. The spectrum is shown in Figure 2C.

Synthesis and Isolation of N^5-CAIR. To AIR (40 μmol) in 1.0 mL of 0.1 M Tris-HCl (pH 7.0) was added 0.11 g (1.14 mmol) of $KHCO_3$. The reaction vessel, chosen to have minimal headspace, was sealed with a rubber septum which was held in place using copper wire. The solution was incubated at 4 °C for 30 min and the pH was then adjusted to the range of 12 and 14 by using 300 μL of 5 N KOH. The solution, diluted to 40 mL, was loaded, subsequent to checking conductivity and pH, onto a 7-mL Dowex 1-X8 (OH^- form) column which had previously been equilibrated with 0.5 M potassium phosphate (pH 12.5) followed by 5 mM potassium phosphate (pH 11.6). N^5-CAIR was eluted, at 4 °C, using a 100 × 100 mL linear gradient from 0 to 300 mM KCl in 5 mM potassium phosphate (pH 11.6). Fractions were monitored by absorbance at 240 nm and the Bratton–Marshall assay; N^5-CAIR eluted at 200 mM KCl. The appropraite fractions, 12 mL, were pooled and concentrated *in vacuo* to 1 mL. (A typical elution profile is available in the supplementary material.) During the concentration, KCl precipitated out of solution. The supernatant containing N^5-CAIR was decanted and the solvent was removed *in vacuo*. The solid remaining was then triturated with CH_3OH, the combined CH_3OH layers were pooled, and the solvent was removed *in vacuo*. The resulting N^5-CAIR was dissolved in H_2O, pH ~14, and stored at −20 °C. The concentration of N^5-CAIR was determined by a modification of the Bratton–Marshall assay in which the sample was incubated in acid for 2 min prior to addition of $NaNO_2$. This step allowed N^5-CAIR to decompose to AIR and thus provides an upper limit for the amount of N^5-CAIR present. Yields ranged from 42% to 57%. ^1H NMR (250 MHz, D_2O, pD 14): δ 7.98 (s, 1 H, C2), 6.83 (s, 1 H, C4), 5.63 (d, J = 4.9 Hz, 1 H, C1′), 4.48 (m, 1 H, C2′), 4.30 (m, 1 H, C3′), 4.21 (m, 1 H, C4′), 3.91 (m, 2 H, C5′). ^{13}C NMR (75 MHz, D_2O, pD 14): δ 167.3 ($KHCO_3$), 161.6 (s, C6), 133.5 (s, C5), 127.7 (s, C2), 122.7 (s, C4), 86.5 (s, C1′), 82.5 (d, J = 8.0 Hz, C4′) 73.7 (s, C3′), 69.3 (s, C2′), 63.1 (d, J = 5.3 Hz, C5′).

Stability of N^5-CAIR. A stock solution of N^5-CAIR was prepared by adding AIR (8.1 μmol) to 500 μL of 100 mM Tris-HCl (pH 7.0) and 600 mM $KHCO_3$. This solution was stored at −20 °C in a tightly stoppered flask on ice. Three different protocols were used to monitor the decomposition of N^5-CAIR.

(i) ΔA_{250} nm. N^5-CAIR decomposes to AIR, which has a UV spectrum previously characterized in detail (Meyer et al., 1992). N^5-CAIR has no absorption maximum, ϵ_{250nm} = 1300 M^{-1} cm^{-1} (pH 7.8). A typical assay in a final volume of 1 mL contained 300 mM Tris-HCl, 20 mM KCl, and 125 μM N^5-CAIR at 25 °C. The pH was varied from 7.6 to 8.4 and the increase in A_{250nm} was monitored by spectrophotometry. The UV spectra of AIR and N^5-CAIR under identical conditions are provided as supplementary material.

(ii) NADH Consumption. To establish the validity of the assay described in (*i*), two end point assays were used. Both are dependent on the observation that N^5-CAIR is a substrate for PurE in the absence of HCO_3^-. A typical assay contained 50 mM HEPES (pH 7.8), 20 mM KCl, 4.5 mM $MgCl_2$, 0.9 mM ATP, 2.0 mM PEP, 7.2 mM aspartate, 0.2 mM NADH, 10 units of PK, 10 units of LDH, and 0.7 unit of PurC in a final volume of 700 μL at 20 °C. N^5-CAIR from the stock solution was diluted into the assay mixture above, where the final concentration ranged from 9 to 44 μM. After variable periods of time, 0.6 unit of PurE was added, and the total change in A_{340nm} was recorded.

(iii) [^{14}C]-5-Aminoimidazole-4-(N-succinylcarboxamide)-ribonucleotide (SAICAR) Formation. The assay mixture was identical to that described above except [1-^{14}C]aspartate (7.5×10^5 cpm/μmol) replaced aspartate, and NADH and LDH were omitted. The reactions were initiated with N^5-CAIR, and 44-μL aliquots were removed at various time points and placed in an Eppendorf tube containing 0.1 unit of PurE. Reactions were quenched 3 min later with 40 μL of 33% (w/v) trichloroacetic acid. The [^{14}C]SAICAR formed was quantitated as previously described (Meyer et al., 1992).

Kinetic Characterization of the Interaction of N^5-CAIR with PurE. The reaction mixture contained, in a final volume of 0.7 mL, 200 mM Tris (pH 7.8), 20 mM KCl, 9.0 mM $MgCl_2$, 0.9 mM ATP, 0.2 mM NADH, 2 mM PEP, 10 units of PK, 10 units of LDH, 7 mM aspartate, 0.08 unit of PurC, and 5×10^{-3} unit of PurE. The mixture was equilibrated for 5 min at 20 °C, and the background rate was recorded. The reaction was initiated with N^5-CAIR (58 μM–1.4 mM) from a stock solution. Given that N^5-CAIR is stored at pH 12–14 in phosphate buffer, great care was taken to ensure that the pH of the reaction mixture was maintained at 7.8. The concentration of phosphate added with N^5-CAIR ranged from 0.15 to 3.5 mM. Data were analyzed using the programs of Cleland (1979). A control reaction mixture containing everything except PurE was used to measure the rate constant for decomposition of N^5-CAIR using method (iii) (preceding section).

Kinetic Analysis by NMR Spectroscopy of PurE-Catalyzed Conversion of CAIR to N^5-CAIR. CAIR (30 μmol) was carefully titrated to pH 7.0 using 1 M HCl and brought to dryness *in vacuo*. The sample was exchanged into 500 μL of D_2O, titrated to pD 8.0 and 20 °C with 1 M NaOD in D_2O, and assayed for CAIR (ϵ_{250} = 10 980 M^{-1} cm^{-1}) and bicarbonate using the PEP-C assay described above.

