Reactions Catalyzed by 5-Aminoimidazole Ribonucleotide Carboxylases from *Escherichia coli* and *Gallus gallus*: A Case for Divergent Catalytic Mechanisms?†

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ABSTRACT: A comparative investigation of the substrate requirements for the enzyme 5-aminoimidazole ribonucleotide (AIR) carboxylase from *E. coli* and *G. gallus* has been conducted using *in vivo* and *in vitro* studies. In *Escherichia coli*, two enzymes PurK and PurE are required for the transformation of AIR to 4-carboxy-5-aminoimidazole ribonucleotide (CAIR). The *Gallus gallus* PurCE is a bifunctional enzyme containing AIR carboxylase and 4-[(*N*-succinylamino)carbonyl]-5-aminoimidazole ribonucleotide (SAICAR) synthetase. The *E. coli* PurE and the C-terminal domain of the *G. gallus* PurCE protein maintain a significant degree of amino acid sequence identity and also share CAIR as a product of their enzymatic activities. The substrate requirements of AIR carboxylases from *E. coli* and *G. gallus* have been compared by a series of *in vitro* experiments. The carbamic acid, N^5-carboxyaminoimidazole ribonucleotide (N^5-CAIR) is a substrate for the *E. coli* PurE (Mueller et al., 1994) but not for the *G. gallus* AIR carboxylase. In contrast, AIR and CO_2 are substrates for the *G. gallus* AIR carboxylase. The recognition properties of the two proteins were also compared using inhibition studies with 4-nitro-5-aminoimidazole ribonucleotide (NAIR). NAIR is a tight-binding inhibitor of the *G. gallus* AIR carboxylase (K_i = 0.34 nM) but only a steady-state inhibitor (K_i = 0.5 μM) of the *E. coli* PurE. These data suggest significant differences in the transition states for the reactions catalyzed by these two evolutionarily related enzymes. Using separate, constitutive overexpression systems for *E. coli purK* or *purE*, or *G. gallus purCE*, the impact of each of these enzymes upon the growth rates of a PurK-deficient strain of *E. coli* was evaluated. The results suggest that a PurK deficiency effects purine metabolism by creating a rate-limiting chemical carboxylation of AIR. Heterologous expression of the *G. gallus* PurCE overcomes this limitation by providing a different pathway for conversion of AIR to CAIR.

The recent characterization of the fungal, yeast, and vertebrate *ADE2*, the bacterial *purK* and *purE*, and the archaebacterial *purE* genes has provided a new perspective on the role of carboxylation reactions in *de novo* purine biosynthesis (Hamilton & Reeve, 1985; Ebbole & Zalkin, 1987; Tiedeman et al., 1989; Watanabe et al., 1989; Chen et al., 1990; Schild et al., 1990; Stotz & Linder, 1990). Mutations in these genes are used as classical genetic markers and have long been associated with altered 5-aminoimidazole ribonucleotide (AIR)[1] metabolism resulting in pigmentation (Fischer, 1969; Yanulaitis et al., 1975; Ishiguro, 1989). All of these genes encode proteins that can be categorized as AIR carboxylases. There are regions of significant amino acid identity among this family of genes, but these relationships are complicated since there is species-dependent integration of other enzymatic activities. For instance, the vertebrate enzyme appears to be a fusion of the related bacterial *purE* and *purC* genes (Chen et al., 1990; Schild et al., 1990). A more detailed understanding of the enzymatic properties of each protein encoded by these genes will allow assignment of their function, and these efforts have recently been initiated (Meyer et al., 1992; Mueller et al., 1994).

In *Escherichia coli*, two proteins, PurK and PurE, are encoded by a single operon (Watanabe et al., 1989; Tiedeman & Smith, 1991). Together, these proteins catalyze the conversion of AIR to CAIR via the unstable intermediate N^5-CAIR (see eq 1 in Scheme 1). PurK is an ATP-dependent carboxylase that forms N^5-CAIR through the proposed intermediate carboxy-phosphate (Meyer et al., 1992; Mueller et al., 1994). A similar function of a PurK enzyme or a related protein in other organisms has been implicated by amino acid sequence homologies with the cyanobacterium and yeast systems. However, comparison of the available amino acid sequences indicates the absence of a related PurK in the vertebrate and archaebacterial proteins (Hamilton & Reeve, 1985; Chen et al., 1990; Minet & Lacroute, 1990). The *Gallus gallus* and *Homo sapiens* forms of AIR carboxylase are bifunctional in *de novo* purine biosynthesis, with the N-terminal region of the protein responsible for the 4-[(*N*-succinylamino)carbonyl]-5-aminoimidazole ribonucleotide

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[1] Abbreviations: AIR, 5-aminoimidazole ribonucleotide; CAIR, 4-carboxy-5-aminoimidazole ribonucleotide; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid); LB medium, Luria–Bertani medium; NAIR, 4-nitro-5-aminoimidazole ribonucleotide; N^5-CAIR, N^5-carboxyaminoimidazole ribonucleotide; PCR, polymerase chain reaction; PEP, phosphoenolpyruvate; SAICAR, 4-[(*N*-succinylamino)carbonyl]-5-aminoimidazole ribonucleotide; Tris, tris(hydroxymethyl)aminomethane; VBC medium, Vogel Bonner medium with 1% casamino acids.

