Purification and Characterization of the *pur*E, *pur*K, and *pur*C Gene Products: Identification of a Previously Unrecognized Energy Requirement in the Purine Biosynthetic Pathway†

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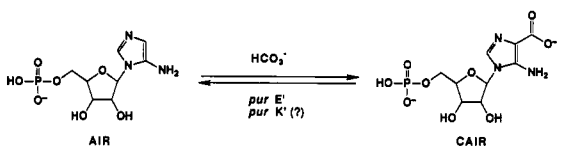
Received February 6, 1992; Revised Manuscript Received March 19, 1992

ABSTRACT: Aminoimidazole riobnucleotide carboxylase, the sixth step in the purine biosynthetic pathway, catalyzes the conversion of aminoimidazole ribonucleotide (AIR) to carboxyaminoimidazole ribonucleotide (CAIR). The gene products of the *pur*E and *pur*K genes (PurE and PurK, respectively) thought to be responsible for this activity have been overexpressed and the proteins purified to homogeneity. PurE separates from PurK in the first ammonium sulfate fractionation during the purification. No evidence for association of the two gene products under a variety of conditions using a variety of methods could be obtained. To facilitate the assay for CAIR production, the *pur*C gene product, 5-aminoimidazole-4-*N*-succinylcarboxamide ribonucleotide (SAICAR) synthetase has also been overexpressed and purified to homogeneity. The activities of PurE, PurK, and PurE·PurK have been investigated. PurE alone is capable of catalyzing the conversion of AIR to CAIR 1 million times faster than the nonenzymatic rate. The K_m for HCO_3^- in the PurE-dependent reaction is 110 mM! PurK possesses an ATPase activity that is dependent on the presence of AIR. No bicarbonate dependence on this reaction could be demonstrated (<100 μM), and AIR is *not* carboxylated during the hydrolysis of ATP. Incubation of a 1:1 mixture of PurE and PurK at low concentrations of bicarbonate (<100 μM) revealed that CAIR is produced but requires the stoichiometric conversion of ATP to ADP and P_i. No dependence on the concentration of HCO_3^- could be demonstrated. A new energy requirement in the purine biosynthetic pathway has been established.

In 1977 Gots et al. proposed, on the basis of genetic analysis, that the *pur*E and *pur*K gene products were responsible for the aminoimidazole ribonucleotide (AIR)[1] carboxylase activity, the sixth step in the de novo biosynthesis of purines (eq 1).



(1)

On the basis of their observations that a putative point mutation in the *pur*K gene resulted in an *Escherichia coli* strain that had an absolute requirement for exogenous purines for growth, but that this auxotrophy for purines could be overcome by high concentrations of CO_2, they proposed that the function of the *pur*K gene product was to provide a binding pocket for the $CO_2(HCO_3^-)$ required for the carboxylation of AIR. We were intrigued, although somewhat skeptical, that the sole function of the 39-kDa protein, the *pur*K gene product (designed PurK) (Tiedman et al., 1989; Watanabe et al., 1989), would be that of a CO_2 carrier. We have therefore overexpressed the *pur*E and *pur*K gene products, purified them to homogeneity, and examined the AIR carboxylase activity in detail. The *pur*C gene product, 5-aminoimidazole-4-*N*-succinylcarboxamide ribonucleotide (SAICAR) synthetase, has also been overexpressed and purified to homogeneity to facilitate the assay for AIR carboxylase activity. In addition, the gene of the putative *pur*K mutant, responsible for the proposal of Gots et al. describing its biochemical function, has been isolated and sequenced. The results reported in the present paper indicate that PurE (the gene product of *pur*E) alone can catalyze the carboxylation of AIR to CAIR (eq 1) in the presence of very high concentrations of HCO_3^-. Carboxylation of AIR to CAIR can also occur nonenzymatically under similar conditions, albeit at a greatly reduced rate. However, if PurE is incubated with PurK, the concentration of HCO_3^- required for AIR-dependent carboxylation is reduced greater than 1000-fold. In addition, this carboxylation now requires the stoichiometric consumption of ATP. Thus, under physiological conditions, ATP must be added as a second substrate for the AIR carboxylation reaction to occur in *E. coli*.

Materials and Methods

Materials

Sephadex G-25, Sephadex G-150, DEAE-Sepharose CL-6B, ampicillin, low molecular weight standards, aminoimidazolecarboxamide ribonucleoside, pyruvate kinase (specific activity 500 units mg^{-1}), lactate dehydrogenase (specific activity 890 units mg^{-1}), malic dehydrogenase (specific activity 3600 units mg^{-1}), glutamic-oxaloacetic transaminase (specific activity 350 units mg^{-1}), and PEP carboxylase (specific activity 3 units mg^{-1}) were obtained from Sigma Chemical Co. Bluescript plasmid M13SK+ was obtained from Stratagene Inc., San Diego. Immobilon P was purchased from Millipore. $POCl_3$

† This work was supported by grant GM 32191 (J. Stubbe) and AI 20068 (J. Smith) from the National Institutes of Health.

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[1] Abbreviations: AIR, aminoimidazole ribonucleotide; SAICAR, 5-aminoimidazole-4-*N*-succinylcarboxamide ribonucleotide; PMSF, phenylmethanesulfonyl fluoride; CAIR, carboxyaminoimidazole ribonucleotide; the corresponding ribonucleoside AIRs, CAIRs, SAICARs.

0006-2960/92/0431-5022$03.00/0

and triethylphosphate were obtained from Aldrich Chemical Co. AffiGel Blue, Dowex 50W-X8, and hydroxylapatite were obtained from Bio-Rad. [^{3}H and ^{14}C]aspartate (specific activity 22.8 and 0.22 Ci/mmol, respectively) were obtained from New England Nuclear. DEAE-A25 Sephadex was obtained from Pharmacia.

Methods

Phosphate was quantitated by the method of Ames and Dubin (1960). Concentrations of proteins were determined by the method of Lowry et al. (1957) using BSA as a standard. The concentration of AIR was determined by the method of Bratton and Marshall (1939) (Schrimsher et al., 1986) and a coupled assay using SAICAR synthetase and NADH consumption (described below). The concentration of aspartate was determined by using the method of Yagi et al. (1985). The concentration of HCO_3^- was determined by using PEP carboxylase (Peled, 1983). One unit of enzyme is the amount of protein required to produce 1 μmol of product min^{-1} at 37 °C.

Preparation of AIR Carboxylase Expression Vector. A λpL expression vector similar to the ones employed previously was constructed for the expression of the *pur*EK genes (Ingelese et al., 1990; Schendel et al., 1989; Cheng et al., 1990). Chromosomal DNA containing the *pur*EK operon was recovered from strain W3110 (Bachmann, 1972) by the PCR protocol described by Saiki et al. (1988). The two PCR primers were designed to introduce new restriction sites to aid in subcloning, subsequent to the recovery of the intact *pur*EK operon. The 3′ primer, 5′-ATAGAGCTGCAGTTAACCGAACTT-3′, introduced a *Pst*I restriction site while the 5′-primer, 5′-TCCGTGGAATTCTCTGTGCCCTCT-3′, introduced an *Eco*RI site. The PCR-generated fragment containing the *pur*EK operon was initially cloned into the *Eco*RI–*Pst*I sites of the Bluescript plasmid, M13SK$^+$, and sequenced. Two differences in the sequence as previously noted were observed (Tiedeman et al., 1989; Watanabe et al., 1989). One difference is the silent mutation ATC to ATT in the Ile 11 codon of AIR carboxylase (*pur*E), while the other was a change in the 5′ untranslated region preceding the *pur*E coding region (AAG(A to T)CGCATG). Neither mutation should have any effect on the primary structure of the *pur*E and *pur*K protein products. We attribute these sequence differences to strain background as the original DNA sequences were determined from clones isolated from different *E. coli* strains (Tiedeman et al., 1989; Watanabe et al., 1989). This DNA fragment was then ligated into the λpL expression vector, pJS338 (Tiedeman and Smith, manuscript in preparation) via the *Eco*RI and *Pst*I restriction sites and transformed in the host strain TX635. A representative plasmid was designated pJS355 and used for the purified of the PurE and PurK proteins.

Purification of purE Gene Product: AIR Carboxylase. E. coli TX635 containing the heat-inducible plasmid pJS355 was grown in medium containing 10 g/L tryptone, 5 g/L yeast extract, 50 μg/mL ampicillin, and 10 g/L NaCl adjusted to pH 7.5. The cells were grown at 30 °C (doubling time 45 min), and when an A_{660nm} of 1.3 was reached, they were heat induced by adding an equal volume of media at 54 °C to raise the temperature to 42 °C. The cells were grown at 42 °C for 30 min and then grown for an additional 5.5 h at 37 °C. The bacteria were harvested to give 2.6 g/L, frozen with liquid nitrogen, and stored at −80 °C.