PurE was exchanged into 300 mM Tris-HCl, pD 8.0, in D_2O using a Centricon-30 (dilution of original buffer salts was 1/100). Enzyme activity was assayed before and after exchange into deuterated buffer.

NMR samples contained, in a final volume of 700 μL, 300 mM Tris-HCl, pD 8.0, 7.5 mM CAIR, and PurE (1.5–15 units) at 20 °C. After an initial spectrum of CAIR was taken, reactions were initiated with PurE and successive 16-scan FIDs were collected over time. Spectra were acquired every 30 s for the first 10 min, every 1 min for the subsequent 15 mins, and finally every 5 min until the reaction had proceeded for 1 h.

The concentrations of substrates and products were determined by integration of the NMR spectra taken at each time point. The kinetic data were analyzed using an IBM-compatible version of GIT [PC version 1.31, 12/87, copyright E. I. Du Pont de Nemours and Company, Inc., by R. McKinney and F. J. Weigert (Stabler & Cheswick, 1978; Weigert, 1987)] and plotted using a MacIntosh version of KINSIM (Barshop et al., 1983), HopKinsim 1.3, provided by Professor Robert Kuchta, University of Colorado, Boulder, CO.

PurK-Catalyzed Transfer of ^{18}O from $NaH^{13}C^{18}O_3$ to P_i. $NaH^{13}CO_3$ (1.61 mg, 18.9 μmol) was placed in an oven-dried Wheaton vial. $H_2{}^{18}O$ (100 μL) was added through the septum using an oven-dried syringe. The concentration of HCO_3^- was confirmed to be 189 mM using the PEP-C assay. The bicarbonate was allowed to incubate at room temperature for 5 h to ensure complete exchange. Isotope ratio mass spectroscopy of the resulting bicarbonate revealed that the $^{18}O/^{16}O$ ratio was 89:11.

AIR (10 μmol) was acidified to pH 5.5 with 1 M HCl, diluted to 1 mL, and degassed using two freeze–pump–thaw cycles followed by bubbling argon through the solution at room temperature for 1 h. The pH of the sample was adjusted to 8.0, and then the sample was assayed for AIR (Bratton–Marshall assay), bicarbonate (PEP-C assay), and total phosphate (Ames & Dubin, 1960).

Reaction samples contained, in a final volume of 500 μL, 100 mM HEPES (pH 8.0), 15 mM KCl, 20 mM $MgCl_2$, 11.4 mM ATP, 1 mM AIR, 11.3 mM $NaH^{13}C^{18}O_3$, and 1.8 units of PurK. A control reaction containing no AIR was also performed. Samples were preincubated for 2 min at 4 °C in the absence of HCO_3^- and PurK, and then the reactions were initiated with bicarbonate, followed 5 s later by PurK. Reactions were allowed to proceed for 2 min and then were quenched by addition of 300 μL of 600 mM CHES and 500 mM EDTA (pH 9.0). These samples were rapidly frozen using liquid nitrogen and stored at −80 °C until NMR analysis was possible.

NMR samples were thawed in an Eppifuge at 4 °C (spun for 10 min), acetone-d_6 was added as a deuterium lock standard to each reaction, and the samples were forced through a 0.45-μm filter by centrifugation (2800*g*). ^{31}P NMR spectra were collected for each sample on a VXR-500 NMR spectrometer (^{31}P 121 MHz) using a sweep width of 7272.7 Hz and an acquisition time of 2.002 s.

PurK-Catalyzed Conversion of AIR to N^5-CAIR and Nonenzymatic Decomposition to AIR Monitored by NADH Consumption. Assays contained, in a final volume of 710 μL, 100 mM Tris-HCl (pH 7.8), 20 mM KCl, 4.5 mM $MgCl_2$, 1.0 mM ATP, 0.2 mM NADH, 2 mM PEP, 10 units of PK, 10 units of LDH, and 0.19 unit of PurK. Reactions were initiated with AIR (3–24 nmol) and run at 20 °C. The stoichiometry of NADH consumption and the steady-state rate of NADH consumption were calculated using ϵ_{340} = 6220 M^{-1} cm^{-1}.

NMR Analysis of PurK-Catalyzed Conversion of AIR to N^5-CAIR. Reactions contained, in a final volume of 560 μL, 50 mM Tris-HCl (pH 8.0), 15 mM KCl, 10 mM $MgCl_2$, 3 mM ATP, 3 mM $KHCO_3$, 3 mM AIR, 3 mM PEP, 8 units of PK, and 0.4 unit of PurK. Samples without enzymes were preincubated at 5 °C for 2 min. To each sample was added PK, after which the reactions were initiated with PurK. Reactions were quenched after 2 or 5 min by adding 140 μL of 1 M NaOD in D_2O (final pH 12.5) and then were rapidly frozen in liquid nitrogen until workup was possible, within 3 h. A control using no enzyme was also run.

Samples were thawed, brought to dryness *in vacuo*, and then exchanged with 2 × 1 mL aliquots of D_2O. After each sample was dissolved in 700 μL of D_2O, ^1H NMR spectra were collected.

Construction of Plasmid pEJM1 (PurE), Transformation To Generate TX209/pEJM1, and Analysis for Growth. A 3-mL culture of *E. coli* strain TX635/pJS355 (Meyer et al., 1992) was grown overnight in LB medium at 37 °C and harvested by centrifugation (3000*g* for 10 min). The cells were subjected to alkaline lysis and their plasmid DNA was isolated using a Magic Miniprep kit. The DNA was incubated for 16 h at 37 °C in 50 mM potassium acetate, 20 mM Tris-HOAc (pH 7.9), 10 mM $Mg(OAc)_2$, 1 mM DTT, 10 units of *Hpa*I and 9 units of *Msc*I in a final volume of 48 μL. The desired fragment of DNA (3.57 kb) was purified on a 1% agarose gel and isolated using U.S. Bioclean. This DNA fragment (~1 μg) was incubated with 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 5% (w/v) polyethylene glycol 8000, and 1 unit of T4 DNA ligase (BRL) in a final volume of 20 μL for 24 h at 14 °C. Half of this mixture was used to transform TX209 made competent with $CaCl_2$ (Sambrook et al., 1989). Transformants were selected on LB plates containing ampicillin. Single colonies from these plates were transferred, using a sterile toothpick, to plates made of minimal medium (M9), minimal medium supplemented with 2 g/L casamino acids (M9CA), and M9CA supplemented with 20 mg/L hypoxanthine (M9CAHX) and analyzed for their ability to grow. A similar transformation and growth experiment was carried out with pZD1 (Chen et al., 1990) containing avian *purE*.

