Nucleotide Complexes of *Escherichia coli* Phosphoribosylaminomimidazole Succinocarboxamide Synthetase*

Received for publication, March 6, 2006, and in revised form, May 8, 2006 Published, JBC Papers in Press, May 9, 2006, DOI 10.1074/jbc.M602109200

Nathaniel D. Ginder, Daniel J. Binkowski, Herbert J. Fromm, and Richard B. Honzatko

From the Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, Iowa 50011

Phosphoribosylaminomimidazole-succinocarboxamide synthetase (SAICAR synthetase) converts 4-carboxyl-5-aminoimidazole ribonucleotide (CAIR) to 4-(N-succinylcarboxamide)-5-aminoimidazole ribonucleotide (SAICAR). The enzyme is a target of natural products that impair cell growth. Reported here are the crystal structures of the ADP and the ADP target of natural products that impair cell growth. Reported are the crystal structures of the ADP and the ADP complex of SAICAR synthetase from *Escherichia coli*, the latter being the first instance of a CAIR-ligated SAICAR synthetase. ADP and CAIR bind to the active site in association with three Mg$^{2+}$, two of which coordinate the same oxygen atom of the 4-carboxyl group of CAIR; whereas, the third coordinates the α- and β-phosphoryl groups of ADP. The ADP-CAIR complex is the basis for a transition state model of a phosphoryl transfer reaction involving CAIR and ATP, but also supports an alternative chemical pathway in which the nucleophilic attack of L-aspartate precedes the phosphoryl transfer reaction. The polypeptide fold for residues 204–221 of the *E. coli* structure differs significantly from those of the ligand-free SAICAR synthetase from *Thermatoga maritima* and the adenine nucleotide complexes of the synthetase from *Saccharomyces cerevisiae*. Conformational differences between the *E. coli*, *T. maritima*, and yeast synthetases suggest the possibility of selective inhibition of *de novo* purine nucleotide biosynthesis in microbial organisms.

*This work was supported in part by National Institutes of Health Research Grant NS 10546. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (codes 2GQR and 2GQS) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

To whom correspondence should be addressed. Tel.: 515-294-6116; Fax: 515-294-0453; E-mail: honzatko@iastate.edu.

1 The abbreviations used are: SAICAR, 4-(N-succinylcarboxamide)-5-aminoimidazole ribonucleotide; CAIR, 4-carboxy-5-aminoimidazole ribonucleotide; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; AIR, 5-aminoimidazole ribonucleotide; eSS, *Escherichia coli* SAICAR synthetase; tSS, *Thermatoga maritima* SAICAR synthetase; ySS, *Saccharomyces cerevisiae* SAICAR synthetase.

Phosphoribosylaminomimidazole-succinocarboxamide synthetase (EC 6.3.2.6, 5′-phosphoribosyl-4-carboxyl-5-aminoimidazole-1-carboxylase) (SAICAR synthetase)$^{2}$ catalyzes the eighth step in bacterial *de novo* purine nucleotide biosynthesis, ATP + L-aspartate + CAIR → ADP + P$_{i}$ + SAICAR. Lukens and Buchanan (1) first described the enzyme in 1959. In 1962 Miller and Buchanan (2) demonstrated its presence in a variety of life forms and reported the purification and properties of the synthetase from chicken liver. More recently, the Stubbe laboratory (3) purified SAICAR synthetase from *Escherichia coli*. The *E. coli* enzyme exhibits a rapid equilibrium random kinetic mechanism (4). SAICAR synthetase from *Saccharomyces cerevisiae* is a monomer (5–8) and that from *Thermatoga maritima* a dimer (9). Comparable enzymes from vertebrates have masses in excess of 330 kDa and possess 6–8 identical subunits of 47 kDa (10, 11). The vertebrate systems are bifunctional, combining 5-aminoimidazole ribonucleotide carboxylase (AIR carboxylase) and SAICAR synthetase activities (10–12).

L-Alanosine can replace L-aspartate as a substrate both *in vitro* and *in vivo* for SAICAR synthetase (4, 13, 14). The product of the SAICAR synthetase reaction, 1-alanosyl-5-amino-4-imidazolocarboxylic acid ribonucleotide, is a potent inhibitor of adenosuccinate synthetase and adenylosuccinate lyase, being the compound responsible for L-alanosine toxicity (13). Many cancers (~30% of all T-cell acute lymphocytic leukemia, for instance) lack a salvage pathway for adenine nucleotides and rely entirely on *de novo* biosynthesis (15). L-Alanosine is toxic to cell lines of such cancers at concentrations well below those that poison cells with intact salvage pathways. Hence, L-alanosine may be effective as a chemotherapeutic agent in combination with other drugs (15).

Differences in subunit size, function, and assembly of microbial and vertebrate SAICAR synthetases suggest the potential for selective inhibition of SAICAR synthetases and, hence, the possibility of new antibiotics. Efforts to further develop specific inhibitors of microbial SAICAR synthetases would benefit from a basic understanding of structure-function relations; however, for SAICAR synthetase such information is lacking. To this end, we report the structures of the ADP and ADP-CAIR complexes of *E. coli* SAICAR synthetase (hereafter, eSS). The latter complex is the first structure of a CAIR-bound SAICAR synthetase and reveals a previously unsuspected requirement for Mg$^{2+}$ in the recognition of CAIR by the synthetase. The CAIR-ADP complex is consistent with a chemical mechanism composed of two partial reactions, a phosphoryl transfer from ATP and a nucleophilic attack by L-aspartate, but the relative order of the two reactions is unclear. Moreover, the conformation of eSS differs significantly from that of ligand-free SAICAR synthetase from *T. maritima* in the region of the CAIR binding site, suggesting the possibility of substrate-induced conformational changes in microbial synthetases.
**Statistics of data collection**