Isolation of protein was carried out at 4 °C. The cells (9.6 g) were suspended in 5 mL/g 100 mM Tris-HCl (pH 7.8), 30 mM $MgCl_2$, 6 mM β-mercaptoethanol, and 0.1% phenylmethanesulfonyl fluoride (PMSF). The cells were broken in a French press pressure cell at 10000 psi, and the cell debris was removed by centrifugation for 20 min at 10000*g*. DNA was removed by adding a solution of protamine sulfate (1.2%) to the supernatant (4.9 mL) over 30 min to give a final concentration of 0.12%. After being stirred for an additional 20 min, the precipitate was removed by centrifugation. The supernatant was adjusted to 50% saturation by the slow addition of solid ammonium sulfate (0.31 g/mL). After the ammonium sulfate had dissolved (30 min), the solution was stirred for 20 min and then centrifuged at 10000*g* for 20 min. The supernatant was then brought to 75% saturation by addition of solid ammonium sulfate (0.176 g/mL over 30 min). The solution was stirred an additional 20 min and centrifuged at 10000*g* for 20 min. The pellet was dissolved in 10 mL of 100 mM Tris (pH 7.8) and desalted by passing it through a (42 × 2.5 cm) Sephadex G-25 column equilibrated in 100 mM Tris (pH 7.8). The fractions containing protein were pooled and loaded directly onto a DEAE-Sepharose CL-6B column (16.5 × 1.5 cm) equilibrated in 100 mM Tris (pH 7.8). The protein was eluted with a 200 × 200 mL linear gradient from 0 to 250 mM KCl in the same buffer. The fractions (3 mL) containing activity (100 mM KCl, 52–82) were pooled and then exchanged into 50 mM potassium phosphate (pH 7.0) by repeated dilution and amicon concentration prior to loading onto a hydroxylapatite column (7 × 2.5 cm) equilibrated in the same buffer. The column was washed with 60 mL of 50 mM potassium phosphate (pH 7.0) followed by a 150 × 150 mL linear gradient from 50 mM to 500 mM potassium phosphate (pH 7.0), resulting in elution of AIR carboxylase with 300 mM potassium phosphate. The fractions (3 mL) containing AIR carboxylase (55–90) were pooled, and the protein was concentrated to 20 mg/mL using an Amicon filtration apparatus with a YM-30 membrane and stored in aliquots at −20 °C.

Purification of purK Gene Product. The steps through the removal of DNA via protamine sulfate precipitation were identical to those described for isolation of the *pur*E gene product. The *pur*K protein precipitates in the 0–50% ammonium sulfate fraction. The pellet was redissolved in 10 mL of 50 mM potassium phosphate at pH 7.0 and desalted on a Sephadex G-25 column (48 × 2.5 cm) equilibrated in the same buffer. The pooled protein fractions were loaded onto a DEAE-Sepharose CL-6B column (16.5 × 1.5 cm) equilibrated in 50 mM potassium phosphate (pH 7.0). The column was eluted with a 150 × 150 mL linear gradient from 0 to 200 mM KCl. The protein eluted at 30 mM KCl and the appropriate fractions (3 mL) were pooled (45–65) and adjusted to pH 7.8 with 50 mM Tris (pH 7.8). This fraction was then loaded onto a second CL-6B column (8 × 1.5 cm) equilibrated in 50 mM Tris (pH 7.8). A 150 × 150 mL linear gradient from 0 to 150 mM KCl was used to elute the protein at 75 mM KCl. Fractions containing protein of high specific activity were pooled and concentrated with a YM-30 Amicon membrane to 10 mg/mL and stored at −20 °C.

Construction of SAICAR Synthetase Expression Vector. The PCR reaction as described by Saiki et al. (1988) was used to recover chromosomal DNA containing the *pur*C gene (Tiedeman et al., 1990) from strain TX337, a derivative of the wild-type strain W3110 (Bachmann, 1972). The two PCR primers were designed to introduce new restriction sites to aid in subcloning as well as changing the ribosomal binding site. The 3′ primer, 5′-CAAGATGATCTGCAGAAAAATCAG-3′, introduced a *Pst*I restriction site while the 5′ primer, 5′-CGGGATCCCGTCTAGAAGACGAGAGACTTATG-

CAAAAGCAAGCTGAG-3′, introduced a *Bam*III restriction site. The ribosomal binding site was adapted from the *pur*L gene, which has served well for other expression constructs (Schendel et al., 1989). Using DNA from a single colony of strain TX337 as the starting material, 25 PCR cycles were performed using cycle conditions of 94 °C (denaturing), 45 °C (annealing), and 72 °C (elongation). The resultant PCR product was electrophoresed on a 0.7% agarose gel, and the 750-bp DNA fragment was recovered with the Prep-A-Gene kit (Bio-Rad, Richmond, CA).

After recovery, the DNA fragment was treated with T4 DNA polymerase to create blunt ends (Flannigan et al., 1990), digested with *Pst*I, and cloned into the *Eco*RV–*Pst*I sites of the Bluescript vector, M13 KS$^+$ (Stratagene, Inc., San Diego, CA). The resulting ligation mix was used to transform strain XL1-Blue with selection for Amp on LB-agar plates supplemented with ampicillin (100 μg/mL), X-Gal, and IPTG (Messing, 1983). Plasmids from colorless colonies were screened for inserts by the PCR reaction using the 3′ primer, and a *lac*Z sequencing primer, 5′-CAGGAAACAGCTATGACCATG-3′. In order to recognize potential transcription errors by *Taq* polymerase, three independent clones were identified and retained for DNA sequencing. The sequences were determined as previously described (Flannigan et al., 1990; Cheng et al., 1990). After confirmation of the DNA sequence, the modified *pur*C gene was then transferred into the λpL expression vector, pJS338, via the *Bam*HI and *Pst*I restriction sites and transformed into the host strain TX635 as described above for the *pur*EK expression vector. The new plasmid was characterized and is designated pJS408.

Purification of purC Gene Product: SAICAR Synthetase. *E. coli* TX635 containing the heat-inducible plasmid pJS408 was grown as described above for pJS355. The bacteria were harvested to give 1.8 g/L and stored at −80 °C.

Isolation of the protein was carried out at 4 °C. The cells (15.8 g) were suspended in 5 mL/g 50 mM Tris-HCl (pH 8.0), 15 mM $MgCl_2$, 6 mM β-mercaptoethanol, and 0.1% PMSF. The cells were broken using a French press pressure cell at 10000 psi, and the cell debris was removed by centrifugation for 20 min at 10000*g*. A solution of protamine sulfate (3.7%) in the same buffer was added to the supernatant over 30 min to give a final concentration of 0.2%. After being stirred for an additional 20 min, the precipitate was removed by centrifugation. The supernatant (66 mL) was adjusted to 50% saturation with ammonium sulfate by addition of 0.31 g/mL over 30 min. The solution was stirred for an additional 20 min and then centrifuged at 10000*g* for 20 min. The pellet, which contained the SAICAR synthetase activity, was redissolved in 10 mL of 50 mM Tris-HCl (pH 8.0) and 15 mM $MgCl_2$ and desalted on a Sephadex G-25 column (42 × 2.5 cm). The fractions containing protein were diluted to 250 mL with 25 mM Tris-HCl (pH 8.0) and 7.5 mM $MgCl_2$ and loaded onto a DEAE-Sepharose Cl-6B anion-exchange column (13.5 × 1.5 cm) equilibrated in the same buffer. The SAICAR synthetase activity was eluted with a 300-mL linear gradient from 0 to 300 mM KCl. The activity eluted at ≈100 mM KCl, and the appropriate fractions were concentrated to 30 mL using an YM30 membrane. The protein was then diluted to 200 mL with 50 mM Tris-HCl (pH 8.0) and 15 mM $MgCl_2$ and loaded onto an Affi-Gel Blue column (12 × 2.5 cm) equilibrated in the same buffer. The enzyme was eluted with a 600-mL linear gradient from 0 to 300 mM KCl in the same buffer. The protein that eluted in fractions 15–45 (6-mL fractions collected) was pooled and concentrated using an YM30 membrane.

Enzyme Assays: PurE·AIR Carboxylase. The assay solution contained 100 mM Tris-HCl (pH 7.8) and 180 mM $KHCO_3$ adjusted to pH 7.8 in a final volume of 400 μL. The AIR concentration was varied between 60 μM and 1.5 mM. At millimolar concentrations of AIR, 0.2-cm path-length cells were utilized. The assay mixtures were equilibrated at 37 °C prior to initiation of the reaction by the addition of PurE (8.8 nM, 0.063 μg). The reaction was monitored at 260 nm [CAIR (260 nm, ϵ = 10500 M^{-1} cm^{-1}) and AIR (260 nm, ϵ = 1570 M^{-1} cm^{-1} at pH 8.0)].

Reverse Direction for AIR Carboxylase. The assay mixture in a total volume of 400 μL contained degassed 100 mM Tris-HCl (pH 7.8) and 8–100 μM CAIR. The cuvette was stoppered with a septum and incubated at 37 °C prior to initiation of the reaction with AIR carboxylase (0.75 nM, 0.005 μg). The decrease in absorbance at 250 nm as a function of time was monitored (Table III).

Coupled Assay for PurE. The assay solution contained 100 mM Hepes (pH 7.8), 180 mM $KHCO_3$, 0.84 mM AIR, 1.1 mM ATP, 2.0 mM PEP, 0.2 mM NADH, 6.0 mM $MgCl_2$, 10 mM KCl, 3.6 mM aspartate, 1.1 units of PurC, 10 units of pyruvate kinase, and 5 units of lactate dehydrogenase in a final volume of 700 μL. The reaction mixture was incubated for 5 min at 37 °C prior to initiation by addition of PurE (0.045 μg, 3.6 nM). The progress of the reaction was monitored by change in absorbance at 340 nm.

Coupled Assay for PurK. The assay solution contained the following in a final volume of 700 μL: 50 mM Hepes (pH 7.8), 20 mM KCl, 6.0 mM $MgCl_2$, 1.1 mM ATP, 2.0 mM PEP, 0.2 mM NADH, 0.42 mM AIR, 10 units of pyruvate kinase, and 5.0 units of lactate dehydrogenase. The reaction mixture was preincubated at 37 °C prior to initiation by addition of PurK (0.16 μg, 5.8 nM). The reaction was monitored by change in absorbance at 340 nm.

Coupled Assay for PurE·PurK. The assay contained the following in a final volume of 250 μL: 50 mM Hepes (pH 7.8), 20 mM KCl, 1.0 mM ATP, 2.0 mM PEP, 4.5 mM $MgCl_2$, 0.5 mM AIR, 5 units of pyruvate kinase, 1.5 units of SAICAR synthetase, 6.0 mM aspartate (8.5 × 10^5 cpm/μmol), PurE (0.28 μg, 63 nM), and PurK (0.67 μg, 68 nM). The reaction was quenched with 15 μL of 30% TCA, and the precipitated protein was removed by centrifugation. A 55-μL aliquot was removed and placed on a 1-mL column of Dowex 50W-X8 (NH_4^+ form). SAICAR was eluted with 5 mL of 50 mM ammonium formate (pH 3.3), and a 1-mL sample was quantitated using scintillation counting.

