Purification and Characterization of Aminoimidazole Ribonucleotide Synthetase from *Escherichia coli*†

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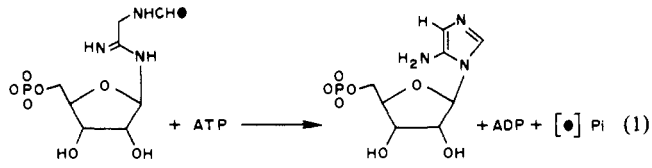
ABSTRACT: Aminoimidazole ribonucleotide (AIR) synthetase has been purified 15-fold to apparent homogeneity from *Escherichia coli* which contains a multicopy plasmid containing the purM, AIR synthetase, gene. The protein is a dimer composed of two identical subunits of M_r 38 500. The N-terminal sequence, amino acid composition, and steady-state kinetics of the protein have been determined. AIR synthetase has been shown to catalyze the transfer of the formyl oxygen of [^{18}O]formylglycinamide ribonucleotide to P_i.

Aminoimidazole ribonucleotide (AIR)[1] synthetase catalyzes the fifth step in de novo purine biosynthesis which is the conversion of formylglycinamidine ribonucleotide (FGAM) and ATP to AIR, ADP, and P_i (eq 1). In 1963, French and



(1)

Buchanan (Flaks & Lukens, 1963) reported a 10-fold purification of AIR synthetase from pigeon liver. Very recently, our laboratory in collaboration with the laboratories of Benkovic, Henikoff, and Patterson reported purification to homogeneity of AIR synthetase from chicken liver and that it is part of a multifunctional protein (M_r 110 000) which also contains glycinamide ribonucleotide (GAR) synthetase and GAR transformylase, the second and third enzymes in the purine biosynthetic pathway, respectively (Daubner et al., 1985; Schrimsher et al., 1986).

In contrast, in bacterial systems, elegant genetic studies of Gots and his collaborators (Gots, 1971; Gots et al., 1977) indicate that the majority of de novo purine biosynthetic genes are unlinked but may act as a single unit of regulation controlled by the "purR" repressor protein. Experiments by Koduri and Gots (1980) suggest that the putative repressor requires either ATP or GTP to effect transcription of the pur genes. More recent studies by Houlberg and Jensen (1983) and Levine and Taylor (1982) suggest that the repressor is regulated by guanine and hypoxanthine.

In order to investigate the regulation of the purine biosynthetic pathway as well as the mechanistic enzymology of the various interconversions, one of us (J. Smith, submitted for publication) cloned a 1.8-kilobase fragment of DNA containing the gene which codes for AIR synthetase (purM$^+$) into the *Hinc*II site of the pUC9 plasmid to produce plasmid pJS24. This plasmid, when grown in *Escherichia coli* strain TX393 [*ara*Δ(purM$^-$upp) *srl*C300:Tn10 *rec*A56], produces AIR synthetase at levels 200 times that found in wild-type *E. coli*. Cloning and overproduction have allowed, for the first time, rapid isolation of homogeneous AIR synthetase using a C-8-linked ATP–agarose affinity column. The physical and kinetic properties of this protein are reported and contrasted with the properties of the recently purified chicken liver enzyme (Schrimsher et al., 1986).

Experimental Procedures

Materials. The [^{14}C]formate (51.5 mCi/mmol) and the [^{32}P]P_i used for [γ-^{32}P]ATP synthesis were obtained from New England Nuclear Corp. [^{13}C]Formic acid was purchased from MSD Isotopes. $H_2{}^{18}O$ (98.1 atom %) was obtained from Monsanto Research Corp. [^{13}C,$^{18}O_2$]Formate was synthesized by the procedure of Hermes et al. (1984). The ^{18}O content of the formate was established by ^{13}C NMR spectroscopy. [^{13}C,^{18}O/^{16}O]FGAR and [^{13}C,^{18}O/^{16}O]FGAM were prepared by the procedure of Schrimsher et al. (1986). ATP, D-ribose 5-phosphate, bovine serum albumin, rabbit muscle L-lactic acid dehydrogenase (920 μmol min^{-1} mg^{-1}), rabbit muscle pyruvate kinase (355 μmol min^{-1} mg^{-1}), phosphoenolpyruvate, molecular weight standards for gel filtration and gel electrophoresis, Sephadex G-25 (fine), Sephadex G-100 (fine), and DEAE-Sephadex A-25 were obtained from Sigma Chemical Co. Hydroxylapatite was purchased from Bio-Rad Laboratories. Azaserine and ATP–agarose (linked through C-8 of the adenine ring via an eight-carbon spacer) were obtained from Pharmacia P-L Biochemicals (Piscataway, NJ). [γ-^{32}P]ATP was synthesized according to the procedure of Selman and Selman-Reimer (1981). FGAM synthetase (0.20 μmol min^{-1} mg^{-1}) from chicken liver was purified by the procedure of