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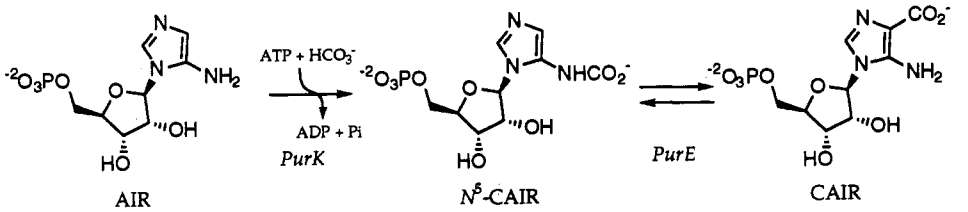
Scheme 1



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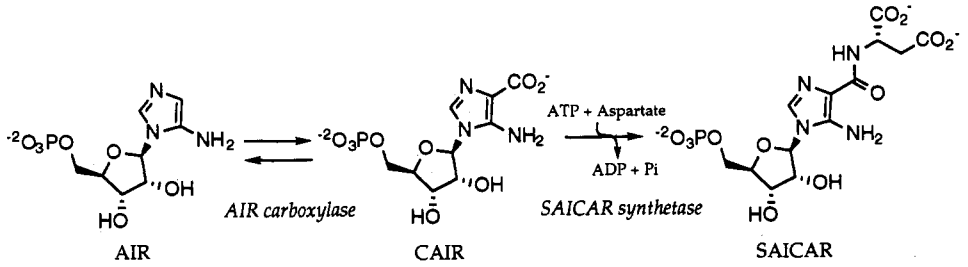

(2)

(SAICAR) synthetase activity (see eq 2 in Scheme 1). The amino-terminal portion of the methanogenic PurE also aligns with the *E. coli* PurE, but the C-terminal region shows no significant homologies with any related proteins. Most interesting is the fact that plasmids bearing the *G. gallus* and methanogen *purE*-related genes have been shown to confer a pur$^+$ phenotype to *E. coli* TX209 (*purK*$^-$) (Hamilton & Reeve, 1985; Chen et al., 1990). This parodox raises questions regarding the biochemical transformations that occur in the *de novo* purine pathways of bacteria and vertebrates.

Our recent investigation of the *G. gallus* AIR carboxylase–SAICAR synthetase (PurCE) has identified several important features (Firestine & Davisson, 1994). This enzyme has no ATP dependence for the carboxylase reaction. The K_m for HCO_3^- (23 mM) in the case of the *G. gallus* AIR carboxylase is 5-fold lower than that observed for the related PurE (K_m = 110 mM) from *E. coli*, suggesting that these two enzymes require different substrates. No common organic or metal cofactors have been identified for the *G. gallus* AIR carboxylase–SAICAR synthetase, indicating a unique catalytic mechanism for this enzyme. The identification of the one-carbon substrate for this protein as CO_2 is reported in this paper. The results further establish the surrogate role that the *G. gallus* AIR carboxylase plays when produced in *E. coli* and compare the unique substrate requirements of the *G. gallus* and *E. coli* PurE enzymes.

MATERIALS AND METHODS

The nucleotide substrates were prepared as described in a previous study (Firestine & Davisson, 1994). Solutions of N^5-CAIR were prepared and quantified by a calibrated Bratton–Marshall assay (Mueller et al., 1994). The content of AIR in the solutions was determined by ^{1}H NMR and found to be ≤20%. The three enzymes PurE, PurC, and PurK were isolated from overproducing *E. coli* strains previously described (Meyer et al., 1992). Protein concentrations were determined by the method of Bradford (Bio-Rad). The construction of plasmid pD3-*pur* and isolation of *G. gallus* AIR carboxylase have been described (Firestine & Davisson, 1994). *E. coli* strain TX209 (*purK*$^-$) and TX257 (*purE*$^-$) were obtained from the laboratory of J. Reeve (Ohio State). *E. coli* strain TX635/pJS355 was provided by the laboratory of J. Stubbe (MIT) (Meyer et al., 1992). The CO_2 solutions were prepared by continuous delivery of CO_2(g) into 100 mL of water at 4 °C, and the concentration was estimated from solubility constants (0.2871 g/100 g of water) (Dean, 1985). All solutions of $KHCO_3$ were prepared immediately before use and filter sterilized when required. Carbonic anhydrase was purchased from Sigma, and 4-nitro-5-aminoimidazole ribonucleotide (NAIR) was prepared as previously described (Firestine & Davisson, 1993). A Varian Cary 3 UV–vis spectrophotometer equipped with temperature controller was used for all enzyme assays. The progress curve data consisted of 18 000 individual points from which 5 min^{-1} was selected for display.

Determination of Substrates for G. gallus AIR Carboxylase and E. coli AIR Carboxylase. Carboxylation assays with CO_2 and HCO_3^-. In stopper-fitted cuvettes containing 200 mM Tris-HCl, pH 8.0, and 0.5–0.55 mM AIR, 0.23 μg of either *G. gallus* AIR carboxylase or *E. coli* PurE was added. The reaction was cooled to 10 °C and initiated by addition of either a CO_2 or $KHCO_3$ solution to a final concentration of 20 mM, and the change in absorbance at 260 nm was recorded. This assay described with *G. gallus* AIR carboxylase was also conducted in the presence of 100 μg of carbonic anhydrase.

Carboxylation Assays with N^5-CAIR. In stopper-fitted cuvettes containing 200 mM Tris-HCl, pH 8.0, either 0.2 μg of *E. coli* PurE or 0.7 μg of *G. gallus* AIR carboxylase was added and the mixture cooled to 10 °C. Reaction initiation was achieved by addition of 0.5 mM N^5-CAIR, and the change in absorbance at 260 nm was recorded. The rate of decomposition of N^5-CAIR was determined by the increase in absorbance at 260 nm using the same protocol, except that enzyme was omitted from the assay.

G. gallus AIR Carboxylase with E. coli PurK and ATP. In a 1-mL total volume, 50 mM HEPES, pH 8.0, 4.0 mM $KHCO_3$, 25 mM $MgCl_2$, 2.0 mM PEP, 1.0 mM ATP, 10 mM aspartate, 0.74 μg (0.027 unit) of *G. gallus* AIR carboxylase, 17 units of pyruvate kinase, and 7.7 μg (0.24 unit) of *E. coli* SAICAR synthetase were incubated at 37 or 10 °C. To the cuvettes were added, in succession, 0–15 μg (0–1 unit) of *E. coli* PurK and 0.3 mM AIR, and the SAICAR production was monitored at 282 nm (Firestine & Davisson, 1994).