Coupled Assay for SAICAR Synthetase. The assay solution contained the following in a final volume of 700 μL: 50 mM Hepes (pH 7.8), 20 mM KCl, 6.0 mM $MgCl_2$, 2.0 mM ATP, 0.2 mM NADH, 2.0 mM PEP, 0.5 mM CAIR, 10 units of pyruvate kinase, 5 units of lactate dehydrogenase, and 6 × 10^{-3} units (0.2 μg, 11 nM) of PurC. The reaction mixture was preincubated at 37 °C and initiated by 10 mM aspartate. The reaction was monitored by change in absorbance at 340 nm.

Radioactive Assay for SAICAR Synthetase. The assay solution contained 50 mM Hepes (pH 7.8), 20 mM KCl, 4.5 mM $MgCl_2$, 1.0 mM ATP, 2.0 mM PEP, 0.5 mM CAIR, 5 units of pyruvate kinase, 0.015 unit of PurC, and 6 mM aspartate (specific activity 1.2 × 10^6 cpm/μmol) in a final volume of 250 μL. Aliquots (45 μL) were withdrawn from the reaction mixture at various times, and the reaction was quenched by the addition of 15 μL of 30% trichloroacetic acid. The protein that precipitated was removed by centrifugation for 1 min using an Eppifuge, and 55 μL was withdrawn and loaded onto a Dowex 50W-X8 (1 cc). The SAICAR was

eluted and quantitated as described above.

Removal of HCO_3^- from Solutions. In an attempt to determine the K_m value for HCO_3^- in the PurK·PurE AIR carboxylase assay and the PurK-dependent ATPase assay, the following protocol was employed. Distilled–deionized H_2O was acidified to pH 2.0 using 6.0 N HCl. The acidic H_2O was then boiled for 30 min and was then cooled to room temperature under N_2. The buffer was prepared using this H_2O under conditions in which titration with base was not required. All of the reagents prepared were sparged with N_2 passed through ascarite (Thomas Scientific Co.) and 50% w/v NaOH overnight prior to use. Only the enzymes were *not* degassed. However, enzyme stock solutions were diluted with degassed buffer solutions directly before use. All of the solutions and cuvettes for assays were sealed with septa and maintained under a positive atmosphere of N_2. All additions and withdrawals from assay mixtures were conducted using Hamilton gas-tight syringes. A lower limit of detection of HCO_3^- of various solutions was obtained using the PEP carboxylase method of Peled (1983).

Determination of the Stoichiometry of SAICAR Synthetase Reaction. A typical assay mixture incubated at 37 °C contained the following in a final volume of 500 μL: 50 mM Hepes (pH 7.8), 20 mM KCl, 0.9 mM ATP, 5.4 mM $MgCl_2$, 0.2 mM NADH, 4.3 mM [^{14}C]aspartate (specific activity, 1.4 × 10^6 cpm/μmol), 0.4 mM CAIR, 1.1 units of PurC, 10 units of pyruvate kinase, and 5 units of lactate dehydrogenase. The amount of ATP consumed was determined spectrophotometrically as a change in absorbance at 340 nm. Samples were removed from the cuvette at fixed times, and the amount of [^{14}C]SAICAR produced was quantitated as described above.

Kinetic Analysis. All kinetic studies were conducted by using coupled assays. The Michaelis constants for AIR, CAIR, and $KHCO_3$, SAICAR, ATP, etc. were determined by varying the concentration of one substrate at saturating levels of the other substrate(s) and fitting the data by the method of Cleland (1979):

$$v = VA/[K_a + A] \quad (2)$$

Native and Subunit Molecular Weight of purE and purK Gene Products. The subunit molecular weight was determined by SDS–polyacrylamide gel electrophoresis (15% acrylamide) performed by the procedure of Laemmli (1970). The molecular weight standards used included bovine serum albumin (66 kDa), egg albumin (45 kDa), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36 kDa), bovine carbonic anhydrase (29 kDa), bovine trypsinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa), and bovine α-lactalbumin (14.2 kDa). The native molecular weight of PurE, PurK, and PurE + PurK was determined by two methods: the first utilized a 83 × 1.5 cm Sephadex G-100 sizing column equilibrated in 50 mM Tris-HCl (pH 7.5). Standards (Stoke's radii, M_r) used to calibrate the column were bovine serum albumin (35.5 Å, 66 kDa), horse heart cytochrome C (17 Å, 12.4 kDa), and yeast alcohol dehydrogenase (46 Å, 150 kDa), and the void volume of the column was determined to be 40 mL using Blue Dextran. The standards (200 μL, 10 mg/mL) were loaded onto the column, and PurE and PurK were loaded individually or in a 1:1 ratio at 0.21 mM. Similar experiments were also carried out in the presence of millimolar ATP.

The second method to establish native molecular weight was sucrose gradient ultracentrifugation (Martin et al., 1961). In polyallomer centrifuge tubes (14 × 89 mm) containing a final volume of 12 mL [100 mM Tris (pH 7.5)], a 5–20% sucrose gradient was generated. The standard curve was generated with proteins of known molecular weight and sedimentation coefficients: catalase (250 kDa, 11.3 S); bovine serum albumin (66 kDa, 4.3 S); lysozyme (17.2 kDa, 2.15 S); and yeast alcohol dehydrogenase (150 kDa, 7.65 S). PurE or a 1:1 mixture of PurE and PurK (0.054 mM) ± 30 mM $MgCl_2$ in 100 mM Tris-HCl (pH 7.5) were loaded into separate tubes. Similar experiments were carried out in the presence of 3 mM $MgCl_2$ and 0.2 mM ATP. The tubes were placed in a Beckman SW-40 rotor and centrifuged for 18 h at 39 000 rpm in a Beckman L7-55 ultracentrifuge. The sedimentation profile was established by removing 150-μL aliquots from the top of the gradient, which was then assayed for protein (Lowry et al., 1957) and for AIR carboxylase activity as described above.

Heat Inactivation of PurK. PurK (0.065 mg) in 50 mM Hepes (pH 7.8) and 20 mM KCl was placed in an Eppendorf tube in a H_2O bath at 55 °C. At various times ranging from 0 to 50 min, 100-μL aliquots were removed and cooled in an ice bath. Each aliquot was then assayed for SAICAR production by the standard procedure and for AIR-dependent ATPase activity.

Equilibrium Constant [CAIR]/[HCO_3^-][AIR]. A 500-μL Eppendorf tube fitted with a rubber septum was filled with 500 μL containing 100 mM Tris-HCl (pH 7.8), variable amounts of $KHCO_3$ (20–180 mM) and either AIR (1.2 mM) or CAIR (1.1 mM). The reaction mixture was incubated at 37 °C prior to addition of the nucleotide. In some cases 0.1 mg of PurE was also included in the incubation mixture. The rate of approach to equilibrium as well as the final equilibrium could be monitored using a reverse-phase HPLC column (Alltech C-18, 10 μm, 25 × 0.46 cm) equilibrated in 80 mM potassium phosphate and 10 mM tetrabutylammonium bromide (pH 7.0). At a flow rate of 1 mL/min, AIR had a retention time of 5 min and CAIR had a retention time of 10 min. Standard curves were prepared using known quantities of AIR and CAIR and an HP integrator. At various times, 10 μL of the reaction mixture was analyzed using this HPLC system.

Rate of Nonenzymatic Carboxylation of AIR. The assay solution of 37 °C contained the following in a final volume of 750 μL: 100 mM Tris (pH 7.8), 180 mM $KHCO_3$, 1.0 mM ATP, 2.0 mM PEP, 6.0 mM $MgCl_2$, 10.0 mM aspartate, 0.2 mM NADH, 10 units of pyruvate kinase, 5 units of lactate dehydrogenase, and 0.3 unit of PurC. To avoid changes in the concentration of bicarbonate, vials were sealed with septa, and the head space in each vial was minimized. The reaction background was determined by monitoring change in absorbance at 340 nm, and the reaction was initiated by addition of AIR ranging from 0.067 to 1.33 mM.

Characterization of SAICAR. SAICAR was prepared for characterization by NMR spectroscopy. The reaction mixture contained a final volume of 1 mL: 2.0 mM ATP, 3.6 mM aspartate, 2.0 mM PEP, 6.0 mM $MgCl_2$, 2 mM NADH, 20 mM KCl, 1.65 mM CAIR, 100 mM Tris-HCl (pH 7.8), 10 units of pyruvate kinase, and 5.0 units of lactate dehydrogenase. After incubation for 1.5 h at 37 °C, the entire sample was loaded onto a semipreparative HPLC reverse-phase Alltech C-18 column (25 × 1.5 cm). The column was equilibrated in 50 mM ammonium formate (pH 3.0) containing 7.5 mM tetrabutylammonium bromide. The flow rate was 2 mL/min, and the products were separated using an isocratic elution from 0 to 20 min followed by a linear gradient from 0 to 5% CH_3OH from 20 to 60 min followed by a linear gradient from 5 to 10% CH_3OH from 60 to 80 min. The compounds and their retention times were as follows: SAICAR, 50–60 min; ADP, 21 min; NAD^+, 33–39 min; ATP and NADH, >90 min. SAICAR was recovered in 85% yield, and

the ammonium formate was removed in vacuo. The SAICAR and remaining salts were then dissolved in 30 mL of 10 mM triethylammonium bicarbonate (pH 7.8) and loaded onto a DEAE-Sephadex A-25 column (3 cc). A 300-mL linear gradient from 0 to 600 mM triethylammonium bicarbonate resulted in elution of SAICAR at 250 mM. The appropriate fractions were pooled, and the volatile buffer was removed in vacuo. NMR (D_2O with DSS standard) δ (pattern, number of hydrogens, assignment) 7.5 (s, 1, C_2H), 5.65 (d, 1, H1′, J = 7 Hz), 4.7 (m, 1, H2′), 4.55 (m, 1, H8), 4.4 (m, 1, H3′), 4.3 (m, 1, H4′), 4.0 (m, 2, H5′, H5″), 2.65 (m, 2, H9).