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[1] Abbreviations: AIR, aminoimidazole ribonucleotide; FGAM, formylglycinamidine ribonucleotide; FGAR, formylglycinamide ribonucleotide; GAR, glycinamide ribonucleotide; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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Schendel and Stubbe (1986). FGAM synthetase (0.70 μmol min^{-1} mg^{-1}) was purified from *E. coli* pJS80 (F. J. Schendel and J. Stubbe, unpublished results). Glycinamide ribonucleotide (GAR) was prepared by the procedure of Chettur and Benkovic (1977). AIR used in the product inhibition studies was prepared by the procedure of Schrimsher et al. (1986). All other reagents used were of reagent grade or better unless otherwise specified. *E. coli* strain PCO135 (purE$^-$) was obtained from the Yale University *E. coli* Genetics Stock Center and grown on minimal media as described by Schrimsher et al. (1986). NMR spectra were recorded on either a Bruker 270-MHz spectrometer or a Nicolet 200-MHz broad-band spectrometer.

Protein Determination. Protein concentrations of the solutions were determined by the method of Lowry et al. (1951). Bovine serum albumin was used as a standard.

Synthesis of β-FGAM. FGAR (a mixture of anomers) was synthesized by a modification of the procedure of Chu and Henderson (1970) (Schendel & Stubbe, 1986). FGAR was converted to FGAM by using FGAM synthetase purified from *E. coli* pJS80 according to the procedure of F. J. Schendel and J. Stubbe (unpublished results). The reaction mixture for the synthesis of β-FGAM consisted of 1.5 mM ATP, 1.0 mM α/β-FGAR, 1.5 mM glutamine, 20 mM Hepes (pH 7.8), 60 mM KCl, 20 mM $MgCl_2$, and 10 units of FGAM synthetase in a volume of 40 mL. The pH of the reaction mixture was adjusted to 8.0 before the addition of FGAR and enzyme.

The reaction mixture was incubated at 37 °C for 10 min and subjected to ultrafiltration for removal of protein. The filtrate was diluted to 250 mL and applied (pH 8.0) to a column of DEAE-Sephadex A-25 (4 × 12 cm), which was washed with 2 column volumes of water. The column was developed with a 1.0-L linear gradient of triethylammonium bicarbonate (pH 8.0), 0–300 mM. The fractions (15 mL) that contained FGAM, detected by assay for total phosphate (Ames & Dubin, 1960), that eluted at 150 mM triethylammonium bicarbonate, were pooled and evaporated to dryness in vacuo. The FGAM was redissolved in 10 mL of H_2O, the pH adjusted to 8.2 with 2 N NaOH, and 60 μmol of $BaBr_2$ added. Five volumes of cold absolute ethanol was added and the solution placed at −20 °C for 4 h. The precipitate which formed was collected, dried over P_2O_5 in vacuo, and dissolved in H_2O, and the Ba^{2+} was exchanged for Na^+ by passage through a Dowex 50W X8 column, Na^+ form. The yield was 17.5 μmol of β-FGAM, 83% from β-FGAR.

Purification of Protein. E. coli strain TX393 containing the multicopy plasmid pJS24 with a DNA insert containing AIR synthetase (J. Smith, submitted for publication) was grown in a medium containing 6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 0.24 g/L $MgSO_4$, 0.011 g/l $CaCl_2$, and 2.0 g/L glucose. The cells were harvested in the late log phase of growth to yield 4.3 g/L *E. coli* cells. The purification of the protein that contained AIR synthetase activity was performed at 4 °C. All buffers contained 10 mM 2-mercaptoethanol, 1 mM EDTA, and 10% (v/v) glycerol in addition to the other components specified below, unless otherwise noted.