Reaction of CAIR with G. gallus AIR Carboxylase. All enzyme reactions monitored by ^{1}H NMR experiments were performed at 20 °C, and the catalytic activity was also assessed at this temperature. NMR reactions were conducted in a total volume of 700 μL containing 300 mM Tris-HCl (pD 7.8), 8.8 mM CAIR (in D_2O), and *G. gallus* AIR carboxylase. The enzyme was exchanged into 300 mM Tris-HCl, pH 7.8, in D_2O using a Centricon-30 prior to use. Bicarbonate present

in a stock CAIR sample was determined as 24 mM final concentration by a PEP-carboxylase assay (Mueller et al., 1994). After initial spectra acquisitions, reactions were initiated with varied amounts of *G. gallus* AIR carboxylase. Three separate experiments were executed with either 5.6 units, 0.6 unit, or 0.06 unit of enzyme. The first spectra were recorded 70 s after addition of enzyme and at 30 s intervals for a total of 5 min. Additional spectra were recorded at various times up to 60 min after enzyme addition. The enzyme was assayed after each NMR experiment to ensure that full catalytic activity was maintained over the time period of the experiment.

Stability of N^5-CAIR in the Presence of G. gallus AIR Carboxylase. The effect of the *G. gallus* AIR carboxylase on the stability of N^5-CAIR was measured by the spectrophotometric assay (Mueller et al., 1994). Assays were performed in a final volume of 1 mL and contained 300 mM Tris-HCl, pH 7.8, and either 0 or 5.6 units of *G. gallus* AIR carboxylase. The reaction was initiated by the addition of 100 nmol of N^5-CAIR, and the absorbance at 250 nm was monitored over 10 min.

Inhibition Studies of E. coli PurE. In a total volume of 1.0 mL, 50 mM Tris-HCl, 0.5 mM EDTA, pH 8.0, CAIR (5–50 μM), and NAIR (0–2.5 μM) were combined and allowed to incubate at 30 °C. To the cuvette was added 0.8 μg of *E. coli* PurE (4.8 nM), and the decrease in absorbance was monitored at 260 nm. The K_i for NAIR was estimated from a series of $1/v$ vs $1/[S]$ plots using the program Enzyme Kinetics from Trinity Software.

Construction of Plasmids for Constitutive Expression: pSP-purE, pSP-purK, pD3-pur. The parent plasmid vector for all three constructs used in this study was derived from pDL-Nde which has been shown to confer constitutive expression in the case of other genes (Davisson et al., 1989). DNA plasmid construct pD3-*pur* was described previously (Firestine & Davisson, 1994). Ligation reactions with this vector were used to transform *E. coli* XL1-blue (Stratagene), and the desired recombinant plasmids were selected by restriction digestion (Sambrook et al., 1989).

Plasmid pJS355 encodes a portion of the *E. coli purKE* operon and served as a template for PCR reactions (Meyer et al., 1992). The sequences of the PCR primers for the subcloning of *purK* were for the sense strand 5′-GTTCGAATTCATATGAAACAGGTTGCGTC-3′ and for the antisense strand 5′-GTTGGCGGATCCTGCAGTTAACCGAACTTA-3′. PCR primers for the subcloning of *purE* were 5′-GTTCGAATTCATATGTCTTCCCGCAATAAT-3′ (sense strand) and 5′-GTTGGCGGATCCTCATGCCGCACCTCGCGG-3′ (antisense strand). The Ampliwax PCR Gem-mediated Hot Start PCR protocol (Perkin Elmer) was followed as prescribed by the manufacturer using 2.5 units of *Pfu* DNA polymerase (Stratagene) for each reaction. Template DNA was varied from 1 to 50 ng with 100 pmol of the appropriate primers and 20 nmol of each dNTP in a total volume of 100 μL. The PCR products were purified by agarose gel electrophoresis and extracted using the Gene Clean protocol (Bio 101) prior to digestion with *Nde*I and *BamH*I restiction enzymes. The purified large fragment from *Nde*I- and *BamH*I-digested pDL-Nde was then ligated with the PCR products. Functional properties of the coding DNA in these constructs were verified by DNA sequencing and functional complementation of the *E. coli* strains TX209 and TX257.

Complementation and Growth of E. coli TX209. VB media was used in all the growth rate studies for *E. coli* TX209 (Vogel & Bonner, 1955). *E. coli* TX209 transformed with each of the plasmids pSP-*purE*, pSP-*purK*, or pD3-*pur* were selected on LB plates with ampicillin (50 μg mL^{-1}). For complementation tests, single colonies were transferred to VB plates supplemented with 0.1% casamino acids (VBC). Stationary-phase cultures (3 mL) were grown in VBC with 50 μg mL^{-1} ampicillin and used to inoculate 25 mL of fresh VBC to an initial $OD_{550} = 0.04$. Growth of triplicate colonies (with the exception of two for pSP-*purE*) was monitored by absorbance at 550 nm. A portion of the stationary cultures (4 mL) was harvested by centrifugation. After resuspension in 50 mM Tris-HCl and 1 mM EDTA, pH 8.0, the cells were lysed by ultrasonication, and cell debris was removed by centrifugation. These cell-free protein extracts were assayed for enzymatic activities following previously described methods (Meyer et al., 1992; Firestine & Davisson, 1994).

Growth in the Presence of Exogenous $KHCO_3$. Stationary cultures of TX209/pSP-*purE* or TX209/pBluescript were prepared from single colonies in LB media containing 100 μg mL^{-1} ampicillin. Cells from a 1 mL sample of each culture were collected by centrifugation and resuspended in VBC buffer (500 μL) to provide a seed culture. Fresh VBC (3 mL) with ampicillin (100 μg mL^{-1}) and 200 mM $KHCO_3$ in 7 mm by 100 mm glass tubes was inoculated with the appropriate amount of seed culture to obtain an initial $OD_{550} = 0.02$. Each tube was stoppered, sealed with Parafilm, and kept at 37 °C with shaking for up to 12 h. The OD_{550} measurements were made at the appropriate time points by direct reading of the stoppered culture tubes.