Phosphorylation of Aminoimidazole Ribonucleoside (AIRs). CAIRs was synthesized from AICARs by the procedure of Srivastava et al. (1974) and converted to AIRs by the procedure of Groziak et al. (1988) and Bhat et al. (1990). A modification of the method of Yoshikawa et al. (1967) was used to convert AIRs to the ribonucleotide, AIR. The riboside (380 μmol) was dried in vacuo over P_2O_5 for 5 h. It was then dissolved in 4 mL (31 mmol) of anhydrous triethylphosphate cooled to 0 °C under Ar. Freshly distilled phosphorus oxychloride (354 μL, 3.8 mmol) was added dropwise over several min, and the reaction was stirred for 1.5 h at 0 °C. The mixture was then poured into 200 mL of anhydrous ether, and the precipitate was separated from the supernatant by centrifugation at 2500g for 20 min. The pellet was washed with an additional 100 mL of ether and the supernatant removed by centrifugation. The pellet was then dissolved in 50 mL of H_2O at 4 °C and the pH immediately adjusted to 8.0 by addition of 1 N NaOH. The sample was diluted to 500 mL and loaded onto a DEAE-Sephadex A-25 column (12 × 4 cm) equilibrated in 0.1 M triethylammonium bicarbonate (pH 8.0). The AIR was eluted with a 750 × 750 mL linear gradient from 0 to 400 mM triethylammonium bicarbonate. Appropriate fractions were pooled subsequent to the Bratton and Marshall (1939) and phosphate assays (Ames & Dubin, 1960). The buffer was removed in vacuo, giving 170 μmol (45% yield) based on the Bratton and Marshall assay. ^{1}H NMR (D_2O, DSS) δ 8.55 (s, 1, H2), 5.85 (d, 1, J = 5 Hz, H1′), 4.62 (m, 1, H2′), 4.45 (m, 1, H3′), 4.35 (m, 1, H4′), 4.08 (m, 2, H5′,5″). The H4 proton has exchanged.

Note: Purification of AIR using a triethylammonium bicarbonate gradient results in conversion of AIR to CAIR by the nonenzymatic carboxylation reaction. In addition, if great care is not taken to remove all of the CO_2 from the triethylammonium bicarbonate buffer, determination of the bicarbonate dependence of the reaction is extremely difficult.

Phosphorylation of CAIRs. The procedure was identical to that described above for AIRs except that the reaction was allowed to proceed for 2 h. The CAIR eluted from the DEAE-Sephadex column at 350 mM triethylammonium bicarbonate (pH 8.0) and was identified by its absorbance at 250 nm. The yield was 48%. ^{1}H NMR (D_2O, DSS) δ 7.53 (s, 1, H2), 5.65 (d, 1, J = 7 Hz, H1′), 4.67 (m, 1, H2′), 4.40 (m, 1, H3′), 4.25 (m, 1, H4′), 3.95 (m, 2, H5′,5″).

Determination of Extinction Coefficient for AIR. AIR was purified directly before use on Sephadex A-25 with triethylammonium bicarbonate elution. The extinction coefficients were determined using three independent methods for quantitation of AIR: Bratton and Marshall assay (1939); Ames and Dubin (1960) assay for phosphate; enzymatic end point assay using PurE, PurC, pyruvate kinase, and lactate dehydrogenase and quantitating the amount of NADH consumed. The extinction coefficients were determined in 100 mM Tris-HCl (pH 8.0), 100 mM potassium phosphate (pH 7.0), and 100 mM Mes (pH 6.0).

Determination of Extinction Coefficient of CAIR. CAIR was purified directly before use. The extinction coefficient was determined using two independent methods for determining the amounts of CAIR: Ames and Dubin (1960) assay for phosphate; enzymatic end point assay using PurC, pyruvate kinase, and lactate dehydrogenase. Buffers were identical to those described above for AIR.

N-Terminal Sequence Analysis. PurE and PurK were run on an SDS gel (Laemmli, 1970) (15% and 10% polyacrylamide, respectively). The proteins were transferred to Immobilon-P (a PVDF filter with a 0.45-μm pore size) using Semi-Phor TE-70 instrument from Hoefer Scientific following the procedure of Matsudaira (1987). The sequencing was performed at the Harvard Microchemistry Facility, Harvard University, Cambridge, MA, using an ABI 470A protein sequencer with a 120A on-line DRH-AA analyzer.

Isolation of "Putative" purK Mutant from E. coli Strain TX209 and Determination of Its Sequence. Strain TX209 was isolated as a spontaneous purine auxotroph from strain TX40 (Smith & Gots, 1980). It was characterized as a *pur*K mutation based on its ability to be complemented with a plasmid containing the *pur*EK operon, pJS131 (Tiedeman et al., 1989), and growth on minimal medium in the absence of added purines and in the presence of "high" CO_2 concentrations. The high CO_2 concentrations were generated with a candle jar, in which the strain was streaked on a minimal plate, a candle lit, and the jar sealed with an air-tight lid. After the candle flame went out, the jar was placed in an incubator at 37 °C, and growth scored after 24–48 h.

While DNA sequencing, complementation, and deletion analysis of the *pur*EK operon have been carried out (Gots et al., 1977; Kamholz et al., 1986; Tiedeman et al., 1989; Watanabe et al., 1989), a fine structure genetic analysis has not been reported for individual *pur*E and *pur*K mutations. Because the present studies with the purified PurE and PurK question the function of the PurK in CO_2 fixation, it was deemed appropriate to characterize by DNA sequence analysis the *pur*K mutation in strain TX209 (designated *pur*K223).

Cloning and DNA Sequence Analysis. As described above, the PCR reaction was used to recover the chromosomal DNA from strain TX209 containing the *pur*K223 mutation. The PCR primers were identical to those described above for construction of the AIR carboxylase expression vector. Using the DNA from a single colony of strain TX209 as the starting material, 25 PCR cycles were performed using cycle conditions of 94 °C (denaturing), 45 °C (annealing) and 72 °C (elongation). The resulting 1.6-bp PCR product was isolated using a Prep-A-Gene kit (Bio-Rad, Richmond, CA) subsequent to electrophoresis on a 0.7% agarose gel. After recovery, the DNA fragment was digested with *Eco*RI and *Pst*I restriction enzymes and cloned into the *Eco*RI–*Pst*I sites of the Bluescript vector, M13 KS$^+$, and transformed into strain BSJ72 with selection for Amp^R on L+Amp+X-Gal+IPTG (Messing, 1983). Plasmids from colorless colonies were screened for inserts by the PCR reaction using the 5′ primer, 5′-TCCGTGGAATTCTCTGTGCCCTCT-3′, and a *lac*Z sequencing primer, 5′-GTTTTCCCAGTCACGAC-3′. In order to recognize potential transcription errors by *Taq* polymerase, three independent clones were identified and retained for DNA sequencing. The sequences were determined as previously described (Flannigan et al., 1990; Cheng et al., 1990).

Does PurE Alone Require ATP? The reaction mixture at 37 °C contained the following in a final volume of 700 μL: 50 mM Hepes (pH 7.8), 20 mM KCl, 50 mM $KHCO_3$, 1.0 mM ATP, 2.0 mM PEP, 4.5 mM $MgCl_2$, 6.7 mM [^{3}H]as-

partate (specific activity, 1.5×10^6 cpm/μmol), 0.55 mM AIR, 0.2 mM NADH, 10 units of pyruvate kinase, 5 units of lactate dehydrogenase, 1.1 units of PurC, and 0.03–0.83 μg (2.4–67 nM) of PurE. The reaction was initiated by the addition of PurE. The progress of the reaction was monitored simultaneously by change in absorbance at 340 nm as a determinant of the ATP consumed and for production of [^{3}H]SAICAR as described above.

Requirement of PurK in Addition to PurE for CAIR Production under Conditions of Low Bicarbonate. A solution containing 1.2 mM ATP, 2.3 mM PEP, 7.0 mM $MgCl_2$, 50 mM Hepes (pH 7.8), and 20 mM KCl was prepared as described above for removal of HCO_3^-/CO_2. The following constituents were then added to 250 μL of this reaction mixture: 5.0 mM [^{14}C]aspartate (specific activity 1.4×10^6 cpm/μmol), 0.8 mM AIR, and 9 μg (1.40 μM) of PurC. Three separate reaction mixtures were incubated at 37 °C and analyzed for product production at 0, 2, 3, 5, and 10 min. Reactions were initiated by the addition of 0.6 μg of PurE, the addition of 1.2 μg of PurK, or the addition of 0.6 μg of PurE and 1.2 μg of PurK. The reactions were monitored for production of [^{14}C]SAICAR.

Requirement for ATP, PurE·PurK in Production of CAIR in Presence of Low Concentrations of Bicarbonate, an HPLC Determination. The reaction mixture contained 50 mM Hepes (pH 7.8), 20 mM KCl, 0.9 mM ATP, 4.5 mM $MgCl_2$, 0.2 mM AIR, no added HCO_3^-, 0.30 μM (4.5 μg) PurE, and 24 nM (0.66 μg) PurK in a final volume of 700 μL. The reaction mixture was incubated at 37 °C for 5 min, and a 200-μL aliquot was removed and analyzed by HPLC chromatography using reverse-phase C-18 column equilibrated with 80 mM Tris (pH 7.8) and 10 mM tetrabutylammonium bromide, flow rate 2 mL/min. AIR eluted with a retention time of 4 min, while CAIR eluted at 6 min. Control experiments minus PurE, minus PurK, and minus ATP were carried out. In addition, standards AIR and CAIR were also chromatographed.