The cells (6.4 g) were resuspended in 25 mL of buffer that contained 20 mM Tris-HCl (pH 8.2) and 100 mM KCl. The cells were broken in a French pressure cell (16 000 psi), and the cell debris was removed by centrifugation at 20000*g* for 15 min. The supernatant (31 mL) was applied to a column of Sephadex G-25 (4.0 × 35 cm) equilibrated with buffer that contained 10 mM Tris-HCl (pH 8.2) and 10 mM KCl. The effluent that contained protein (72 mL) was diluted 1:1 (v/v) with water and applied (4–6 mL/min) to a column of DEAE-cellulose (2.5 × 24 cm, Whatman DE-52) previously equilibrated with buffer that contained 20 mM Tris-HCl (pH 8.2) and 50 mM KCl. The column was then washed with the sample buffer until the absorbance (280 nm) of the effluent was less than 0.1 and developed with a 800-mL linear gradient of KCl (50–300 mM) in the equilibration buffer.

Fractions (10 mL) from the DEAE-cellulose column that contained the AIR synthetase activity, determined by the Bratton–Marshall assay, that eluted at 150 mM KCl, were pooled (257 mL) and concentrated to 5 mL by using an Amicon ultrafiltration apparatus with a PM 30 membrane. The concentrated enzyme was applied to a column (2.5 × 115 cm) of Sephadex G-100 equilibrated with a buffer that contained 100 mM potassium phosphate (pH 7.4) and 5% (v/v) glycerol. The flow rate was maintained at 5–6 mL/h. Fractions (4 mL) were collected, and those that contained the AIR synthetase activity were pooled (49 mL) and concentrated to give a protein solution of 15 mg/mL (10 mL). This solution was desalted on a column of Sephadex G-25 equilibrated with a buffer containing 8 mM $MgCl_2$, 10 mM Hepes (pH 7.7), and 50 mM KCl (EDTA excluded). The protein-containing fractions were applied directly to a column containing (2.5 × 10 cm) ATP–agarose (C-8 linked via an eight-carbon spacer) and subsequently washed with the sample buffer until the A_{280} was less than 0.05. AIR synthetase was then eluted from the column with 100 mL of the sample buffer that contained 1 mM EDTA but lacked $MgCl_2$. Fractions (4 mL) were collected, and those containing AIR synthetase were concentrated to give a protein concentration of 2 mg/mL and stored at −20 °C.

Native Molecular Weight Determination. The native molecular weight of *E. coli* AIR synthetase was determined through the use of gel filtration chromatography and sucrose density gradient ultracentrifugation. The procedure of Siegel and Monty (1966) was used in the correlation of the Stokes radius and sedimentation coefficient of the protein for the calculation of the molecular weight.

The column of Sephadex G-100 (described above) was calibrated with blue dextran (average molecular weight of 2 × 10^6) and proteins with known Stokes radii in order to obtain that of the *E. coli* AIR synthetase. The proteins used were yeast alcohol dehydrogenase, sweet potato β-amylase, bovine serum albumin, carbonic anhydrase, and cytochrome *c*.

Sucrose density gradient ultracentrifugation was performed according to the procedure of Martin and Ames (1961) utilizing 5–20% sucrose gradients (4.8 mL) in 100 mM potassium phosphate (pH 7.5) and 10 mM β-mercaptoethanol. Gradients were poured at room temperature and allowed to equilibrate at 4 °C overnight. Centrifugation was performed on 0.1 mL of enzyme (2.0 mg/mL) at 4 °C in a Beckman Model L5-50 ultracentrifuge (SW-50.1 rotor) at 40 000 rpm for 5–20 h. The sedimentation coefficient was determined by using lysozyme, egg albumin, yeast alcohol dehydrogenase, and catalase as standards.

The data from these experiments were fit to eq 2 to obtain the molecular weight where M_r = molecular weight, $\eta_{20,w}$ =

$$M_r = 6\pi\eta Nas/(1 - \nu\rho) \quad (2)$$

viscosity of media, N = Avogadro's number, a = Stokes radius (38.2 × 10^{-8} cm), $s_{20,w}$ = sedimentation coefficient (4.56 × 10^{-13} s), ν = partial specific volume (assumed to be 0.725 for AIR synthetase), and $\rho_{20,w}$ = density of medium.

Polyacrylamide Gel Electrophoresis. SDS gel electrophoresis (7.5% acrylamide) was performed according to the

Tabel I: Purification of *E. coli* AIR Synthetase

step	protein (mg/mL)	total protein (mg)	total act. (units)	sp act. (units/mg)	x-fold purification	recovery (%)
crude[a]	3.83	1191	268	0.225		100
DE-52	2.03	521	180	0.346	1.54	67
G-100	3.06	149	148	0.990	4.40	55
ATP–agarose	0.57	39.4	132	3.35	14.9	49

[a] 6.4 g of *E. coli* strain TX393 containing plasmid pJS24.

procedure of Laemmli (1970). The molecular weight standards used included bovine serum albumin, egg albumin, glyceraldehyde-3-phosphate dehydrogenase, and trypsinogen.