RESULTS

Nonenzymatic Conversion of AIR to N^5-CAIR in the Presence of High Concentrations of $NaHCO_3$: Isolation and Characterization of N^5-CAIR. Alenin et al. (1987) observed that incubation of N^1-methyl-5-aminoimidazole with 0.6 M potassium bicarbonate resulted in rapid formation of a new product in equilibrium with the starting material (eq 3). This new compound proved to be unstable; upon dilution it was rapidly converted back to starting material. Both ^{13}C and ^1H NMR spectroscopy were used to characterize this product. The presence of both C2 and C4 protons suggested that a carboxylation had most likely occurred on the exocyclic amine group. A similar reaction was reported to occur with AIR, although no experimental data were provided. A second set of experiments reported by these workers involved incubation

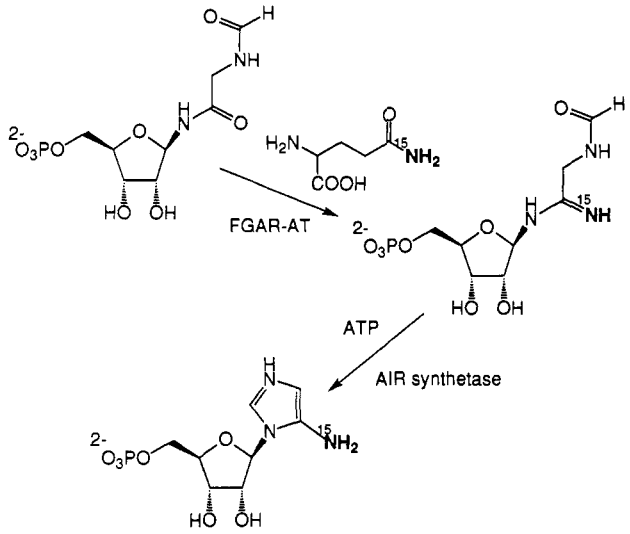

FIGURE 1: Enzymatic synthesis of [5-*amino*-^{15}N]AIR from [γ-*amido*-^{15}N]glutamine.

of N^1-methyl-4-carboxy-5-aminoimidazole or CAIR with 0.6 M $KHCO_3$ at 70 °C for 20 min. New products were observed by ^{1}H NMR and again assigned as the carbamates of their corresponding exocyclic amines.

These intriguing results provided the impetus for us to examine this process in detail using AIR and 1M HCO_3^-. Upon incubation of these compounds, a new product was generated so rapidly that the reaction was complete before the first NMR spectrum could be acquired. After an extended period of time (>70 h), CAIR was also observed (data not shown). Under the conditions of the experiment (1 M bicarbonate, 4 °C), the production of CAIR is pseudo-first-order in HCO_3^-, with a rate constant of $(1.0 \pm 0.03) \times 10^{-2}$ h^{-1} ($t_{1/2}$ = 69 h). This rate constant is similar to our previously reported value for the conversion of AIR to CAIR (4.2×10^{-2} h^{-1}) when the differences in reaction conditions are considered (Meyer et al., 1992). At that time we did not realize that AIR was rapidly converted to a new product now designated N^5-CAIR, which is converted directly or indirectly to CAIR.

The amount of N^5-CAIR produced is proportional to the concentration of HCO_3^- in solution (data not shown). The observation that 75% of the AIR can be converted to N^5-CAIR at 1 M bicarbonate suggested to us that N^5-CAIR might in fact be isolated. Stability studies of a variety of carbamate analogs indicate that at pH 12–14 they are moderately stable (the majority of carbamates have half-lives ranging from 2 to 200 h) (Caplow, 1968; Ewing et al., 1980; Johnson & Morrison, 1972). Our strategy was therefore to incubate AIR with 1 M HCO_3^-, rapidly adjust the solution to pH 12–14, and separate the carbamate from the starting material using anion-exchange chromatography on Dowex-1-X8 (OH^- form). N^5-CAIR was isolated in nearly homogeneous form using this methodology. The ^{1}H and ^{13}C spectra of the purified samples were as expected (Materials and Methods).

While the studies of Alenin et al. (1987) strongly suggested that N^5-CAIR was a carbamate, and our ^{1}H and ^{13}C NMR spectra (Figure 2A) are consistent with this proposal, further evidence was sought to define its structure. We therefore prepared [5-*amino*-^{15}N]AIR biosynthetically by the procedure outlined in Figure 1. This compound was incubated with $H^{13}CO_3^-$, and a ^{13}C NMR spectrum was taken (Figure 2C). A signal is observed at 161.6 ppm with a ^{13}C–^{15}N coupling constant of 19.7 Hz. These results are consistent

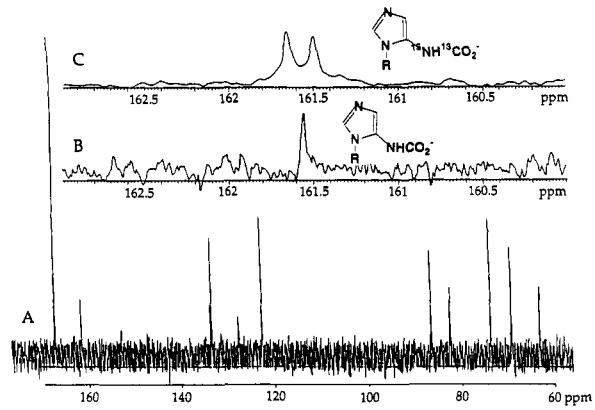

FIGURE 2: ^{13}C NMR of [*amino*-^{15}N]AIR with [^{13}C]HCO_3^-. (**A**) Natural abundance ^{13}C NMR spectrum of N^5-CAIR prepared by incubating HCO_3^- with AIR. Assignments: δ 167.5 (s, HCO_3^-), 161.6 (s, carbamate C), 135.1 (d, J = 12 Hz, C5), 131.5 (s, C2), 111.6 (s, C4), 86.4 (s, C1′), 83.8 (d, J = 8.4 Hz, C4′), 72.5 (s, C3′), 69.9 (s, C2′), 62.8 (d, J = 3.8 Hz, C5′). (**B**) An expansion of the spectrum recorded in (**A**) in the region of the carbon of the putative carbamate of N^5-CAIR. (**C**) The spectrum of the carbamate carbon produced from [*amino*-^{15}N]AIR incubated with [^{13}C]HCO_3^- (J_{C-N} = 19.7 Hz).

Table 1: Stability of N^5-CAIR versus (A) pH and (B) Temperature[a]

(A)		(B)	
pH	k (min^{-1})	temp (°C)	k (min^{-1})
7.6	0.32		
7.8	0.23	5	0.03
8.0	0.17	10	0.062
8.2	0.11	20	0.225
8.4	0.08	30	0.75

[a] (A) Reactions were run in 200 mM Tris-HCl, 20 °C. These rate constants are very sensitive to pH, ionic strength, and metal ion concentrations. (B) Reactions were run in 200 mM Tris-HCl (pH 7.8) and 20 mM KCl.

with one-bond coupling and unambiguously define the structure as the predicted carbamate (Levy & Lichter, 1979).