RESULTS

Nucleotide Substrate for G. gallus AIR Carboxylase. The recent observation that N^5-CAIR is the substrate for *E. coli* PurE (Mueller et al., 1994) raised questions regarding the reaction catalyzed by the *G. gallus* AIR carboxylase. Under the assay conditions for *G. gallus* AIR carboxylase, N^5-CAIR has been shown to exist in equilibrium with AIR, leading to ambiguity regarding the substrate in the previously described assay (Firestine & Davisson, 1994). The first approach used to identify the nucleotide substrate(s) for *G. gallus* AIR carboxylase explored the possible requirement for protein–protein interactions. The existence of a *G. gallus* PurK was initially considered since it could have eluded isolation in the original cDNA screening strategies. The utilization of N^5-CAIR by the *G. gallus* AIR carboxylase was tested in an assay with *E. coli* PurK present to generate the nucleotide *in situ* from AIR, ATP, and HCO_3^-. Addition of PurK to the *G. gallus* AIR carboxylase in the presence of nonsaturating concentrations of HCO_3^- (4 mM) resulted in no increase in specific activity over that of the *G. gallus* enzyme alone (data not shown). The rate of N^5-CAIR production in these assays is 37-fold greater than the rate of the enzymatic SAICAR production that was observed.[2] Although the analysis does not rule out possible PurK dependent protein–protein interactions, the effect of *in situ* generation of N^5-CAIR on *G. gallus* AIR carboxylase turnover was negligible.

The second approach was a direct comparison of N^5-CAIR as a substrate for *E. coli* PurE and the *G. gallus* AIR carboxylase. Since the chemical stability of N^5-CAIR is limited under the previous experimental conditions, the rate of CAIR production was measured at 10 °C under conditions

[2] This amount of PurK activity is based upon the ATPase assay for PurK (Meyer et al., 1992). The half-life of N^5-CAIR under these conditions ($t_{1/2} \approx 1$ min) should be suitable to have achieved >50 μM concentrations of this intermediate. The enzyme concentrations were chosen to have up to a 20-fold excess of the PurK enzyme.

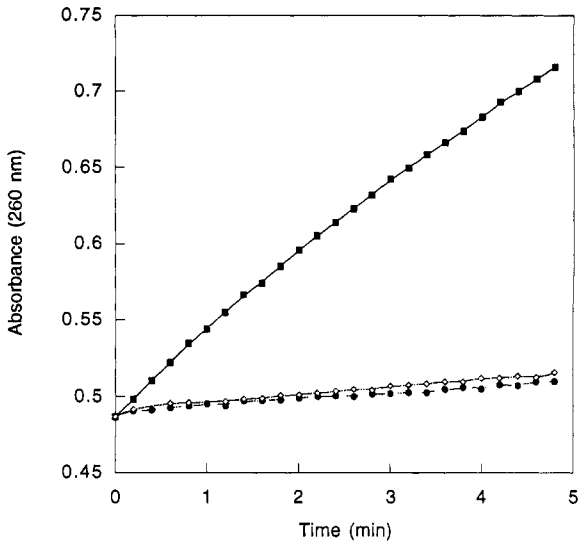

FIGURE 1: Production of CAIR at 10 °C by the addition of 0.5 mM N^5-CAIR to (■) 0.2 μg (0.007 unit) of *E. coli* PurE; (◊) 0.7 μg (0.2 unit) of *G. gallus* AIR carboxylase; (●) no protein present.

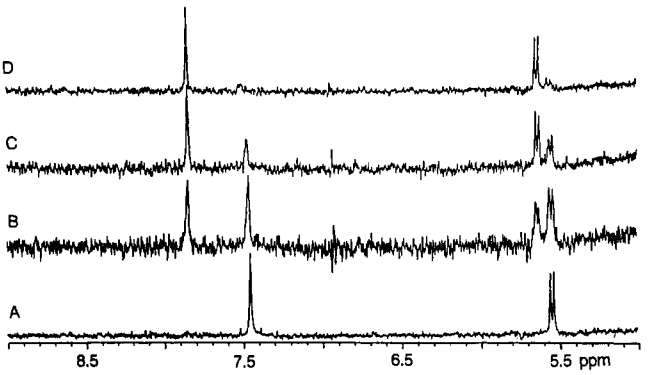

FIGURE 2: A portion of the ^{1}H NMR spectra for the *G. gallus* AIR carboxylase reaction with CAIR. The resonance at ≈5.5 ppm is the H1′, and the aromatic resonances from 7.8 to 7.5 ppm are for H2. (A) CAIR at t = 0; (B) t = 1 min after addition of 0.6 unit of *G. gallus* AIR carboxylase; (C) t = 2 min after enzyme addition; (D) t = 4 min after enzyme addition. In previous experiments with the *E. coli* PurE, the H2 resonance for N^5-CAIR was observed at a chemcial shift of Δ0.2 ppm than that for AIR. The H1′ resonsance for N^5-CAIR is observed at an intermediate value between those for AIR and CAIR (Mueller et al., 1994).

with increased enzyme concentrations. In a previous controlled study, a 10-fold difference in the half-life of N^5-CAIR was observed between 30 and 10 °C. Final addition of the correct nucleotide substrate is expected to provide an immediate formation of CAIR. Shown in Figure 1 are data comparing the *E. coli* PurE and *G. gallus* AIR carboxylase enzymes. An immediate production of CAIR is observed upon addition of the *E. coli* PurE, confirming that N^5-CAIR is a substrate. In the case of the *G. gallus* AIR carboxylase, the increase in absorbance could be accounted for by spontaneous decarboxylation. No CAIR formation was observed even with catalytic units 3-fold greater than were used with the *E. coli* PurE. In total, these results indicate that the *G. gallus* AIR carboxylase and the *E. coli* PurE require different nucleotide substrates but produce the same nucleotide product. Since these substrates are structurally unique, these two enzymes must have different catalytic activities.