Enzyme Assays. One unit of activity is defined as the amount of enzyme required to produce 1 μmol/min of product at 37 °C. AIR synthetase was quantitated by one of three methods: production of [^{32}P]P_i, ADP, or AIR (Schrimsher et al., 1986). GAR synthetase assays were carried out as described by Schrimsher et al. (1986).

Kinetic Studies. Initial velocity and product inhibition studies were carried out as described by Schrimsher et al. (1986), and the data were analyzed by using the BASIC versions of the computer programs of Cleland (1979).

^{18}O Transfer Experiment. To a 5-mm NMR tube containing 20 μmol of ATP, 40 μmol of magnesium acetate, 150 μmol of KCl, and 30 μmol of Hepes (pH 7.8) in a final volume of 1 mL (30% D_2O) was added 0.8 unit of AIR synthetase from *E. coli* (3.4 units/mg). An 80-MHz ^{31}P NMR spectrum was taken, then 5 μmol of [^{18}O,^{16}O]FGAM was added, and the NMR tube was incubated at 37 °C for 30 min. When the reaction was complete as determined by ^{31}P NMR, the reaction was applied to a DEAE-Sephadex A-25 column (0.4 × 2 cm), and the column was washed with 5 mL of water. The inorganic phosphate was eluted with 200 mM triethylammonium bicarbonate and concentrated to dryness in vacuo.

A ^{31}P NMR spectrum was taken at 80 MHz using D_2O as the lock solvent with a ±800-Hz sweep width (quadrature phase detection), a 90° pulse angle, and a 16K data block. The acquisition time was 2.8 s, and the pulse delay was 1 s. To enhance resolution, exponential multiplication with a line-broadening factor of 0.1 Hz was applied to the free induction decay before Fourier transformation.

N-Terminal Sequence. N-Terminal sequence analysis and amino acid composition were performed by the University of Wisconsin Protein Sequence and DNA Synthesis Facility. For N-terminal sequence analysis, 1 mg of purified AIR synthetase was subjected to carboxymethylation as described by Work and Burdon (1981). The analysis was performed on 40 μg of the modified protein. The purified protein was prepared for amino acid composition analysis by desalting 0.3 mg on a column of Sephadex G-25 equilibrated with doubly distilled, deionized water. The protein (100 μg) was lyophilized in a hydrolysis tube in preparation for the analysis.

Results and Discussion

Isolation of AIR Synthetase. AIR synthetase has been purified to homogeneity from *E. coli* strain TX393 which contains the multicopy plasmid pJS24 with a DNA insert which codes for this protein (Table I). From 15 g of bacteria, 90 mg of homogeneous protein can be isolated in 49% yield (Figure 1). A number of ATP–agarose affinity columns under a variety of conditions were investigated for their potential to aid in the purification of AIR synthetase. Only the C-8 linked ATP affinity column with an eight-carbon spacer proved useful. This contrasts with the results from isolation of AIR synthetase from chicken liver in which only the N-6-linked ATP–agarose column with a six-carbon spacer worked successfully (Schrimsher et al., 1986).

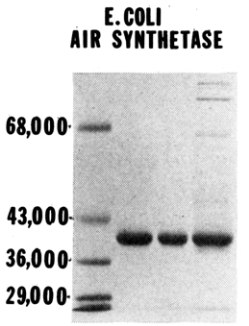


FIGURE 1: SDS gel electrophoresis (7.5% acrylamide) of *E. coli* AIR synthetase. (Lanes numbered from left to right.) Lane 1, molecular weight standards; lane 2, 30 μg of ATP affinity column purified protein (Table I); lane 3, 20 μg of ATP affinity column purified protein (Table I); lane 4, 21 μg of Sephadex G-100 column purified protein.