Stability of N^5-CAIR. Once N^5-CAIR had been characterized structurally, several assay methods were developed to examine its chemical stability. This knowledge is a prerequisite for examining the interaction of N^5-CAIR with PurE and PurK. N^5-CAIR, in the absence of HCO_3^-, is rapidly converted to AIR as determined by NMR spectroscopy (Figure 4). Furthermore, the molar absorptivity of N^5-CAIR at 250 nm is substantially different from that of AIR ($\Delta\epsilon$ = 2000 M^{-1} cm^{-1} at pH 7.8) (Meyer et al., 1992; Alenin et al., 1987) and has provided the basis for a spectrophotometric assay to study stability. As discussed subsequently, N^5-CAIR is a substrate for PurE and therefore several different coupled assays with PurC, 5-aminoimidazole-4-(*N*-succinylcarboxamide)ribonucleotide synthetase, which monitor [^{14}C]SAICAR production or NADH consumption, can also be used to measure the rate of decomposition of N^5-CAIR (Meyer et al., 1992). The results of these three methods are similar and the rate constants for decomposition of N^5-CAIR as a function of both pH and temperature, using the assay monitoring Δ 250 nm, are reported in Table 1. The $t_{1/2}$ for N^5-CAIR at 30 °C and pH 7.8 is ~0.9 min, with its stability decreasing with decreasing pH. The chemical instability explains the lack of its previous characterization as an intermediate in the purine biosynthetic pathway.

PurE-Catalyzed Conversion of CAIR to N^5-CAIR. With the availability of large amounts of PurE and our knowledge

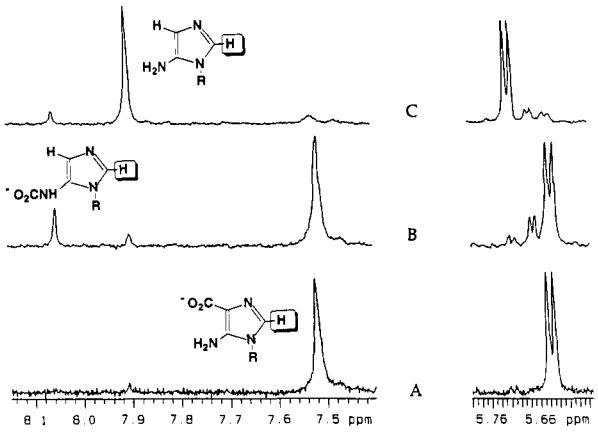

FIGURE 3: PurE-dependent generation of N^5-CAIR from CAIR at 25 °C. (A) A zero time point in which only CAIR (and a small amount of contaminating AIR) is present. (B) A 1.5-min time point after addition of PurE reveals that N^5-CAIR has formed along with a small amount of breakdown product, AIR. (C) A 20-min time point after addition of PurE reveals a nearly completed reaction, with AIR as the major species.

of the stability of N^5-CAIR, we set out to examine by NMR spectroscopy the PurE-catalyzed conversion of CAIR to N^5-CAIR (eq 4), the reverse of the biosynthetic reaction. We predicted that N^5-CAIR, following its formation catalyzed by PurE, would rapidly decompose to form AIR in a non-enzyme-dependent fashion. As indicated in Figure 3, the C1′ proton resonances of CAIR, N^5-CAIR, and AIR (δ 5.6–5.8 ppm) are separately observable using 500-MHz ^{1}H NMR spectroscopy. In addition, the C2 protons (7.5–8.1 ppm) of all these species are separable, as are the C4 protons[2] (data not shown) of N^5-CAIR and AIR. The number of units of PurE and the temperature of the reaction were chosen to facilitate kinetic analysis by NMR spectroscopy. Typical NMR spectra are shown in Figure 3 and the kinetics of CAIR decomposition, N^5-CAIR formation and decomposition, and AIR production are shown in Figure 4. The data have been fit by computer to eq 5, giving values of $k_1 = 0.18$ min^{-1}, $k_{-1} = 0.12$ min^{-1}, $k_2 = 0.26$ min^{-1}, and $k_{-2} = 2.8 \times 10^{-4}$ min^{-1} in 300 mM Tris-HCl (pD 8.0). The rate of conversion of CAIR

$$\text{CAIR} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{N}^5\text{-CAIR} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{AIR} + \text{HCO}_3^- \qquad (5)$$

to N^5-CAIR was shown to be dependent on the concentration of PurE, while the rate of conversion of N^5-CAIR to AIR was found to be independent of PurE (data not shown). The rate constant for the decomposition of N^5-CAIR (k_2) under these conditions, is virtually identical to that determined as described above, when pH differences, temperature, and ionic strength are taken into account. Thus PurE catalyzes the conversion of CAIR to N^5-CAIR as predicted by our model (eq 4).

Is N^5-CAIR a Substrate for PurE? Our previous kinetic studies of the PurE-catalyzed conversion of AIR to CAIR were carried out in the presence of 180 mM HCO_3^- and various amounts of AIR at 37 °C. Under these conditions we determined a K_m for AIR of 430 μM and a V_{max} of 70 μmol min^{-1} mg^{-1}. These experiments have been repeated at 20 °C and give numbers of 110 μM and 16 μmol min^{-1} mg^{-1}, respectively. NMR spectroscopic analysis of products formed when AIR is incubated with 180 mM HCO_3^- (20 °C) revealed the presence of 16% N^5-CAIR in solution. Thus, if N^5-CAIR

[2] The C4 proton of AIR exchanges with solvent with a $t_{1/2}$ ~8 min in 50 mM phosphate at pH 7.0 (Schendel, 1987).