Product from Reaction of G. gallus AIR Carboxylase with CAIR. The enzyme-catalyzed decarboxylation of CAIR was monitored by ^{1}H NMR to determine the structure of the nucleotide substrate for the *G. gallus* AIR carboxylase. A similar experimental approach was employed to detect the formation of N^5-CAIR from CAIR by the *E. coli* PurE (Mueller et al., 1994). The *G. gallus* enzyme was found to catalyze the conversion of CAIR to AIR. Figure 2 displays the ^{1}H resonances for the H1′ and H2 of AIR and CAIR formed upon addition of 0.6 unit of *G. gallus* AIR carboxylase. The half-life of N^5-CAIR under the conditions of this reaction was previously determined to be ~2 min. If N^5-CAIR was released by *G. gallus* AIR carboxylase, the concentrations should be sufficient to observe the appropriate ^{1}H resonances at chemical shift values greater than AIR over the time course of the experiment (Mueller et al., 1994). A reaction in the presence of 5.6 units of *G. gallus* AIR carboxylase showed complete conversion of CAIR to AIR within 70 s, the first time point, without any evidence of N^5-CAIR formation (data not shown). Control experiments revealed that *G. gallus* AIR carboxylase does not catalyze the decomposition of N^5-CAIR. These findings indicate that the product of *G. gallus* AIR carboxylase-catalyzed decarboxylation of CAIR is in fact AIR.

One-Carbon Substrate: CO_2 vs HCO_3^-. The one-carbon unit is of key importance in understanding the reaction mechanism and physiological role of any carboxylase in primary metabolism. A major feature which distinguishes carboxylases is whether CO_2 or HCO_3^- is the actual substrate (O'Leary, 1992). Information regarding the one-carbon substrate for *G. gallus* AIR carboxylase takes on added importance since the enzyme is not dependent upon a cofactor and appears to catalyze a chemical reaction distinct from the HCO_3^--dependent *E. coli* PurE or PurK enzymes.

Using a method modified from Cooper (Cooper et al., 1968), the one-carbon substrates for the *G. gallus* AIR carboxylase and the *E. coli* PurE were compared. This method relies on the reduced rate of the approach to equilibrium for CO_2 and HCO_3^- at 10 °C ($t_{1/2}$ = 1.5 min). Under conditions of appropriate enzyme concentrations, reaction initiation with the preferred one-carbon substrate (CO_2 or HCO_3^-) is expected to result in immediate CAIR formation, while the other would only form product as the equilibrium is achieved. Figure 3A shows the results with the *G. gallus* AIR carboxylase in the presence of AIR and CO_2 or HCO_3^-. Rapid CAIR formation was observed upon addition of the CO_2 solution. Using an excess of carbonic anhydrase in these reactions rapidly establishes the CO_2/HCO_3^- equilibrium and, as shown in Figure 3A, reduces the rate of carboxylation from CO_2 addition. In the same way, inclusion of carbonic anhydrase increases the rate of carboxylation when HCO_3^- is used as the substrate.[3] Figure 3B displays the carboxylation reaction progress curves catalyzed by *G. gallus* AIR carboxylase at varied amounts of added CO_2 and demonstrates the dependence of the rate and the percent conversion of AIR upon the initial concentration of CO_2.

Figure 4 compares the CO_2 or HCO_3^- dependence of the *G. gallus* AIR carboxylase and the *E. coli* PurE. As described above, at 10 °C addition of CO_2 to the *G. gallus* AIR carboxylase and AIR mixture led to immediate production of CAIR. For *E. coli* PurE, a decrease in absorbance followed by a much slower increase was observed upon addition of CO_2. The apparent decrease in absorbance is attributed to the rapid formation of N^5-CAIR which is reported to have a reduced extinction coefficient at 260 nm (Mueller et al., 1994), and the subsequent absorbance increase is attributed to CAIR

[3] The fact that parallel rates are observed in the presence of carbonic anhydrase confirms the concentration estimates of the stock CO_2 and $KHCO_3$ solutions used in these experiments.

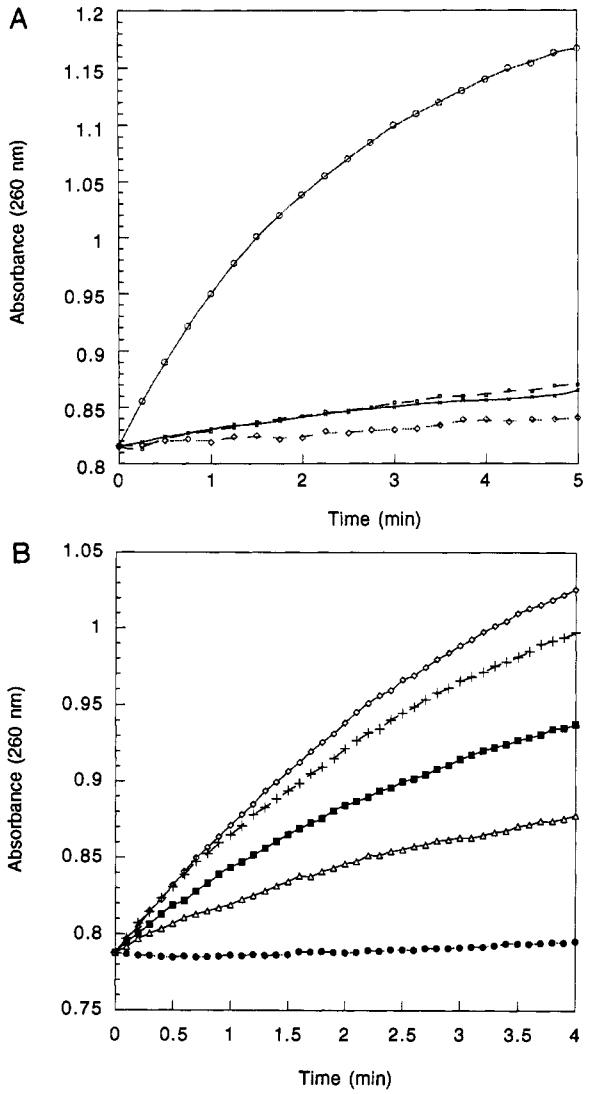

FIGURE 3: Comparison of $KHCO_3$ and CO_2 as substrates for *G. gallus* AIR carboxylase in the presence of AIR at 10 °C. (A) (○) *G. gallus* AIR carboxylase and 20 mM CO_2; (◇) *G. gallus* AIR carboxylase and 20 mM HCO_3^-; (×) *G. gallus* AIR carboxylase and 20 mM HCO_3^- in the presence of carbonic anhydrase; (□) *G. gallus* AIR carboxylase and 20 mM CO_2 in the presence of carbonic anhydrase. (B) The dependence of initial rate and extent of CAIR formation by *G. gallus* AIR carboxylase as a function of added CO_2. (●) 20 mM $KHCO_3$; (▲) 5 mM CO_2; (■) 10 mM CO_2; (+) 20 mM CO_2; (◇) 30 mM CO_2.

formation. No significant CAIR formation is observed upon addition of HCO_3^- to the *E. coli* PurE, showing that this enzyme does not use either one-carbon substrate.