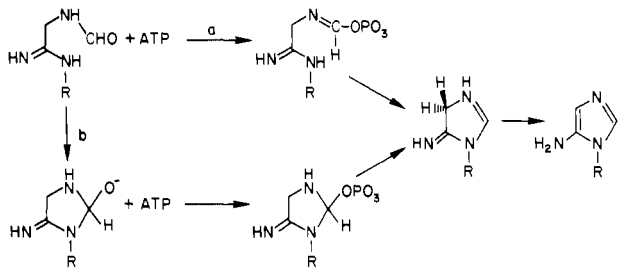
Characterization of AIR Synthetase. SDS gel electrophoresis of *E. coli* protein exhibits a single polypeptide of M_r 38 500 when compared with standards of known molecular weights (Figure 1). An apparent native molecular weight of the protein of 71 700 (Stokes radius = 32.2 Å, $s_{20,w}$ = 4.56 S) was obtained by using Sephadex G-100 chromatography and sucrose density ultracentrifugation. Thus, AIR synthetase from *E. coli* appears to be a dimer composed of two equivalent subunits. These results are quite different from those obtained by using similar methods to study the chicken liver AIR synthetase. The subunit molecular weight for this trifunctional protein is 110 000, and the native molecular weight is 330 000 by Sephadex chromatography and 133 000 on the basis of sucrose density ultracentrifugation. While the specific activity of the *E. coli* protein (3.35 μmol min^{-1} mg^{-1}) is 8 times higher than that of the chicken liver enzyme (0.4 μmol min^{-1} mg^{-1}), the turnover numbers of AIR synthetase from these sources are quite similar: 111 min^{-1} (*E. coli*) and 44 min^{-1} (chicken liver).

Since this protein had not been previously purified, we established that incubation of FGAM, ATP, and protein resulted in the production of a 1:1:1 mixture of P_i, ADP, and AIR. The P_i was quantitated by using [γ-^{32}P]ATP in the incubation mixture, isolating [^{32}P]P_i, and quantitating by scintillation counting. The ADP was quantitated by meauring the A_{340nm} ($\Delta\epsilon$ = 6200 M^{-1} cm^{-1}) using the coupled assay with pyruvate kinase and lactate dehydrogenase. The AIR was quantitated by using $\Delta\epsilon$ = 26 400 M^{-1} cm^{-1} and the Bratton–Marshall assay.

Intriguingly, the AIR synthetase isolated from chicken liver was recently reported by Daubner et al. (1985) to be a trifunctional protein containing in addition GAR synthetase and GAR transformylase activities. Therefore, the *E. coli* protein was assayed for these two activities and shown to possess neither. The fact that AIR synthetase is not physically linked with the GAR synthetase and GAR transformylase is consistent with the genetic mapping of these proteins (Bachmann, 1983).

Transfer of ^{18}O of Formyl-Labeled FGAM to P_i. In 1981, Satterthwait and Westheimer proposed the following general

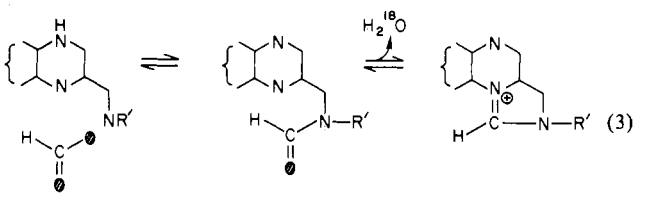
Scheme I: Proposed Mechanism for Conversion of FGAM and ATP to AIR, ADP, and P_i Catalyzed by AIR Synthetase



mechanism for amidotransferase enzymes which catalyze the conversion of a C=O to a C=N concomitant with cleavage of ATP to ADP and P_i (Scheme Ia,b). In Scheme Ia, ATP plays a kinetic role and attacks the carbonyl of the amide and activates it for nucleophilic attack, whereas in Scheme Ib a tetrahedral intermediate is formed which is subsequently trapped by ATP. Van der Saal et al. (1985) studying the amidotransferase CTP synthetase, using positional isotope exchange methodology, have provided evidence in support of Scheme Ia involving a phosphorylated nucleotide intermediate. Both of the proposed mechanisms postulate that the ^{18}O from the formyl moiety of FGAM will be transferred stoichiometrically to P_i.

To test this postulate, [*formyl*-^{18}O]FGAM was prepared from [^{18}O]FGAR by utilizing purified FGAM synthetase, which in turn was prepared biosynthetially by use of [^{18}O,^{13}C]formate. In addition to determining if ^{18}O from FGAM is transferred to phosphate during the conversion of FGAM to AIR catalyzed by AIR synthetase, analysis of the ^{18}O content of FGAR allowed us to draw some conclusions about the mechanism of the GAR formylation reaction and hence the enzymes involved in the conversion of GAR to FGAR in *E. coli*.