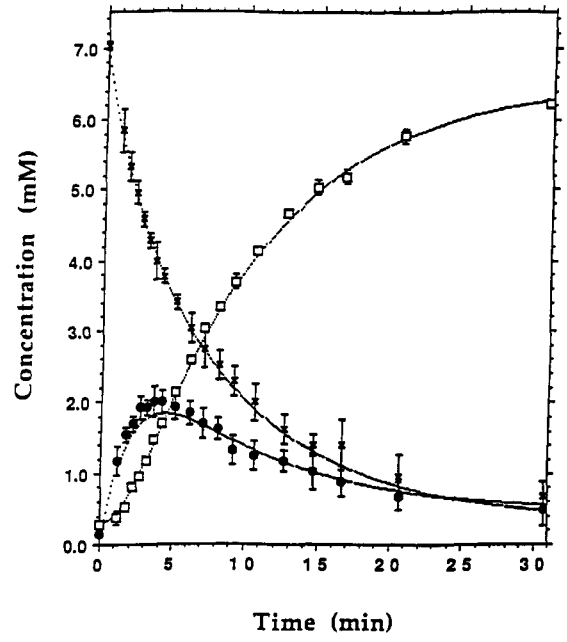

FIGURE 4: Kinetics of PurE-dependent conversion of CAIR to N^5-CAIR at 20 °C followed by nonenzymatic conversion of N^5-CAIR to AIR. NMR spectroscopy (Figure 3) allows monitoring of the reaction species: ×, CAIR; ●, N^5-CAIR; □, AIR. The solid lines are a nonlinear least-squares fit to eq 5 where $k_1 = 0.18$ min^{-1}, $K_{-1} = 0.12$ min^{-1}, $K_2 = 0.26$ min^{-1}, and $k_{-2} = 2.8 \times 10^{-4}$ min^{-1}. Error bars reflect integration errors.

is the actual substrate for PurE and AIR is a competitive inhibitor of the PurE-dependent reaction, the actual K_m would be expected to be less than the apparent K_m measured above. We therefore purified N^5-CAIR and determined its kinetic parameters as a substrate for PurE using the PurC, PK, LDH coupled assay. These studies are complex in that N^5-CAIR is isolated in high salt at very basic pH (12–14), and control of the pH and ionic strength of the assay mixture was somewhat problematic. The assays were carried out at 20 °C, in an attempt to limit the problems associated with decomposition of N^5-CAIR. The half-life for decomposition of N^5-CAIR in the assay mixture is ~3.0 min (Note: This number differs from that reported in Table 1. The reasons for this difference are not understood but may be related to the presence of metal ions such as Mg^{2+}). The K_m was measured to be 140 ± 50 μM and the specific activity was determined as 70 μmol min^{-1} mg^{-1}. Although the K_m for N^5-CAIR is larger than predicted, these studies support the contention that N^5-CAIR is the substrate for PurE, which catalyzes its conversion to CAIR. The error associated with these kinetic parameters is, in part, a consequence of the decomposition rate of the substrate and its sensitivty to pH and ionic strength.

PurK-Catalyzed Transfer of [^{18}O] from $HC^{18}O_3^-$ to P_i. The model in eq 2 predicts that, as in biotin-dependent reactions (Knowles, 1989), the function of ATP and HCO_3^- is to generate the elusive compound carboxyphosphate. $HC^{18}O_3^-$ was prepared by incubating bicarbonate in $H_2{}^{18}O$ using the methods of Knoche (1980) and Faurholt (1925) and its ^{18}O content was established (Materials and Methods). Incubation of $HC^{18}O_3^-$ with ATP and PurK in the presence of 1 mM AIR gave the ^{31}P NMR spectra shown in Figure 5. The spectrum in Figure 5B displays the γ-phosphate at ATP (–4.30 ppm) and β-phosphate of ADP (–4.81 ppm) and shows that 8% of the ATP has been hydrolyzed during the reaction. The amount of ADP is stoichiometric with respect to the amount of AIR consumed. Figure 5C displays an expanded version of the inorganic phosphate region of the spectrum and indicates

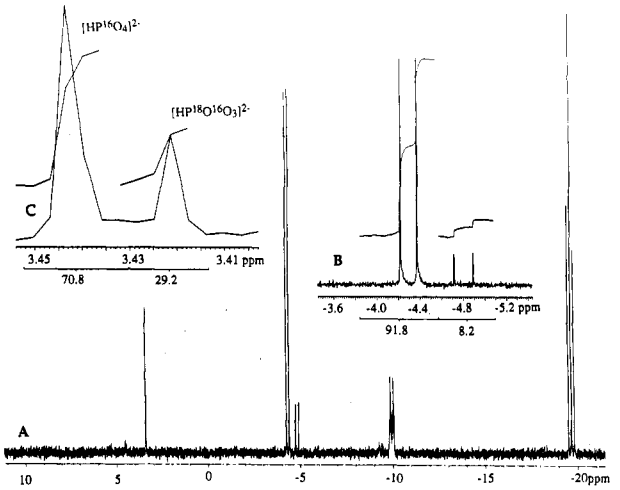

FIGURE 5: ^{31}P NMR spectrum of the products produced when AIR is incubated with ATP, $HC^{18}O_3^-$, and PurK. (A) Spectrum on completion of the reaction of 1 mM AIR, $HC^{18}O_3^-$, and 11.4 mM ATP with 1.8 units of PurK. The sample also contained 2.3 mM carrier P_i. δ 4.54 (s, AIR), 3.44 (P_i), –4.30 (d, J = 31 Hz, γ-P of ATP), –4.81 (d, J = 36 Hz, β-P of ADP), –9.32 (d, J = 36 Hz, α-P of ADP), –9.92 (d, J = 31 Hz, α-P of ATP), –19.64 (t, J = 31 Hz, β-P of ATP). (B) Expanded region at –3 to –5 ppm reveals that ~8% of the ATP has been hydrolyzed to ADP. (C) Expanded region at 3.4 ppm reveals 71% $[HP^{16}O_4]^{2-}$ (3.44 ppm) and 29% $[HP^{18}O^{16}O_3]^{2-}$ (3.42 ppm).

$[HP^{16}O_4]^{2-}$, which was added as carrier and a reference to the reaction mixture, at 3.44 ppm and $[H^{18}OP^{16}O_3]^{2-}$, the product of the reaction, at 3.42 ppm. Quantitation of the amount of $[H^{18}OP^{16}O_3]^{2-}$ generated revealed that ~1 atom of ^{18}O was transferred from bicarbonate to phosphate for every ADP generated. Control reactions containing 0.1 mM AIR or no PurK revealed no perturbation of the chemical shift of the phosphate in the ^{31}P NMR spectrum. These results are consistent with the intermediacy of carboxyphosphate (eq 4).

PurK-Catalyzed Conversion of AIR to N^5-CAIR in the Presence of ATP. The following experiment was designed to show the formation of N^5-CAIR catalyzed by PurK (eq 6).

$$\text{ATP} + HCO_3^- + \text{AIR} \xrightarrow[\text{nonenzymatic}]{\text{PurK}} N^5\text{-CAIR} + P_i + \text{ADP} \xrightarrow[\text{NADH, PEP}]{\text{PK/LDH}} \text{ATP} \quad \text{NAD}^+, \text{pyruvate} \qquad (6)$$

Our hypothesis predicts that incubation of a small amount of AIR with an excess of ATP, HCO_3^-, and PurK should produce a burst of N^5-CAIR, P_i and ADP. The latter can be monitored using a PK/LDH coupled assay which follows NADH consumption. If sufficient PurK is present, then the rate-determining step in the reaction will be the nonenzymatic decomposition of N^5-CAIR to AIR. This rate constant can be measured by the slow rate of consumption of NADH subsequent to the burst and can be compared to the rate of decomposition determined as described above. The results of an actual experiment in which the consumption of AIR (12 nmol) is monitored by the ΔA_{340} of NADH oxidation is shown in Figure 6. The initial burst is followed by a slower rate of NADH consumption. The results of a variety of experiments in which the amount of AIR and PurK were varied are summarized in Table 2. The observed bursts are equivalent to the amount of AIR present, while the slow rates of NADH consumption are independent of the concentration of PurK and are equivalent to the rate constant determined independently for the breakdown of N^5-CAIR, 0.40 s^{-1}, under identical conditions. These results support our hypothesis that PurK catalyzes the formation of N^5-CAIR.