Inhibition by NAIR. Another test to differentiate *E. coli* PurE and *G. gallus* AIR carboxylase enzymes involved the use of the nucleotide 4-nitro-5-aminoimidazole ribonucleotide (NAIR). This compound has been shown to behave as a tight-binding inhibitor of the *G. gallus* AIR carboxylase with K_i = 0.34 nM (Firestine & Davisson, 1993). Because of the structural relationship of NAIR to CAIR, the enzyme inhibitory properties of this material led us to postulate that the compound is a transition-state analog for the final step in the carboxylation reaction catalyzed by the *G. gallus* AIR carboxylase. Since the reactions for the *G. gallus* AIR carboxylase and *E. coli* PurE are related by their products, a comparative study of the inhibitory properties of NAIR with PurE was conducted. In contrast to the time-dependent inhibition of the *G. gallus* enzyme, NAIR behaved as a steady-state inhibitor (K_i = 0.5 μM) of PurE in the standard

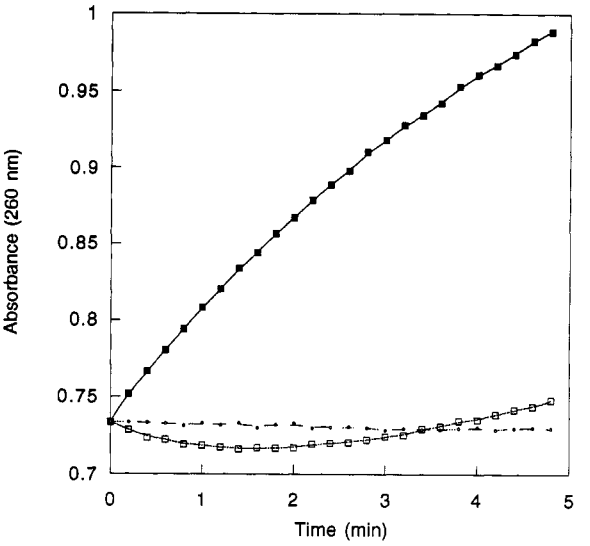

FIGURE 4: Direct comparison of *G. gallus* AIR carboxylase and *E. coli* PurE in $KHCO_3$- or CO_2-dependent carboxylase reactions at 10 °C. (■) *G. gallus* AIR carboxylase and 20 mM CO_2; (●) *E. coli* PurE and 20 mM $KHCO_3$; (□) *E. coli* PurE and 20 mM CO_2.

decarboxylase assay. The ratio of K_i to enzyme concentration is considered for comparison; for the *G. gallus* system, this ratio is 0.7 while in the *E. coli* PurE it is 104. For the single-substrate reaction, a simple comparison of K_i values can be used to relate the dissociation constants of NAIR from each protein. This 300-fold difference in binding properties with NAIR for the *G. gallus* and *E. coli* enzymes suggests that the major recognition features are unique for each protein despite their significant degree of amino acid sequence identity (26%). Furthermore, the transition-state mimetic property proposed for NAIR is clearly not as important with *E. coli* PurE.

Complementation of purK and purE Auxotrophs. A series of DNA plasmid constructs were prepared to evaluate the potential for PurE to complement a PurK deficiency in *E. coli* TX209. Our approach involved an *in vivo* system that allows direct *in vivo* comparison of the chemistry catalyzed by the *E. coli* PurE, PurK, and *G. gallus* PurCE proteins. The nature of the mutation in *E. coli* TX209 was previously characterized and found to be a nonsense codon within the coding region of PurK (Meyer et al., 1992), which suggests that *purE* expression in the host cell is not affected. Genes encoding the three proteins were cloned into a constitutive vector with the identical context of the start codons to confer comparable levels of expression in TX209. Stationary cultures of transformed TX209 were grown on defined media lacking purines and analyzed for enzyme activity. The data in Table 1 indicate that CAIR decarboxylase activities were overproduced to similar levels for both the *E. coli* and *G. gallus* enzymes. On the basis of the specific activities for the purified proteins, all three enzymes are present at >100-fold over the background levels in TX209.

The effect of overproduction of either PurK, PurE, and *G. gallus* PurCE on the growth rates of *E. coli* TX209 on media lacking purines was assessed. As shown in Figure 5, the growth characteristics of the host are dependent upon the plasmid construct. The differences in the initial growth phases for cells harboring plasmids encoding PurK and *G. gallus* PurCE are significant, although similar doubling times of 50 min were observed in the logarithmic phase. These data are consistent with earlier results in which a lacZ protein fusion with the *G. gallus* purCE was able to confer a pur$^+$ phenotype to this strain (Chen et al., 1990). Despite the lack of any amino acid sequence homology or enzyme activity associated

Table 1: AIR Carboxylase Activity in Transformed *E. coli* TX209[a]

plasmid	$KHCO_3$	protein (mg/mL)	sp act.[b] (units/mg)	total units
pSP-*purE*	+	0.134	2.1	0.28
pSP-*purE*[c]	–	0.159	4.5	0.72
pD3-*pur*	–	0.159	1.4	0.22
pBluescript[d]	+	0.138	–	–
pSP-*purK*[e]	–	0.155	1.3	0.20

[a] Saturated cultures were harvested at the end of the growth curves in Figure 5 and are averages of four assays. [b] CAIR decarboxylase activity. [c] Harvested from air-equilibrated cultures at $OD_{550} = 0.5$ after 96 h. The growth is attributed to the PurE overproduction which is greater than the previously described low copy expression system (Mueller et al., 1994). [d] A rate could not be detected that was above that contributed by spontaneous decarboxylation of CAIR. [e] Assay is the AIR-dependent ATPase activity (Meyer et al., 1992).