One Equivalent of ATP Is Required for the PurE·PurK AIR Carboxylase Reaction. The reaction mixture at 37 °C contained the following in a final volume of 400 μL: 50 mM Hepes (pH 7.8), 20 mM KCl, 0.9 mM ATP, 4.5 mM $MgCl_2$, 2.0 mM PEP, 0.2 mM NADH, 0.045–0.11 mM AIR, 6.0 mM [^{3}H]aspartate (specific activity 2.1×10^6 cpm/μmol), 10 units of pyruvate kinase, 5 units of lactate dehydrogenase, 0.8 μM (5.7 μg) PurE, and 4.8 μM PurC. The reaction mixture was monitored spectrophotometrically at 340 nm prior to initiation by addition of 8 nM (0.13 μg) PurK. The total amount of ATP consumed was determined by the burst of NADH consumption. All of the AIR is consumed under these conditions. Once the NADH consumption returned to its base-line value, a 100-μL aliquot was withdrawn from the cuvette and assayed for [^{3}H]SAICAR by the method described above. The ratios of AIR and ATP consumed to SAICAR produced was determined.

Results

Isolation of PurE and PurK. The *pur*E and *pur*K genes are part of an operon regulated by the *pur*R repressor protein (Rolfes & Zalkin, 1988). Genetic studies of Gots and coworkers (1977) suggested that both PurE and PurK are required for AIR carboxylase activity and that PurK functions as the CO_2 carrying domain for this reaction. The proposal that a 39-kDa protein, PurK, functions as a CO_2 carrier is in fact unusual and unprecedented, and therefore we decided to investigate the AIR carboxylase reaction in detail. To assist in this goal, the *pur*E and *pur*K genes were cloned into a λP_L expression vector to produce a new plasmid pJS355. Heat induction of pJS355 in *E. coli* host cell TX635 revealed that both PurE (17 kDa) and PurK (39 kDa) were overproduced (Figure 1). It was anticipated that PurE and PurK would copurify. PurE was purified to homogeneity as summarized in Table I. In contrast to expectations, however, analysis by SDS gel electrophoresis of each step of the purification (Figure 1) revealed that the 39-kDa protein separated from the 17-kDa protein during the ammonium sulfate fractionation. Furthermore, addition of the desalted 50% ammonium sulfate fraction containing the 39-kDa protein, back to the desalted 50–75% ammonium sulfate fraction containing the 17-kDa protein (PurE) had no effect on the AIR carboxylase activity, using an assay in which the increase in absorbance at 260 nm due to the production of CAIR is monitored. Addition of this same fraction, 50% ammonium sulfate, to homogeneous PurE likewise had no measurable effect on AIR carboxylase activity or the requirement for HCO_3^-.[2] Given that PurK did not have the anticipated function, no catalytic assay was available to assist in its purification. The 39-kDa protein was therefore initially purified to homogeneity using SDS gel electrophoresis as an assay. However, it was subsequently discovered serendipitously that PurK possesses an AIR-dependent ATPase activity. This observation permitted use of a coupled assay with pyruvate kinase and lactate dehydrogenase in the purification of PurK to homogeneity as shown in Table II.

Assay for AIR Carboxylase Activity. The assay employed by previous investigators for AIR carboxylase monitored the

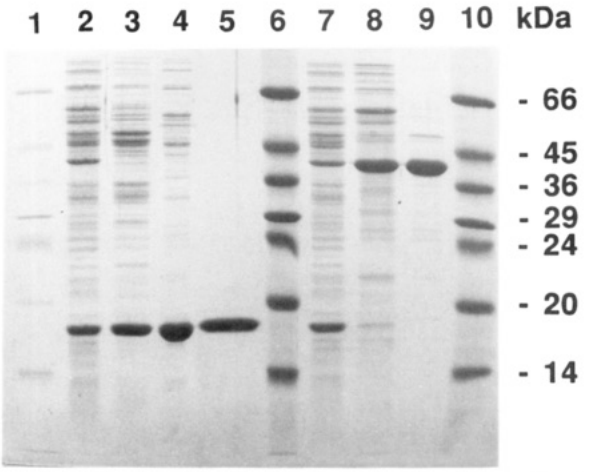


FIGURE 1: SDS gel analysis (12.5% polyacrylamide) of the purification of PurE and PurK, respectively. Molecular weight standards (lane 1); crude *E. coli* TX635/pJS355 (lane 2); Sephadex G-25 50–75% ammonium sulfate (lane 3); Sepharose CL-6B (lane 4); hydroxylapatite (lane 5); molecular weight standards (lane 6); crude *E. coli* TX635/pJS355 (lane 7); Sephadex G-25 0–50% ammonium sulfate (lane 8); Sepharose CL-6B (lane 9); molecular weight standards (lane 10).

Table I: Purification of AIR Carboxylase (PurE)

step[a,b]	protein, mg	volume, mL	total activity, units	specific activity, units/mg^{-1}
crude	1750	56	6010	3.4
G-25 (50–75%) ammonium sulfate	271	80	3940	14.5
DEAE CL-6B	148	100	3470	23.4
hydroxylapatite	90.4	8	3610	40.0

[a] 10 g of TX635/pJS355. [b] All assays were carried out using 0.84 mM AIR and 180 mM HCO_3^-, using the coupled assay procedure in the absence of PurK.

[2] In our original assay for AIR carboxylase activity, no ATP was added to the assay mixtures as there was no evidence for its requirement as a substrate.

Table II: Purification of PurK

step[a]	protein, mg	volume, mL	total activity, units	specific activity,[b] units/mg^{-1}
crude	1320	65	13090	9.9
G-25 (of 50% $(NH_4)_2SO_4$)	464	90	7555	22
DEAE CL-6B (pH 7.0)	117	78	5400	46
DEAE CL-6B (pH 7.8)	64	6.4	4410	68

[a] 10 g of TX635/pJS355. [b] The coupled assay measuring AIR-dependent ATPase with pyruvate kinase and lactate dehydrogenase was used.

Table III. Extinction Coefficients for AIR and CAIR as a Function of pH

	AIR[a]		CAIR[a]	
pH	250 nm	260 nm	250 nm	260 nm
6	4170	2900	9440	9080
7	3830	2400	10580	10080
8	3270	1570	10980	10500

[a] Values are ±100 (M^{-1} cm^{-1}).

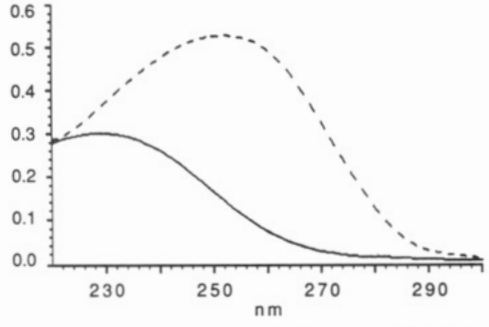


FIGURE 2: Absorbance spectra of AIR (—) and CAIR (---) [0.05 mM] in 100 mM Tris (pH 8.0).

increase or loss in absorbance of CAIR at 260 nm as it was formed from or decomposed to AIR (Patey & Shaw, 1973; Lukens & Buchanan, 1959). These previous assays failed to take into account that AIR as well as CAIR absorb in the 250–260 nm region. Furthermore, obtaining accurate extinction coefficients for AIR and CAIR has been, until recently, problematic due to both the difficulty in obtaining homogeneous nucleotides and the instability of these compounds. Alteration in synthetic procedures has now allowed synthesis of both homogeneous AIR and CAIR (Groziak et al., 1988; Bhat et al., 1990). Their absorption spectra are shown in Figure 2, and the extinction coefficients at various wavelengths as a function of pH are given in Table III. The interconversion of AIR to CAIR can be monitored at 260 nm using $\Delta\epsilon$ = 9000 M^{-1} cm^{-1} (pH 8.0).

Purification of PurC: SAICAR Synthetase. The assay for CAIR production from AIR monitoring the change in absorbance at 260 nm is also problematic due to the unfavorable equilibrium constant for the reaction at low HCO_3^- concentrations.[3] In addition, if ATP is required for this enzymatic reaction, its absorbance at 260 nm would also dramatically decrease the sensitivity of the assay. To overcome these problems, the gene for SAICAR synthetase was cloned, overexpressed, and purified to homogeneity. SAICAR synthetase

[3] The equilibrium constant was determined using both nonenzymatic and enzymatic equilibration of AIR and CAIR and CAIR to AIR at various concentrations of HCO_3^-. K_{equil} = 1.8 ± 0.4.

Table IV: Purification of PurC

step[a]	protein, mg	volume, mL	total activity, units	specific activity, units/mg^{-1}
crude	2156	70	1230	0.57
Sephadex G-25 (of 50% ammonium sulfate)	512	80	2180	4.26
DEAE CL-6B	122	30	1477	12.1
Affi-Gel Blue	58	1.9	1800	31

[a] 15.8 g of TX635 containing pJS408.