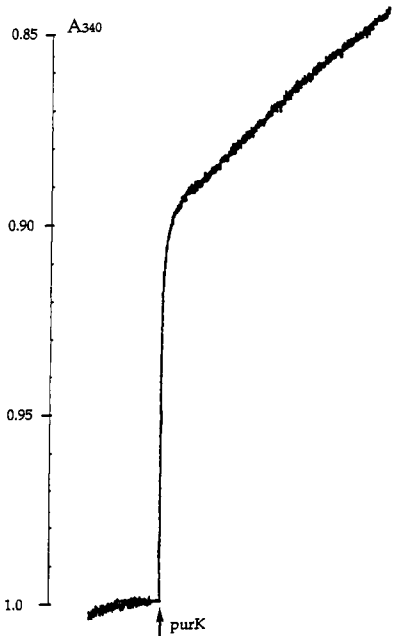

FIGURE 6: PurK-catalyzed conversion of AIR to N^5-CAIR as monitored by a coupled assay using PK/LDH and consumption of NADH. The kinetics initially exhibit a rapid, AIR-dependent burst of NADH consumption (equivalent to 12 nmol, the amount of AIR added) followed by a slower rate which reflects nonenzymatic conversion of N^5-CAIR to AIR.

Table 2: Incubation of Limiting Amounts of AIR with PurK, ATP, LDH, and PK and Monitoring of NADH Consumption

nmol of AIR[a] added	nmol of NADH rapidly consumed	steady-state constant for NADH consumed, k (min^{-1})
3.2	3.5	0.44
5.9	5.2	0.40
11.9	12.9	0.39
23.9	23.6	0.40

[a] 100 mM Tris-HCl (pH 7.8, 20 °C).

Detection of PurK-Catalyzed Formation of N^5-CAIR by NMR Spectroscopy. The experiments described in the previous sections all corroborate the model proposed in eq 4. It remained to be shown, however, that the compound whose formation is responsible for the burst of NADH consumption is in fact N^5-CAIR. Thus, an experiment was set up under conditions identical to the "burst" experiments described, so that N^5-CAIR could be detected by NMR spectroscopy. The reaction was quenched with base to a final pH of 13 to decrease the rate of N^5-CAIR decomposition. The results of this experiment are shown in Figure 7. The observation of new resonances at 7.96, 6.82, and 5.58 ppm (Figure 7B, arrows), identical to those associated with N^5-CAIR at 7.98, 6.83, and 5.63 ppm (Figure 7C), provides direct evidence that PurK catalyzes formation of N^5-CAIR.[3]

Characterization of pEJM1 and Growth of E. coli TX209/pEJM1. The avian and methanobacterial genes for PurE were cloned by complementation to an *E. coli purK*$^-$ strain,

[3] The chemical shifts are very sensitive to changes in both the pH and the ionic strength of the solutions. The pH of the solution, the spectrum of which is shown in Figure 7B, is ~13, while that in Figure 7C is ~14. The C4 proton of AIR (starting material) had been partially exchanged in D_2O during its characterization. Consequently, the appearance of less than 1 equiv of a proton at C4 in N^5-CAIR is a consequence of the exchanged starting material and not additional exchange during the experiment or workup.

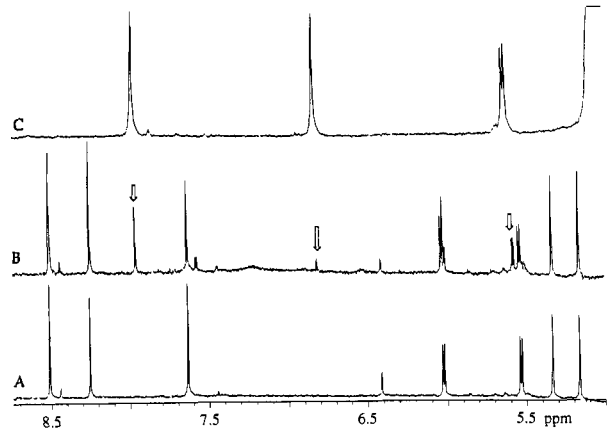

FIGURE 7: PurK-catalyzed conversion of AIR to N^5-CAIR monitored by NMR spectroscopy subsequent to quenching at pH 12.5. (**A**) The reaction mixture with everything present prior to the addition of enzyme: δ 8.56 (C8, ATP), 8.28 (C2, ATP), 7.69 (C2, AIR), 6.45 (C4, AIR)[3], 6.06 (d, J = 6 Hz, C1′ of ATP), 5.56 (d, J = 6.9 Hz, C1′ of AIR), 5.39 (s, vinyl protons of PEP), 5.21 (s, vinyl protons of PEP). (**B**) PurK was added to initiate the reaction, which was quenched after 2 min by adjusting the pH to 13. New resonances (indicated by arrows) are observed at δ 7.96 (s, C2 of N^5-CAIR), 6.82 (s, C4 of N^5-CAIR), 6.02 (d, J = 6 Hz, C1′ of ADP), 5.58 (d, J = 5.0 Hz, C1′ of N^5-CAIR). (**C**) Spectrum of N^5-CAIR at pH 14, δ 7.98 (s, C2 of N^5-CAIR), 6.83 (C4, N^5-CAIR), 5.63 (d, J = 4.8 Hz, C1′ of N^5-CAIR). Differences in the new resonances and the N^5-CAIR standard are attributed to the pH differences in the two samples. In addition, as indicated in footnotes 2 and 3, the C4 proton of AIR and consequently N^5-CAIR has undergone partial exchange.