Previous studies by Dev and Harvey (1978) concluded that 10-formyltetrahydrofolate and not 5,10-methenyltetrahydrofolate was involved in the formyl transfer to GAR to produce FGAR in *E. coli*. Similar conclusions can be drawn from our biosynthetic preparation of labeled FGAR from labeled formate. Incubation of a 1:1 mixture of [^{16}O,^{13}C]formate and [^{18}O,^{13}C]formate with crude "enzymes" prepared from *E. coli*, which contain no folate source added, produced FGAR whose ^{13}C NMR spectum is shown in Figure 2A. The spectrum indicates the ^{16}O:^{18}O ratio in the formyl group is 1:1, identical, within experimental error, with that of the starting formate. If 5,10-methenyltetrahydrofolate was involved in the formylation, the formyl oxygen would have been lost during amidine formation from 10-formyltetrahydrofolate and replaced by an oxygen derived from the solvent (eq 3). Furthermore, since our present preparation of crude enzymes presumably contained 5,10-methylenetetrahydrofolate cyclohydrolase, the 10-formyltetrahydrofolate produced by 10-formyltetrahydrofolate synthetase must be more effectively trapped by GAR transformylase than the cyclohydrolase, or washout of ^{18}O label from FGAR would have been observed. Alternatively, the synthetase might exist in a complex with the transformylase so that channeling of 10-formyltetrahydrofolate directly to GAR may have occurred.



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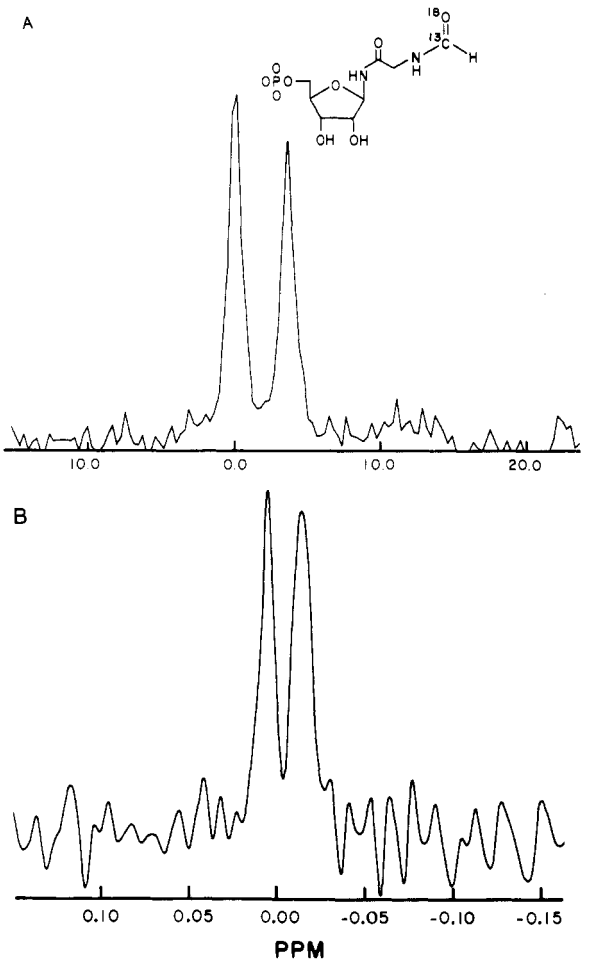

FIGURE 2: (A) Proton-decoupled 125-MHz ^{13}C NMR spectrum of [^{18}O,^{16}O,^{13}C]FGAR (structure) showing the extent of ^{18}O incorporation into the formyl group position. (B) 80-MHz ^{31}P NMR spectrum of P_i showing the extent of ^{18}O incorporation into the P_i isolated from the incubation of [^{16}O,^{18}O,^{13}C]FGAM, ATP, and AIR synthetase. The [^{16}O,^{18}O,^{13}C]FGAM was prepared biosynthetically from [^{16}O,^{18}O,^{13}C]FGAR.

Table II: Amino Acid Composition of *E. coli* AIR Synthetase[a]

amino acid	predicted[b]	experimental[c]
Asx	36	36.1
Thr	17	14.9
Ser	20	21.0
Glx	31	31.1
Pro	14	14.0
Gly	40	40.0
Ala	29	29
Val	37	20.8
Cys	7	5.2
Met	7	6.0
Ile	21	12.1
Leu	33	29.1
Tyr	9	8.0
Phe	7	6.2
Lys	18	17.0
His	8	7.7
Arg	11	11.2

[a] Expressed as residues per mole obtained from a 24-h hydrolysis in 6 N HCl and 0.2% (v/v) phenol. [b] From gene sequence data. [c] All values are normalized to alanine.