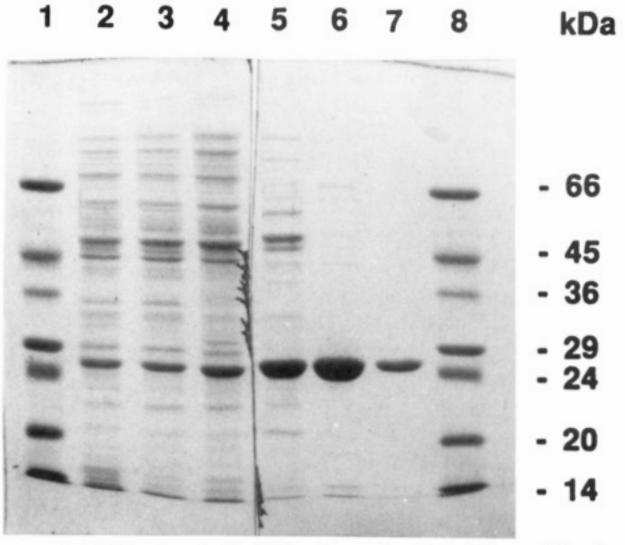


FIGURE 3: SDS gel analysis (10% polyacrylamide) of the purification of PurC. Molecular weight standards (lane 1); crude *E. coli* TX635/pJS355 (lane 2); protamine sulfate (lane 3); Sephadex G-25, 0–50% ammonium sulfate (lane 4); Sepharose CL-6B (lane 5); Affi-Gel Blue 51 μg (lane 6); Affi-Gel Blue 9 μg (lane 7); molecular weight standards (lane 8).

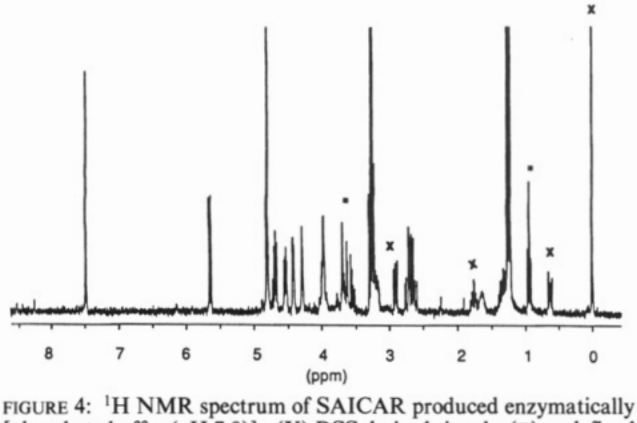


FIGURE 4: ^1H NMR spectrum of SAICAR produced enzymatically [phosphate buffer (pH 7.0)]. (X) DSS derived signals; (■) undefined buffer impurities.

activity was then used in a coupled assay with pyruvate kinase and lactate dehydrogenase, allowing the conversion of AIR to CAIR to be followed continuously by consumption of NADH.

The gene for SAICAR synthetase was cloned into a λP_L expression vector to give plasmid pJS408 in a fashion similar to that described above for AIR carboxylase and plasmid pJS355. Heat induction of the plasmid in host cell TX635 resulted in overexpression of a 27-kDa protein. The protein was purified to homogeneity in 82% overall yield (Table IV), and the purification monitored by SDS gel electrophoresis indicated in Figure 3. Incubation of SAICAR synthetase with ATP, CAIR, and aspartate resulted in the stoichiometric production of SAICAR identified by NMR spectroscopy

(Figure 4). The K_m values for the substrates aspartate, MgATP, and CAIR for SAICAR synthetase were determined to be 1.3 mM, 40 μM, and 36 μM, respectively, using the coupled assay with pyruvate kinase and lactate dehydrogenase. This protein used in a variety of coupled assays has proven invaluable in unraveling the chemistry catalyzed by PurE·PurK and PurE alone.

Molecular Weight Determinations. The subunit molecular weights of the PurE and PurK are 17 and 39 kDa, respectively, determined by using SDS polyacrylamide electrophoresis (Figure 1) (Laemmli, 1970) and are consistent with the predictions from the gene sequence. The native M_r of PurE, PurK, and a combination of the PurE and PurK proteins have also been examined using several methods. Conventional gel-sizing chromatography has revealed that PurE has a M_r of 126 000, an apparent octamer, while PurK has a M_r of 79 000, an apparent dimer. Chromatography of a 1:1 mixture of PurE and PurK gave no evidence for coelution. FPLC Superose chromatography also failed to reveal any interaction between these two proteins. Sucrose gradient ultracentrifugation has been utilized as an additional method of analysis to establish (Martin et al., 1961) the native molecular weights of these proteins and to determine if these proteins associate. The apparent molecular weight of PurE using this method is 136 kDa and that of PurK is 78 kDa, consistent with the size-exclusion chromatography results. As in the latter case, no evidence for association between the two proteins under a variety of conditions was obtained.

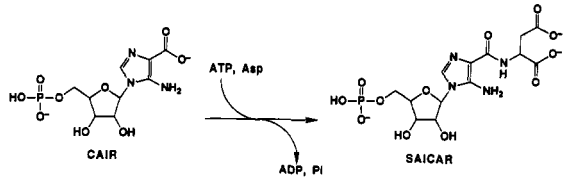
The subunit M_r of 27 000 was established for SAICAR synthetase using SDS page gel electrophoresis (Figure 3) (Laemmli, 1970) as predicted from the gene sequence. Analysis of the native M_r by ultracentrifugation revealed a protein of 76 000, and thus SAICAR synthetase appears by this method to be a trimer.

Characterization of Protein Products: N-Terminal Sequence Analysis. To establish that the 17- and 39-kDa proteins are the desired products of the *pur*E and *pur*K genes, respectively, the proteins were blotted onto Immobilon and subjected to N-terminal sequencing. A comparison of the gene sequences shown below establishes the identity of these proteins as the desired gene products.

PurE protein	S S R N N P A R V A
gene	M S S R N N P A R V A

*pur*K protein	M K Q V C V L G N G Q L G R M L R Q A G
gene	M K Q V C V L G N G Q L G R M L R Q A G

Characterization of Catalytic Activities of PurE·PurK and 1:1 Mixture of PurE·PurK: PurE-Dependent AIR Carboxylase Activity. Because of the problems with monitoring CAIR production directly, the coupled assay using SAICAR synthetase pyruvate kinase and lactate dehydrogenase was used to measure AIR carboxylase activity



(3)

This coupled assay revealed that PurE can catalyze the conversion of AIR to CAIR in the absence of both PurK and ATP.[4] The K_m values for PurE-catalyzed carboxylation of

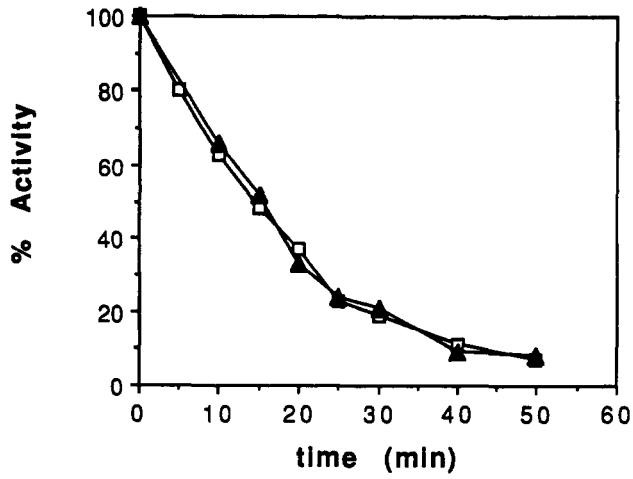


FIGURE 5: Heat-dependent inactivation of PurK. (□) Loss of AIR-dependent ATPase; (▲) loss of ability to produce SAICAR.

AIR were found to be 430 μM for AIR and 110 mM for HCO_3^-! This extremely high K_m for bicarbonate is substantially above the concentrations of HCO_3^- (Schloss, 1990) that probably occur in vivo, and thus this PurE activity appears not to be physiologically important.

Interestingly, AIR can also be converted to CAIR nonenzymatically in the presence of very high concentrations of bicarbonate such as those described above for the PurE-catalyzed carboxylation. The pseudo-first-order rate constant for CAIR production from AIR at 180 mM HCO_3^- is 7.0×10^{-4} min^{-1}. This compares with a turnover number of 1200 min^{-1} for PurE under identical conditions. Thus, PurE is capable of catalyzing the carboxylation reaction 10^6-fold over the nonenzymatic rate. In spite of this acceleration, some factor or factors appeared to be missing which would lower the apparent K_m value for HCO_3^- into a concentration range observed under normal physiological conditions. One obvious candidate for the missing factor is PurK, initially suggested by genetic analysis of Gots et al. (1977) to be a component of the AIR carboxylase reaction.

PurK Possesses an Unusual AIR-Dependent ATPase. If PurK is in fact a "CO_2" carrier, it might be anticipated that its function would be to convert HCO_3^- in an ATP-dependent reaction to carboxyphosphate, which could then be directly delivered to PurE. In fact, PurK alone does catalyze a substantial ATPase activity (Table II), but it only does so in the presence of AIR. Under these conditions AIR is not carboxylated to CAIR. Using a coupled assay with pyruvate kinase and lactate dehydrogenase, a K_m value of 90 μM was determined for ATP and 26 μM for AIR. However, we were unable to demonstrate any convincing dependence of the ATPase activity on the concentration of HCO_3^-. Even when the concentration of HCO_3^- was 50 μM, no reproducible effect on the rate of ATP hydrolysis could be observed. While this ATPase may be due to a contaminating enzyme in the PurK protein preparation, its strict dependence on the presence of AIR makes this possibility seem unlikely. To establish that the PurK-dependent ATPase is not a contaminant, a heat inactivation study was carried out on the purified protein. The ATPase activity and the ability of PurK to support CAIR production measured as SAICAR at low concentrations of bicarbonate in the presence of PurE were measured (Figure 5) (see next section). Both activities were lost simultaneously

[4] Only a single ATP is consumed in the production of SAICAR from AIR, not the two expected if both PurE and PurC require ATP for activity.