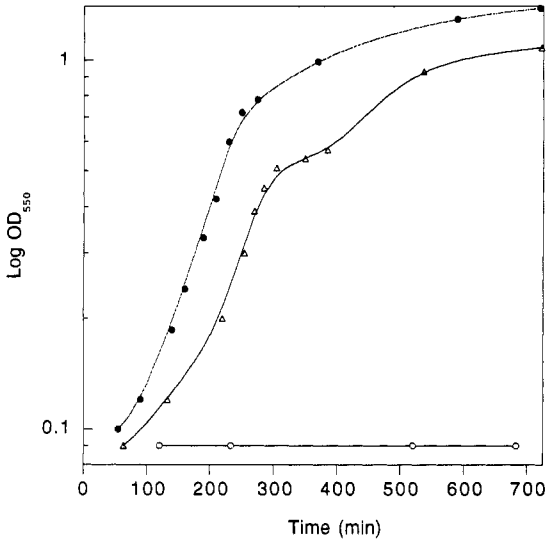

FIGURE 5: Growth data for transformed *E. coli* TX209 (PurK$^-$) strains in air-equilibrated flasks at 37 °C: (●) pSP-*purK*/TX209; (△) pD3-*pur*/TX209; (○) pSP-*purE*/TX209. Each curve was performed in triplicate.

with PurK, heterologous expression of the *G. gallus purCE* is sufficient to compensate at least partially for the PurK deficiency. In light of the *in vitro* data for the *G. gallus* AIR carboxylase, these growth characteristics indicate that sufficient CO_2 concentrations are present to allow for the direct carboxylation of AIR by the heterologous enzyme.

In striking contrast are the growth characteristics of the cells harboring the plasmid pSP-*purE*. Overproduction of PurE under these conditions was not sufficient to compensate for the lack of PurK under ambient air conditions (Figure 5). However, very slow growth of pSP-*purE*/TX209 was measured after 3 days, and these cultures clearly had overproduced PurE encoded by the plasmid as detected by enzyme assay (Table 1). Growth of nontransformed TX209 was not observed under these conditions.

An explanation for these *in vivo* results became apparent with the knowledge of the enzymatic function of PurK. PurK catalyzes the formation of N^5-CAIR from AIR, ATP, and HCO_3^-, which in turn is a substrate for PurE. Formation of N^5-CAIR from AIR and CO_2 also occurs nonenzymatically and led to the hypothesis that PurK deficiency in *E. coli* results in a rate-limiting chemical synthesis of N^5-CAIR. To test the *in vivo* effects of PurE overproduction, the growth characteristics of the transformed *E. coli* TX209 were assessed in the presence and absence of 200 mM $KHCO_3$. In order to maintain elevated concentrations of CO_2/HCO_3^- in the culture media, stoppered culture tubes were used for these studies.

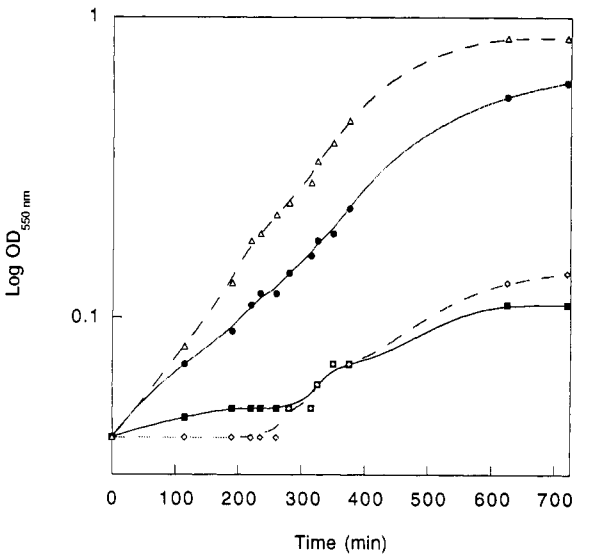

FIGURE 6: Growth data for transformed *E. coli* TX209 (PurK$^-$) strains in stoppered culture tubes at 37 °C: (△) pSP-*purE* with $KHCO_3$; (●) pBluescript/TX209 with $KHCO_3$; (◇) pSP-*purE* without $KHCO_3$; (■) pBluescript/TX209 without $KHCO_3$. Each curve was performed in duplicate.

The results in Figure 6 show that these modifications in growth conditions stimulate the growth of pBluescript/TX209, although the plasmid does not harbor any purine gene, and pSP-*purE*/TX209. The fact that these strains can grow slowly in stoppered flasks is consistent with the solid media complementation experiments of Gots (Gots et al., 1977) with *E. coli* TX209. Inclusion of added $KHCO_3$ enhances the growth rates of pBluescript/TX209 and pSP-*purE*/TX209 by 5- and 8-fold, respectively. These effects are attributed in both cases to increased intracellular concentrations of CO_2/HCO_3^-. Furthermore, the effect of PurE overproduction is now observed since pSP-*purE*/TX209 displayed doubling times >2-fold over those cells without plasmid-encoded PurE (pBluescript/TX209).

These data in Figures 5 and 6 show that HCO_3^- alone can complement the PurK deficiency in *E. coli* TX209. The interpretation of this effect is that the elevated CO_2 concentrations shift the *in vivo* equilibrium of AIR to N^5-CAIR. The elevated levels of N^5-CAIR result in PurE-catalyzed turnover to CAIR. When additional plasmid-encoded copies of PurE are present from pSP-*purE*, the efficiency of this process is increased. The combined effects are manifested in the enhanced growth rate of the organism. These data support the hypothesis that *E. coli* TX209 growth is limited by the chemical synthesis of N^5-CAIR and that it cannot be complemented by overexpression of *purE* in an ambient CO_2 environment. These results further exemplify the differences *in vivo* of the *E. coli* PurE and *G. gallus* PurCE.