TX209 (Chen et al., 1990; Hamilton & Reeve, 1985a,b). A plasmid containing *E. coli purE* was constructed and transformed into TX209 to determine if it could overcome purine auxotrophy. pJS355, containing both *purE* and *purK*, was digested with two restriction enzymes and the resulting 3.75-kb fragment was religated, subsequent to purification, to form a new plasmid, pEJM1. This plasmid contains *purE* and 84 bp of *purK*, verified by restriction digest analysis. When pEJM1 was transformed into a *purK*$^-$ strain, TX209 growth occurred on purine-supplemented M9CAHX, producing visible colonies after only 12 h at 37 °C. TX209/pEJM1 was also able to grow very slowly at 37 °C on M9CA solid medium. Small colonies were visible after 3 days; however, all efforts to grow these colonies in liquid culture failed. The host TX209 did not show colony formation under the same conditions. This result suggests that *E. coli* cells transformed with a high-copy plasmid cannot compensate for the *purK*$^-$ phenotype. A similar experiment with the avian clone, pZD1, of Chen et al. (1990) transformed into *E. coli* TX209 followed by growth in liquid culture in M9CA medium revealed a doubling time of 50 min. Thus, the avian *purE* appears to easily complement the *E. coli purK* deletion.

DISCUSSION

In 1992, we reported that PurK catalyzes ATP hydrolysis at a high rate, 80 μmol min^{-1} mg^{-1} at 37 °C (Meyer et al., 1992). The intriguing, but at the time inexplicable, observation was that this reaction was absolutely dependent on the presence of AIR, which did not appear to be consumed during this reaction. This observation remained confusing until we became aware of the important experiments reported by Alenin et al. in 1987. Their studies suggested that N-substituted carbamates of 5-aminoimidazole derivatives could be rapidly generated in a chemical process from aminoimidazole derivatives in 1 M HCO_3^- solutions. Furthermore, their studies revealed that, on dilution of these HCO_3^- solutions, the putative carbamates decomposed back to starting aminoimidazole derivatives. Their observations provided an explanation for our observation of AIR-dependent ATPase activity and allowed us to formulate a new biosynthetic pathway for CAIR production in *E. coli* (eq 4), which has been established by the experiments reported herein.

Our initial efforts focused on the isolation and structural characterization of the compound produced when 1 M HCO_3^- was incubated with millimolar concentrations of AIR. Studies of Caplow (1968) on model carbamates for biotin-dependent decarboxylations provided the insights needed about carbamate stability to successfully complete these tasks. ^{1}H, ^{15}N, and ^{13}C NMR experiments (Figure 2) indicate that this species is the N^5-carbamate of AIR, and kinetic analyses have shown that its half-life in solution at 30 °C, pH 7.8, is 0.9 min.

We were thus in a position to characterize the role of N^5-CAIR in purine biosynthesis. The availability of large amounts of PurK and PurE (Meyer et al., 1992) and of homogeneous N^5-CAIR, albeit at pH 12, allowed us to test two hypotheses using NMR spectroscopic methods. The first is that PurK catalyzes conversion of AIR, ATP, and HCO_3^- to N^5-CAIR, ADP, and P_i and the second is that PurE catalyzes the reversible interconversion of N^5-CAIR and AIR.

The instability of N^5-CAIR made the experiments to test these hypotheses challenging. As shown in Figure 7, however, PurK does catalyze the formation of a new, "unstable", species with a ^{1}H-NMR spectrum identical to that of N^5-CAIR. An explanation for our previously reported AIR-dependent ATPase catalyzed by PurK is now readily apparent. PurK catalyzes the stoichiometric conversion of ATP and AIR to ADP, P_i, and N^5-CAIR (Table 2). The N^5-CAIR, however, rapidly undergoes nonenzymatic breakdown to regenerate AIR, which can then be used to promote the hydrolysis of additional molecules of ATP. Thus, millimolar quantities of ATP can be consumed in the presence of catalytic quantities of AIR.

The instability of N^5-CAIR also rendered detection of the PurE-catalyzed conversion of N^5-CAIR to CAIR challenging. Both ^{1}H-NMR and kinetic experiments were undertaken to examine this reaction. Using NMR spectroscopy, we examined the PurE-catalyzed conversion of CAIR to N^5-CAIR. CAIR is stable, and conditions of temperature and pH were carefully chosen so that N^5-CAIR formation would be detectable using this methodology. These studies (Figures 3 and 4) unambiguously established that PurE catalyzes the conversion of CAIR to N^5-CAIR, which nonenzymatically and irreversibly decomposes to AIR.

To examine the reaction in the biosynthetic direction, attempts were made to determine a K_m and V_{max} for N^5-CAIR with PurE. Assay conditions required appropriate pH and ionic strength control, as well as selection of a temperature that minimized interference by the decomposition of N^5-CAIR. The assays were carried out in 100 mM Tris buffer (pH 7.8) and 20 °C. The half-life for decomposition of N^5-CAIR in the assay mixture is ~3.0 min. Under these conditions the K_m for N^5-CAIR is 140 μM and the V_{max} is ~70 μmol min^{-1} mg^{-1}.

The observation that N^5-CAIR is a substrate for PurE seems contradictory to our original report (Meyer et al., 1992) that AIR in the presence of high concentrations of HCO_3^- (180 mM) is a substrate for PurE. The recent revelation, however, that N^5-CAIR is the product of the rapid nonenzymatic reaction between AIR and HCO_3^- provides an explanation for these results. Under these conditions, ^{1}H NMR spec-

Table 3: Homology of *E. coli* PurE and PurK in other organisms

organism	genetic construction	% homology PurE identity	% homology PurE similarity	% homology PurK identity	% homology PurK similarity
Synechococcus sp.[a]	purK			28	61
B. subtilis[b]	purE purK purB	58	83	24	52
S. pombe[c]	purK purE	48	84	23	57
S. cerevisiae[d]	purK purE	43	80	26	57
human[e]	purC purE	22	59		
chicken[f]	purC purE	23	64		
M. thermoautotrophicum[g]	purE	38	71		
M. smithii[h]	purE	38	74		

[a] Schwarz et al. (1992), accession number M91187. [b] Ebbole & Zalkin (1987), accession numbers P12044 (purE) and P12045 (purK). [c] Szankasi et al. (1988), accession number P15567. [d] Sasnauskas & Janulaitis (1992), accession number M58324. [e] Minet & Lacroute (1990), accession number P22234. [f] Chen et al. (1990), accession number A35641. [g] Hamilton & Reeve (1985b), accession number X03250. [h] Hamilton & Reeve (1985a), accession number P22348.

troscopy reveals that the ratio of N^5-CAIR to AIR is 0.16:0.84, suggesting that the actual substrate for PurE is N^5-CAIR and not AIR.

A comparison of the kinetic parameters for these two different assays (N^5-CAIR vs AIR at 180 mM HCO_3^-) reveals some discrepancies that still remain to be resolved. In part these discrepancies arise from the problems associated with the instability of N^5-CAIR. However, the consequences of the presence of 180 mM HCO_3^- on the kinetic parameters and an understanding of the mechanism of this putative mutase reaction catalyzed by PurE are still unknown as well. Further analysis of the PurE reaction should eventually provide a satisfactory resolution to these differences.