[^{16}O]FGAR/[^{18}O]FGAR (1:1) was then converted to FGAM by using purified FGAM synthetase from chicken liver (Schendel & Stubbe, 1986). The FGAM produced was not analyzed via ^{13}C NMR spectroscopy due to its instability but was isolated by anion-exchange chromatography to remove

Table III: Inhibition Patterns and Constants Obtained for Product Inhibition of the Reaction Catalyzed by *E. coli* Aminoimidazole Ribonucleotide Synthetase

variable substrate	constant substrate	inhibitor	inhibition type[a]	K_{ii} (mM)	K_{is} (mM)
ATP	FGAM (28 μM)	ADP	C		0.0110 ± 0.0018
FGAM	ATP (30 μM)	ADP	NC	0.0069 ± 0.0006	0.0165 ± 0.0033
ATP	FGAM (25 μM)	AIR	NC	0.119 ± 0.23	0.0404 ± 0.0083
FGAM	ATP (43 μM)	AIR	NC	0.0647 ± 0.0057	0.0654 ± 0.0031
ATP	FGAM (30 μM)	P_i	NC	176 ± 39	62 ± 14
FGAM	ATP (40 μM)	P_i	NC	54.5 ± 4.6	118 ± 26

[a] Abbreviations: C, competitive; NC, noncompetitive.

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(S,A,G,T)-D-K-T-(S>A)-L-(s)-Y-K-D-A-G-V-D-i-(d)-A-G-N-A      protein

V-T -D-K-T-  S  -L- S -Y-K-D-A-G-V-D-I- D -A-G-N-A      gene
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FIGURE 3: N-Terminal sequence of AIR synthetase.

the P_i and then incubated immediately with AIR synthetase. The reaction was followed by ^{31}P NMR spectroscopy, and upon completion, the P_i was isolated by anion-exchange chromatography and analyzed by ^{31}P NMR spectroscopy (Figure 2B). The spectrum indicates a 1:1 mixture of $[^{16}O]P_i/[^{18}O]P_i$, identical with the ratio of the starting labeled FGAR. Thus, AIR synthetase from *E. coli*, as in the case of the chicken liver protein, catalyzes transfer of the oxygen of the formyl group to inorganic phosphate.

N-Terminal Sequence. The N-terminal sequence of AIR synthetase, prepared as described under Experimental Procedures, was determined by automated Edman degradation. The results are indicated in Figure 3. In Figure 3, the lower case letters show less confidence, and the parentheses indicate little confidence. Comparison of the N-terminal protein sequence with the DNA sequence allowed Smith et al. (unpublished results) to define the start of the open-reading frame for the gene coding for AIR synthetase. The N-terminal amino acid sequence derived from this gene sequence (Figure 3) is in excellent agreement with that obtained by Edman degradation. Furthermore, these data suggest that the start of the open-reading frame is at the valine codon at base pairs 780–782 of the 1.8-kilobase *Hin*cII fragment and that the protein may be processed by an exopeptidase to give an N-terminal threonine, observed by N-terminal analysis of the protein.

In addition, the amino acid composition data (Table II) obtained for AIR synthetase were in agreement with those predicted from the gene sequence. The values obtained for the branched-chain hydrophobic amino acids, isoleucine and valine, were lower than predicted, due to the short time of the acid hydrolysis. Previous studies have shown that Val–Val, Ile–Ile, and Ile–Val are the most difficult peptide bonds to hydrolyze under acidic conditions (Mitchel et al., 1970; Christensen, 1943; Blackburn, 1978). There are 11 of these sequences in this protein.

Kinetic Studies. In order to determine the optimum conditions for kinetic analysis of AIR synthetase, the pH dependence and metal ion specificity were investigated. The activity of the protein did not vary significantly between pH 7.5 and 8.3, with an optimum at pH 7.8. In addition, both K^+ and free Mg^{2+} ions were found to be absolutely required for catalytic activity with apparent K_m values of 96 and 0.3 mM, respectively. These results are quite similar to those for the chicken liver enzyme.

Initial velocity studies on the AIR synthetase reaction using β-FGAM and MgATP revealed a series of intersecting lines. The kinetic constants derived from these studies are K_m = 0.065 mM for MgATP, K_m = 0.027 mM for β-FGAM, and V_{max} = 3.5 μmol min^{-1} mg^{-1}. Product inhibition by AIR, P_i, and ADP was also investigated and gave the results shown in Table III.