DISCUSSION

Metabolic Impact of PurK Deficiency. The characterization of genes associated with AIR carboxylases from a variety of sources reveals regions of amino acid sequence homology and implicate functional significance by correlation with the bacterial PurK or PurE proteins. However, complementaion of *E. coli* TX209 by a seemingly unrelated *G. gallus* gene led us and others to question how this enzyme functions (Hamilton & Reeve, 1985; Chen et al., 1990). One explanation for this paradox was that overproduction of PurCE leads to complementation of the PurK deficiency. The fact that N^5-CAIR is formed in a nonenzymatic reaction from CO_2/HCO_3^- and

AIR supports this hypothesis, and elevated levels of PurE should result in conversion to CAIR. Growth of TX209 in a CO_2-enriched environment had previously been observed (Gots et al., 1977) and motivated the quantification of bacterial growth in liquid cultures under elevated CO_2 conditions. Acceleration of TX209 growth in a CO_2 enriched environment provides elevated concentrations of N^5-CAIR since a genomic copy of PurE was present. Consistent with this observation is the additional increase in growth rate under conditions of elevated CO_2 concentrations upon overproduction of PurE. Despite the short *in vitro* half-life of N^5-CAIR, it must be available to PurE *in vivo* regardless of the PurK status. Therefore, a role for PurK is to provide a growth advantage to the organism by accelerating purine metabolism using ATP and HCO_3^- to produce N^5-CAIR. A similar functional property is expected for the fungal and yeast proteins which appear as fusions of PurK and PurE; however, their catalytic properties are not fully described at this time. Preliminary data with the *ADE 2* gene from *Cryptococcus neoformans* indicate that this protein complements both PurK and PurE deficiencies (Klem and Davisson, unpublished results).

Comparison of Catalytic Properties. In contrast to the *E. coli* PurE, the *G. gallus* PurCE does not require elevated levels of CO_2 to complement the PurK deficiency, which suggested to us a catalytic property unique for the *E. coli* PurE. Since *de novo* purine biosynthesis is central to all life forms except protozoa, the general themes for enzyme catalysis are expected to be similar. A 26% amino acid sequence identity exists between the *E. coli* PurE and the related C-terminal domain in the *G. gallus* PurCE. Despite this relationship, several lines of experimental evidence show that the purine pathways in vertebrates and bacteria differ at this point in metabolic intermediates. A comparative study with *E. coli* PurE and the *G. gallus* AIR carboxylase shows that the products from enzymatic decarboxylation of CAIR are different for each protein (Mueller et al., 1994). Direct comparison of *E. coli* PurE and *G. gallus* PurCE in N^5-CAIR-dependent experiments reveals that this material is not a substrate for the *G. gallus* AIR carboxylase enzyme. Further *in vitro* comparisons have identified AIR and CO_2 as substrates for the *G. gallus* enzyme. These differences are consistent with the *in vivo* results and explain the ability of the *G. gallus* enzyme to complement the PurK deficiency, since a surrogate pathway from AIR to CAIR is introduced through heterologous expression of *G. gallus* PurCE.

N^5-CAIR vs AIR. The species differences in *de novo* purine biosynthesis are exemplified by the fact that eubacteria and vertebrates have evolved alternative carboxylation strategies for CO_2 incorporation. Both systems have evolved mechanisms that are suited to their cellular CO_2 environments. The *in vivo* concentrations of HCO_3^-/CO_2 are difficult to mimic *in vitro,* which places increased significance on comparative studies of these reactions. AIR carboxylation presents a particularly challenging problem since the equilibria are not favored for either carboxylation at nitrogen to form N^5-CAIR, or at carbon, to form CAIR. Enzymes in vertebrates that use CO_2 without a cofactor or prior metabolic activation of the nucleophilic substrate have not been described until characterization of the *G. gallus* AIR carboxylase (Firestine & Davisson, 1994). This feature has interesting implications for the binding of CO_2 by the vertebrate AIR carboxylases. The steady-state kinetic data for the *G. gallus* enzyme implicate a Michaelis complex with a K_m = 0.8 mM. Alternatively, eubacteria make use of HCO_3^- through metabolic activation events that resemble the biochemical strategies for biotin-dependent carboxylations and require the coexpression of a second gene (O'Leary, 1992; Mueller et al., 1994).

Divergent Catalysts? An obvious evolutionary relationship exists among the vertebrate AIR carboxylases and the bacterial PurE. These proteins serve similar functions in *de novo* purine biosynthesis, yet they are unique at the level of substrate recognition and their catalytic properties. Eubacteria have evolved a mechanism for efficient utilization of HCO_3^- at the expense of ATP to provide the unstable carboxyl donor N^5-CAIR for the PurE reaction. In contrast, the vertebrate enzyme uses AIR and CO_2 without cleavage of a high-energy phosphate bond or the use of a cofactor. These differences dictate alternate chemical mechanisms at least in the initial substrate binding and/or CO_2 activation stages of the reactions. Furthermore, the studies with the tight-binding inhibitor NAIR implicate differences in the last step of the chemical reaction involving either carbon–carbon bond formation or carbon–hydrogen bond cleavage. Alternatively, the mechanisms for binding of AIR/CAIR and CO_2 could contribute to the differences observed with NAIR between the two enzyme systems. Regardless of the final mechanistic paths, the evidence suggests that these two enzymes are evolutionarily related and divergent at the level of their catalytic chemistry. While primary metabolic enzymes are not expected to be identical, such striking examples of divergent chemistry are rarely revealed in other primary biosynthetic pathways. The differences between AIR carboxylases at the amino acid level and how they influence their catalytic properties are subjects for future consideration.

CONCLUSION

The comparison of the related AIR carboxylases from *E. coli* and *G. gallus* show that two unique catalytic strategies exist for CO_2 incorporation in *de novo* purine biosynthesis. PurK in eubacteria accelerates the growth of the organism by coupling ATP hydrolysis to HCO_3^- activation and subsequent *N*-carboxylation of AIR, forming N^5-CAIR. The *C*-carboxylated nucleotide CAIR is formed in a subsequent PurE- and N^5-CAIR-dependent reaction. A PurK function is not present in vertebrates since the AIR carboxylase forms CAIR directly from AIR and CO_2. This catalytic property distinguishes the vertebrate enzyme from the bacterial PurE despite the significant degree of amino acid sequence identity and their related roles in purine metabolism.

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