Our previous studies defining the requirement for ATP in the "AIR carboxylase" reaction in conjunction with the results presented herein require the addition of a previously unrecognized intermediate designated N^5-CAIR and two new enzymatic activities to the purine biosynthetic pathway in *E. coli*. We propose to designate PurK as N^5-CAIR synthetase and PurE as N^5-CAIR mutase or isomerase, depending on the chemical mechanism, which remains to be established.

This new understanding of the roles of PurE and PurK in the *E. coli* pathway leads to the question of their significance in the purine biosynthetic pathways of all organisms. The answer to this question remains to be determined unambiguously, but information described subsequently allows us at present to favor the hypothesis that, at least in avian systems and methanogens, alternate methods of CO_2 fixation may have evolved.

Almost all of the avian genes for various steps in the purine biosynthetic pathway have been cloned by complementation of the appropriate *E. coli* mutant (Chen et al., 1990). These observations originally led us to favor similar mechanisms for the carboxylation of AIR to CAIR in both eukaryotes and prokaryotes. However, a comparison of the amino acid sequences of PurE and PurK and the organization of these genes from a variety of organisms, Table 3, reveals some striking differences. The genes for AIR carboxylase from avian liver and methanobacteria (Chen et al., 1990; Minet & Lacroute, 1990; Hamilton & Reeve, 1985a,b) were cloned by complementation of the *E. coli purK*$^-$ strain TX209. In the case of avian liver carboxylase, however, only ~23% identity exists with the *E. coli* PurE sequence (Table 3). Amazingly, although the gene could complement *purK* deletions, no statistically significant complementarity with *E. coli* PurK was found. Similarly, the putative carboxylase from several different methanogens exhibits ~38% sequence identity with *E. coli* PurE and no complementary with *E. coli* PurK. Thus, the observations that these genes for PurE were cloned by complementation of the *purK* mutant, TX209, suggested to Chen et al. (1990) and to us that these organisms have evolved alternatives to the PurK-dependent carboxylation mechanism.

Studies of Minet and Lacroute (1990) using cDNA from HeLa cells and of Schild et al. (1990) using cDNA from human HepG2 cells reveal that human AIR carboxylase can be cloned by complementation of appropriate mutants of *Saccharomyces cerevisiae*. *S. cerevisiae* contain an AIR carboxylase (Table 3) which is a single polypeptide with both *purE* and *purK* domains. In contrast to expectations based on the work of Chen et al. (1990) with the avian DNA and *E. coli purK*$^-$ mutant, Minet and Lacroute found that the human gene for *purE* was unable to complement yeast mutants thought to be deficient in the *purK* domain of the yeast AIR carboxylase. Furthermore, in the case of several yeast mutants from *Schizosaccharomyces pombe* which required purines for growth, the auxotrophy was suppressed by increased concentrations of CO_2 (Szankasi et al., 1988). These results suggest that a *purK* domain is required for AIR carboxylase to function in humans. This apparent contradiction with the results from the avian system requires further characterization of the yeast mutant alleles and the yeast AIR carboxylase itself.

One possible explanation for the successful cloning experiments of Chen et al. (1990) and Hamilton and Reeve (1985a,b) is that expression of heterologous PurE by transformed *E. coli* TX209 allowed nonenzymatically produced N^5-CAIR to be converted to CAIR in amounts sufficient to overcome purine auxotrophy. To test this hypothesis, *E. coli* PurE was reengineered into a multicopy plasmid, transformed into *E. coli* TX209, and examined for its ability to supress this strain's requirement for purines. The results indicate that purine auxotrophy cannot be overcome. Thus the successful complementation of *E. coli* TX209 with a Bluescript vector containing avian DNA for PurE is in striking contrast to the results observed with *E. coli* PurE (Chen et al., 1990). We have isolated the vector, pZD1, constructed by Chen et al.

(1990) and shown that *E. coli* TX209 can grow on minimal medium subsequent to transformation with a doubling time of ~50 min. These preliminary results again support the proposal that an alternative carboxylation pathway is operating in the avian system.

The cloning and expression of the avian AIR carboxylase (Chen et al., 1990) allowed its overexpression and purification to homogeneity (Davisson, unpublished results). An NMR experiment was therefore designed to monitor interaction of CAIR with this protein. Preliminary results indicate that CAIR rapidly decomposed in the presence of avian PurE, directly to AIR. Control experiments revealed that under these conditions N^5-CAIR should have been detected had it been produced. These results provide the strongest evidence that carboxylation, in at least the avian system, is different from *E. coli*. Biochemical studies of carboxylases from avian liver and other organisms are essential to address their mechanistic similarities and differences with the carboxylation system from *E. coli*.

Regardless of the mechanism of CAIR biosynthesis in eukaryotes, this new twist in the *E. coli* pathway returns us to a long-standing unresolved problem in carboxylation chemistry: the mechanism by which carboxybiotin and now N^5-CAIR delivers CO_2 to its substrate(s). There are striking similarities and a few differences in these two CO_2 delivery systems (Knowles, 1989). Both are chemically unstable: carboxybiotin has a half-life of 103 min (Tipton & Cleland, 1988), while N^5-CAIR has a half-life of 0.9 min at 30 °C. Carboxybiotin and N^5-CAIR are both generated in ATP-dependent processes which presumably generate carboxyphosphate. In both cases the existence of carboxyphosphate has been inferred by incorporation of ^{18}O from $HC^{18}O_3^-$ into P_i. In biotin-dependent reactions, the biotin-dependent carboxylase activity is on a separate subunit from that required to catalyze the carboxybiotin-dependent carboxylation of substrate. The two subunits, as well as the biotin carboxyl carrier protein, are part of a multienzyme complex. While a second protein (PurE) is required to catalyze the formation of CAIR from N^5-CAIR as the substrate, in contrast to the biotin-requiring systems, there is no evidence that PurE and PurK are subunits of the same enzyme.

Finally, the mechanisms by which carboxybiotin and N^5-CAIR deliver their CO_2 to their respective substrates are unprecedented chemically. In the case of the N^5-CAIR system the problems associated with removing HCO_3^- from solutions, due to the acid instability of both CAIR and N^5-CAIR, will make mechanistic studies very challenging. Whether the CO_2 is transferred from N^5-CAIR to produce CAIR through a CO_2 intermediate or an enzyme-bound carbamate and whether the carboxylate of the carbamate of N^5-CAIR becomes the C4 carboxylate of CAIR are still unresolved questions that will require further experimentation.

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SUPPLEMENTARY MATERIAL AVAILABLE

Two figures, showing UV spectra of AIR and N^5-CAIR and isolation of N^5-CAIR (3 pages). Ordering information is given on any current masthead page.

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