The results of these product inhibition and initial velocity studies suggest a sequential mechanism in which ATP binds first to the enzyme and ADP is released last. Unfortunately, due to the irreversibility of the overall reaction and the lack of FGAM analogue inhibitors, further dissection of the kinetic mechanism is presently not possible. The results of these kinetic investigations, however, contrast with those found with the chicken liver enzyme. In the trifunctional enzyme case, the initial velocity studies gave parallel lines, and ADP was an uncompetitive inhibitor with respect to FGAM. Interpretation of the chicken liver data is complicated by the existence of an additional ATP binding site involved in the GAR synthetase reaction. However, ADP was a competitive inhibitor with respect to ATP, analogous to the results with the *E. coli* protein. An additional similarity between *E. coli* and chicken liver AIR synthetase is their ability to utilize ATPγS as substrate. In the case of the *E. coli* protein, V_{max} is 0.0003 the rate of turnover with ATP; K_m is 172 μM. These results suggest that phosphorus stereochemistry may be of use to investigate the possible existence of a phosphorylaed enzyme intermediate in this reaction.

SUMMARY

AIR synthetase from *E. coli* has been purified to homogeneity for the first time. The enzyme is a dimer (M_r 71 700) composed of two equivalent protomers. Although the turnover number for the conversion of FGAM to AIR is nearly identical with that reported for the recently purified chicken liver protein, the similarities end there. The chicken liver AIR synthetase is a trifunctional protein with a subunit molecular weight of 110 000, containing GAR synthetase and GAR transformylase activities in addition to AIR synthetase activity. Furthermore, the kinetic properties of these two AIR synthetases are substantially different. The role of each of these enzymes in the purine biosynthetic pathway and how these structures relate to their function are presently under investigation.

Registry No. P_i, 14265-44-2; β-FGAM, 6157-85-3; AIR, 25635-88-5; ADP, 58-64-0; MgATP, 1476-84-2; AIR synthetase, 9023-53-4.

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Covalent Modification of the Inhibitor Binding Site(s) of *Escherichia coli* ADP-Glucose Synthetase: Specific Incorporation of the Photoaffinity Analogue 8-Azidoadenosine 5′-Monophosphate†

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ABSTRACT: The photoaffinity agent 8-azidoadenosine 5′-monophosphate (8-N_3AMP) is an inhibitor site specific probe of the *Escherichia coli* ADP–glucose synthetase (ADPG synthetase). In the absence of light, 8-N_3AMP exhibits the typical reversible allosteric kinetics of the physiological inhibitor AMP. In the presence of light (254 nm), the analogue specifically and covalently modifies the enzyme, and photoincorporation is linearly related to loss of catalytic activity up to at least 65% inactivation. The substrate ADPG provides nearly 100% protection from 8-N_3AMP photoinactivation, while the substrate ATP provides approximately 50% protection and the inhibitor AMP, approximately 30% protection. These three adenylate allosteric effectors of *E. coli* ADPG synthetase also protect it from photoincorporation of 8-N_3AMP. A structural overlap of the inhibitor and substrate binding sites is proposed which explains the protection data in light of the known binding and kinetic properties of this tetrameric enzyme.

Adenosine diphosphate glucose (ADPG)[1] synthetase (EC 2.7.7.27) catalyzes the first committed reaction in the metabolic synthesis of glycogen in *Escherichia coli*, ATP + α-glucose-1-P $\rightleftharpoons$ ADPG + PP_i (Preiss, 1973, 1978). An allosterically regulated enzyme, its physiological activator is Fru-P_2, and it is inhibited by AMP, ADP, and P_i (Gentner et al., 1969; Govons et al., 1973; Haugen et al., 1974; Preiss et al., 1966). The complete amino acid sequence of this enzyme was deduced by sequencing of various CNBr peptides and complete nucleotide sequencing of the structural *glg* C (Baecker et al., 1983). The enzyme consists of four identical subunits with a molecular weight each of 48 762 (Baecker et al., 1983; Haugen et al., 1976).

In previous chemical modification studies, the allosteric activator pyridoxal phosphate was used as an affinity label to

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[1] Abbreviations: 8-N_3AMP, 8-azidoadenosine 5′-monophosphate; 8-N_3ATP, 8-azido-ATP; 8-N_3ADPG, 8-azido-ADP-glucose; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Fru-P_2, fructose 1,6-bisphosphate; BPA, bovine plasma albumin; EDTA, ethylenediaminetetraacetic acid; ADPG, adenosine diphosphate glucose; DTE, dithioerythritol; HPLC, high-performance liquid chromatography; MSAP, mouse submaxillary arginyl protease; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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