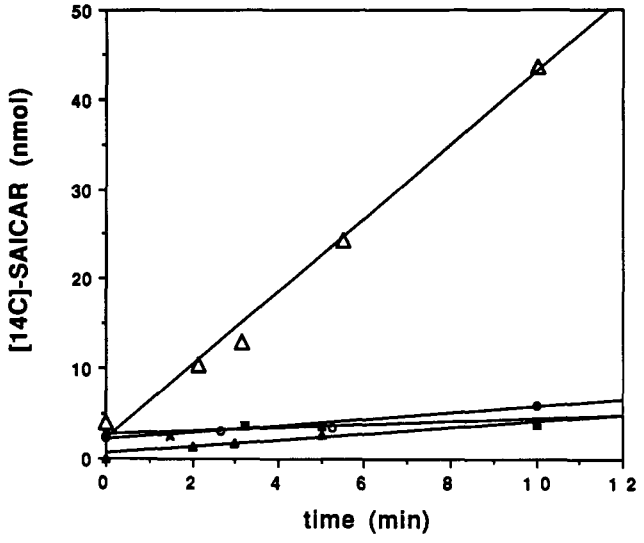


FIGURE 6: Dependence of CAIR production measured as SAICAR, on the presence of PurK at low bicarbonate concentrations (2 mM). (▲) No (PurE + PurK); (X) only PurK; (O) only PurE; (△) PurE + PurK.

in time, suggesting that they are associated with the same protein. While the physiological significance of this AIR-dependent ATPase is questionable, these results suggest that PurK and ATP together might be required for the PurE-dependent conversion of AIR to CAIR in a reasonable time in the present physiological concentrations of bicarbonate.

PurK Is Required for AIR Carboxylase Activity under Low [HCO_3^-]. The high level of AIR-dependent ATPase of PurK has complicated the demonstration of the ATP requirement for the PurE·PurK-dependent AIR carboxylase activity. The first experiment therefore was undertaken to demonstrate that at low concentrations of HCO_3^- (10 mM) PurK was indeed required for carboxylation of AIR. The coupled assay with PurC, ATP, and aspartate was used to measure SAICAR production. The results are shown in Figure 6. Under these conditions neither PurK nor PurE alone catalyzes production of any SAICAR. However, together in a 1:1 molar ratio, SAICAR is produced, and the rate of SAICAR production is proportional to the concentration of the enzymes (data not shown). Thus PurK is required for CAIR production under low bicarbonate concentrations. The levels of ATP consumed in this experiment, however, are substantially higher (10-fold) than the amount of SAICAR produced, presumably due to the PurK-dependent ATPase.

Demonstration of Stoichiometric Requirement for ATP in PurE·PurK-Dependent Carboxylase Reaction. An experiment was devised to minimize the PurK–AIR-dependent ATPase activity, so that the stoichiometry of the requirement of ATP in the PurE·PurK-catalyzed conversion of AIR to CAIR could be investigated. Using a limiting amount of AIR in the presence of large amounts of PurE, PurC, [^{14}C]aspartate, ATP, PEP, pyruvate kinase, lactate dehydrogenase, NADH, and <100 μM HCO_3^-, the reaction was initiated by a small amount of PurK. A burst of NADH consumption is observed at the end of which all of the AIR is turned over and the product is quantitated as [^{14}C]SAICAR. NADH consumption is a measure of the ATP utilized. The stoichiometry observed, the average of six experiments, revealed that for every mole of AIR consumed, 1 mol of SAICAR is produced and 2 mol of ATP are consumed. Thus, given the previously demonstrated need for 1 mol of ATP in the SAICAR synthetase reaction, 1 mol of ATP is required for the conversion of AIR to CAIR.

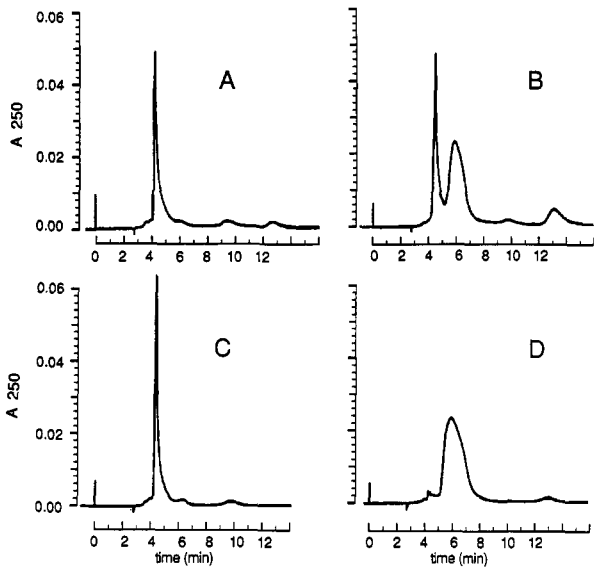


FIGURE 7: Requirement for ATP in the conversion of AIR to CAIR determined by HPLC analysis. Each reaction mixture contained 50 mM Hepes (pH 7.8), 20 mM KCl, 4.5 mM $MgCl_2$, 0.2 mM AIR, and <100 μM HCO_3^-. In addition, in (A) PurE and 0.9 mM ATP were added; (B) PurE, PurK, and 0.9 mM ATP were added; (C) PurE and PurK were added. Panel D is the standard CAIR in 50 mM Hepes (pH 7.8) and 20 mM KCl. The retention time for AIR is 4 min.

This requirement for ATP in the PurE·PurK-dependent conversion of AIR to CAIR was further demonstrated by the direct analysis of AIR to CAIR interconversion by HPLC (Figure 7A–D). Under the conditions of low concentrations of HCO_3^-, PurE alone produced no CAIR (Figure 7A). If PurK is added, AIR disappears and CAIR is produced (Figure 7B). If ATP is removed from the PurE·PurK-dependent reaction, no CAIR is produced (Figure 7C). Figure 7D is the authentic CAIR. These two experiments demonstrate for the first time that ATP is stoichiometrically consumed in the PurE·PurK conversion of AIR to CAIR.

Isolation and Sequencing of Putative PurK Mutant. Studies described above suggest that PurK does play a key role in preparing "CO_2" in a palatable form for the consumption by PurE under physiological conditions. Therefore, to investigate the nature of the *pur*K mutation (purK223) further, three independent clones of the *pur*K mutant gene were isolated from *E. coli* TX209 and characterized by sequence analysis. Their sequences were found to be identical and contained a single-base transversion at nucleotide 1268 in which a C is changed to an A in comparison to the wt gene. This mutation converts tyrosine 147 (TAC) to a termination codon (TAA) in the middle of the gene. Subsequent termination during translation would result in the production of a truncated PurK polypeptide of M_r 16 383 without any apparent in vivo activity. Thus, the purine auxotrophy and the CO_2 conditional phenotype of strain TX209 can be ascribed to a single nonsense mutation in the *pur*K gene. How high concentrations of CO_2 can overcome the requirement for PurK is a mystery to be unraveled. However, PurE mutants with a similar phenotype have not been detected (J. Smith, unpublished results). These results are even more intriguing given that Chen et al. (1990) recently used this mutant to clone the avian AIR carboxylase. Sequence of this gene, discussed below, revealed a PurE domain but no PurK domain!

DISCUSSION

The isolation and characterization of SAICAR synthetase for use in the coupled assay to measure AIR carboxylase

activity (eq 1) was essential for deconvolution of the catalytic capabilities of PurE, PurK, and PurE·PurK. The ability of AIR to be carboxylated nonenzymatically, the ability of CAIR to be decarboxylated nonenzymatically, the unfavorable equilibrium constant for the conversion of AIR to CAIR, and the fact that both of these species absorb light in the ultraviolet region between 250 to 260 nm have all made the direct assay for AIR carboxylase activity difficult. Although AIR carboxylase in various states of purity from a variety of organisms has been previously investigated, neither the requirement of the reaction for ATP nor the dependence of the reaction on physiological concentrations of HCO_3^- has been demonstrated (Patey & Shaw, 1973; Lukens & Buchanan, 1959; Nikolaeva et al., 1975, 1982; Yanulaitis et al., 1975; Ahmad et al., 1965). In fact, most previous investigators of the avian, yeast, and mammalian systems have used >100 mM HCO_3^- when assaying activity in the forward direction.

The coupled assay with SAICAR synthetase, pyruvate kinase, and lactate dehydrogenase (eq 3) has allowed us to measure the PurE-catalyzed conversion of AIR to CAIR by monitoring change in absorption at 340 nm. Kinetic analysis has revealed a K_m value for HCO_3^- of 110 mM and has further revealed that this carboxylation process does not require ATP. The coupled assay has also been used to measure the nonenzymatic rate of carboxylation of AIR to CAIR under similar conditions. PurE appears to accelerate the rate of the reaction over the nonenzymatic rate approximately 10^6-fold. It is obvious that for PurE to function in vivo under physiological concentrations of HCO_3^-, however, some missing factor must be supplied to this system. The genetics studies of Gots et al. (1977) suggested that this factor might be PurK.

Initial attempts to purify PurK revealed unexpectedly that it separated from PurE during the first ammonium sulfate fractionation. PurK was shown to possess an AIR-dependent ATPase activity. This observation provided a convenient assay which facilitated purification of the protein to homogeneity. However, the very high turnover number for this uncoupled activity (Table II) has made difficult the characterization of the PurE·PurK AIR carboxylase activity discussed below. Three experiments indicate that the AIR-dependent ATPase is associated with PurK and not with a contaminating protein. The first is that both activities, the AIR-dependent ATPase and the ability of PurK to support CAIR production in the presence of PurE and ATP, copurify. The second is that both these activities are lost simultaneously during heat inactivation (Figure 5). The third is that the apparent K_m of this ATPase for AIR is 26 μM.

The requirement of AIR for observation of ATP hydrolysis catalyzed by PurK remains unexplained. However, the ATPase activity itself suggests a role for PurK as the missing factor required to make PurE function under physiological conditions. Specifically, PurK could catalyze the reaction of ATP and HCO_3^- to produce carbonyl phosphate, which could then be delivered to PurE as a source of CO_2 required to carboxylate AIR. All efforts thus far, however, to demonstrate a HCO_3^- requirement for this ATPase, even at concentrations of HCO_3^- as low as 50 μM, have failed. Furthermore, AIR is not carboxylated by PurK alone, nor can CAIR substitute for AIR in this process. The presence of PurE was initially anticipated to restrain this uncoupled ATPase activity and harness the energy of ATP to stoichiometrically produce CAIR.

Further studies were therefore undertaken using homogeneous PurE and PurK. These investigations revealed, using a coupled assay system (eq 3), that both proteins are required to produce SAICAR and thus CAIR in the presence of low HCO_3^- concentrations. In addition, a variety of methods (Figures 6 and 7) have shown for the first time that ATP is stoichiometrically consumed in the conversion of AIR to CAIR.

Determination of the mole ratio of PurE to PurK required for the maximum rate of CAIR formation, however, has proven problematic due to the large turnover number of PurK-dependent hydrolysis of ATP. The high rate of this uncoupled process is not suppressed by the presence of PurE or by addition of a variety of metal ions. Furthermore, methods including sedimentation velocity and size-exclusion chromatography in the presence of ATP and a variety of metals have all thus far failed to reveal any association between these two proteins. Whether the factor or factors required to eliminate the AIR-dependent ATPase of PurK will also enhance the affinity of PurE for PurK is the subject of ongoing investigation.

Given the requirement of PurK for AIR carboxylase activity in the presence of physiological bicarbonate concentrations, the recent report of Chen et al. (1990) using the PurK mutant (*E. coli* TX209) to clone the avian AIR carboxylase is particularly intriguing. The cDNA isolated by Chen et al. using this selection procedure encodes a bifunctional protein containing SAICAR synthetase at the N-terminus (1–259) and AIR carboxylase at the C-terminus (260–426). Sequence analysis of the chicken liver AIR carboxylase reveals 27% and 30% exact identity with the *E. coli* and *Bacillus subtilis* PurE, respectively (Tiedeman et al., 1989; Watanabe et al., 1989; Ebbole & Zalkin, 1987). Further sequence analysis, however, fails to reveal any PurK-encoded subunit. Thus, how an *E. coli* mutant deficient in PurK could be used to isolate the clone for AIR carboxylase activity which contains no PurK domain is an unresolved mystery.

A number of possible explanations can be put forth to explain this apparent paradox. The first is that putative *pur*K mutant TX209 is not in fact defective in PurK since this mutant had not been previously characterized in any detail. We therefore isolated the gene for *pur*K from *E. coli* TX209 and established by sequence analysis that there is a single base-pair transversion at nucleotide 1268 which results in the conversion of Y147 in the wt PurK to a stop codon. Thus the mutation is in fact in *pur*K and presumably results in the production of a truncated protein. The paradox remains unresolved. A second possible explanation is that the chicken liver AIR carboxylase could stabilize the putative truncated PurK polypeptide in strain TX209 to restore the CO_2 fixing ability. The two highly conserved regions of PurK as revealed by a comparison of their sequences in *E. coli*, *B. subtilis*, and *Saccharomyces cerevisiae*, however, appear at the N-terminus and C-terminus. Thus, given that the C-terminus of the *pur*K mutant is missing, this explanation seems unlikely. A third possibility is that the function of the 39-kDa PurK has been incorporated directly into the PurE domain of the chicken liver protein. This possibility also seems unlikely given that there is a high degree of sequence homology and similarity in size between the PurE domain of *E. coli* and the chicken liver protein. In addition, the conserved regions of PurK from a variety of organisms appear to have no counterpart in the avian AIR carboxylase. A fourth possible explanation, and the one we presently favor, is that the avian AIR carboxylase has evolved to produce a CO_2 or HCO_3^- binding site with a much lower K_m for the substrate than *E. coli* PurE. Recent cloning of the chicken liver bifunctional protein should, subsequent to expression, allow assessment of the ATP requirement and the determination of K_m for HCO_3^-(CO_2?) for this protein.

Perhaps one of the most intriguing aspects of PurK is revealed by its strong sequence identity and similarity (27% exact 55% conserved) with the newly discovered gene product of *pur*T, a second GAR transformylase in *E. coli*. Recent studies (Smith and Benkovic, unpublished results) have revealed that N^{10}-formyl tetrahydrofolate is not required for this transformylase activity, but that ATP and formate are required. Formyl phosphate is proposed to be the reactive formylating agent. The observed sequence homology with *pur*K thus gives parallel support to the proposal that the PurK catalyzes the formation of carbonyl phosphate. [*Note*: PurK does not possess a formate-dependent ATPase.] Identification of sequence homology between PurK and other putative carbonyl phosphate generating enzymes, acetyl and pyruvate carboxylase, and carbamoyl phosphate synthetase have thus far failed. Further studies will be required to establish if PurK can generate carbonyl phosphate.

References

Ahmad, F., Missimer, P., & Moat, A. G. (1965) *Can. J. Biochem. 43*, 1723–1731.

Ames, B. N., & Dubin, D. T. (1960) *J. Biol. Chem. 235*, 764.

Bachmann, B. J. (1972) *Bacteriol. Rev. 36*, 525–557.

Bhat, B., Groziak, M. P., & Leonard, N. J. (1990) *J. Am. Chem. Soc. 112*, 4891.

Bratton, A. C., & Marshall, E. K. (1939) *J. Biol. Chem. 128*, 537.

Chen, Z., Dixon, J. E., & Zalkin, H. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3097–3101.

Cheng, Y. S., Rudolph, J., Stern, M., Stubbe, J., Flannigan, K. A., & Smith, J. M. (1990) *Biochemistry 29*, 218–227.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 103–138.

Ebbole, D. J., & Zalkin, H. (1987) *J. Biol. Chem. 262*, 8274–8287.

Flannigan, K. A., Hennigan, S. H., Vogelbacker, E. E., Gots, J. S., & Smith, J. M. (1990) *Mol. Microbiol. 4* (3), 381–392.

Gots, J. S., Bensen, C. E., Jochimsen, B., & Koduri, K. R. (1977) Purine and Pyrimidine Metabolism. *CIBA Found. Symp. 48*, 23–41.

Groziak, M. P., Bhat, B., & Leonard, N. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7174.

Ingelese, J., Johnson, D. L., Benkovic, S. J., Shiau, A. & Smith, J. M. (1990) *Biochemistry 29*, 1436–1443.

Kamholz, J., Keyhani, J., & Gots, J. S. (1986) *Gene 44*, 55–62.

Laemmli, U. K. (1970) *Nature (London) 227*, 680.

Lowry, O. H., Rosenberg, N. J., Farr, A. L., & Randall, R. I. (1957) *J. Biol. Chem. 193*, 265.

Lukens, L., & Buchanan, J. M. (1959) *J. Biol. Chem. 234*, 1799.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular cloning. A laboratory manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Martin, R. G., & Ames, B. N. (1961) *J. Biol. Chem. 236*, 1372.

Matsudaira, P. (1987) *J. Biol. Chem. 262*, 10035.

Messing, J. (1983) *Methods Enzymol. 101*, 20–78.

Nikolaeva, Z. K., Alenin, V. V., Domkin, V. D., & Smirnov, M. N. (1975) *Biokhimiya 40*, 751–754.

Nikolaeva, Z. K., Pushnova, E. A., Aronshtam, A. A., Subbotina, M. F., & Smirnov, M. N. (1982) *Vestn. Leningr. Univ., Ser. 3: Biol. 1*, 92–98.

Patey, C. A., & Shaw, G. (1973) *Biochem. J. 135*, 543.

Peled, N. (1983) in *Methods of Enzyme Analysis* (Bergmeyer, N. U., Ed.) 3rd ed., Vol. 7, pp 572–577, VCH Publishers, Cambridge.

Rolfes, R. J., & Zalkin, H. (1988) *J. Biol. Chem. 263*, 19653–19661.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Schurf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., & Erlich, H. A. (1988) *Science 239*, 487–491.

Schendel, F. J., Mueller, E., Stubbe, J., Shiau, A., & Smith, J. M. (1989) *Biochemistry 28*, 2459–2471.

Schloss, J. V. (1990) *Enzymatic and Model Carboxylation and Reduction Reactions for Carbon Dioxide Utilization* (Anesta, M., & Schloss, J. V., Eds.) Kluwer, The Netherlands.

Schrimsher, J. L., Schendel, F. J., & Stubbe, J. (1986) *Biochemistry 25*, 4356–4365.

Smith, J. M., & Gots, J. S. (1980) *J. Bacteriol. 143*, 1156–1164.

Smith, M., Hamilton, P. T., & Reeve, N. J. (1985) *J. Mol. Evol. 22*, 351–360.

Srivastava, P. C., Mancuso, R. W., Rousseau, R. J., & Robins, R. K. (1974) *J. Med. Chem. 17*, 1207.

Szankosi, P., Heyer, W. D., Schuchert, P., & Kohl, J. (1988) *J. Mol. Biol. 204*, 917–925.

Tiedeman, A. A., Keyhani, J., Kamholz, J., Daum, III, H. A., Gots, J. S., & Smith, J. M. (1989) *J. Bacteriol. 171*, 205–212.

Tiedeman, A. A., De Marini, D. J., Parker, J., & Smith, J. M. (1990) *J. Bacteriol. 172*, 6035–6041.

Watanabe, W., Sampei, G., Aiba, A., & Mitzobuchi, K. (1989) *J. Bacteriol. 171*, 198–204.

Yagi, T., Kagamiyama, H., Nozaki, M., & Soda, K. (1985) *Methods Enzymol. 113*, 83–89.

Yanulaitis, A. A., Nikolaeva, Z. K., & Smirnov, M. N. (1975) *Biokhimiya 40*, 588–591.

Yoshikawa, M., Kato, T., & Takenishi, T. (1967) *Tetrahedron Lett. 50*, 5065.