|             | Inflection (E1) | Peak (E2) | Remote (E3) |
|-------------|----------------|-----------|-------------|
| Wavelength (Å) | 0.97900 | 0.97884 | 0.98671 |
| Resolution (Å)  | 46.4–2.00 (2.07–2.00) | 46.4–2.20 (2.28–2.00) | 46.4–2.00 (2.07–2.00) |
| Reflections measured  | 276,309 | 215,947 | 286,076 |
| Reflections unique  | 40,967 | 31,041 | 40,775 |
| Redundancy  | 6.74 (5.28) | 6.96 (7.05) | 7.02 (5.42) |
| % Completeness | 99.7 (97.2) | 100.0 (100.0) | 99.6 (95.8) |
| Rmerge  | 0.133 (0.515) | 0.107 (0.345) | 0.067 (0.331) |
| Rmerge (i/i) | 7.9 (2.6) | 10.3 (4.5) | 16.3 (4.7) |
| Rmerge (f/f) | −15.2 | −9.45 | −4.8 |
| Rmerge (g/g) | 6.4 | 10.5 | 0.5 |

\[ R_{merge} = \sum_i \sum_j |I_{ij} - \langle i\rangle|/\sum_i \sum_j I_{ij} \]
\[ R_{merge}^{\text{merge}} = \sum_i \sum_j |I_{ij} - \langle i\rangle|/\sum_i \sum_j I_{ij} \]

**Statistics of refinement**

|                      | ADP complex | ADP-CAIR complex |
|----------------------|-------------|------------------|
| Space group          | P2₁ 2₁ 2₁  | P2₁ 2₁ 2₁        |
| Unit cell parameters |             |                  |
| a (Å)                | 59.42       | 59.43            |
| b (Å)                | 67.13       | 67.12            |
| c (Å)                | 148.5       | 149.3            |
| Resolution           | 25–2.00     | 25–2.05          |
| No. of reflections   | 286,076     | 205,397          |
| No. of unique reflections | 40,775     | 34,593          |
| % Completeness       | 99.6 (95.8) | 90.4 (61.7)      |
| Rmerge               | 0.067 (0.331) | 0.059 (0.280) |
| No. of atoms         | 4207        | 4095             |
| No. of solvent sites | 363         | 197              |
| Rmerge (i/i)         | 24.0        | 26.3             |
| Rmerge (f/f)         | 25          | 31               |
| Mean B for protein (Å²) | 1.3        | 1.3              |
| Mean B for ligands (Å²) | 22.5       | 22.6             |
| Mean B for waters (Å²) | 1.98       | 1.86             |

**Experimental Procedures**

**Materials**—ATP, l-aspartate, NADH, phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase were purchased from Sigma. CAIR was synthesized as described previously (4). E. coli strain BL21(DE3) came from Invitrogen.

**Enzyme Preparation**—Selenomethionine substitution in eSS employed the inhibition of methionine biosynthesis coupled with selenomethionine supplementation (16). BL21(DE3) cells were transformed with a pET 28b vector containing the eSS insert with an N-terminal His₆ tag (4). All bacterial cultures contained 30 µg/ml kanamycin sulfate (Invitrogen). An overnight culture was prepared in LB media (Sigma), and the cells were isolated by centrifugation (1500 × g for 10 min). The pellet was re-suspended in 24 ml of M9 media, supplemented with 1 mM MgSO₄, 0.3 mM FeSO₄, and 0.5 µM thiamin. Four ml of inoculant culture was added to each flask containing 650 ml of supplemented M9 media. The flasks were shaken at 37 °C to an A₆₀₀ of 0.8. The temperature was adjusted to 16 °C, and 35 mg each of L-leucine, L-isoleucine, and L-valine, and 65 mg each of L-phenylalanine, L-lysine, and L-threonine were added as solids to each flask. After shaking for 20 min, 2 ml of a 20 mg/ml solution of L-selenomethionine was added to each flask. Isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM after an additional 15 min of agitation. Cells were isolated after 18 h by centrifugation (1500 × g, 10 min), re-suspended in 10 mM KP, (pH 7.0), centrifuged again, and finally re-suspended in 100 ml of lysis buffer containing 50 mM KP, 300 mM NaCl, and 10 mM imidazole (pH 8.0). Cells were disrupted by sonication in the presence of 0.25 mg/ml lysozyme, 50 µg/ml DNase I, 1 ml of 100 mM phenylmethanesulfonyl fluoride in isopropyl alcohol, and 5 µg/ml leupeptin. The lysate was centrifuged (33,000 × g, 1 h) and the supernatant fluid loaded onto 25 ml of nickel-nitrioteracetic acid-agarose (Novagen), pre-equilibrated in lysis buffer. The column was washed sequentially with 2 column volumes each of lysis buffer, lysis buffer containing 20 mM imidazole, and lysis buffer containing 40 mM imidazole. eSS was subsequently eluted from the column with lysis buffer containing 250 mM imidazole. Immediately upon elution, dithiothreitol and EDTA were added to the fractions to final concentrations of 5 and 10 mM, respectively. Fractions were pooled and dialyzed overnight in buffer containing 15 mM Tris-HCl, 25 mM KCl, 5 mM.
MgCl₂, 5 mM dithiothreitol, and 5 mM EDTA (pH 8.0). Native protein was prepared using an identical protocol, except cell growth and expression was done in LB media without amino acid supplements.

Protein concentration was determined by the method of Bradford (17) using bovine serum albumin as a standard. Protein purity was confirmed by SDS-PAGE (18). Mass determinations of purified protein were done by the Iowa State University core facility using an Applied Biosystems Voyager System 6075 matrix-assisted laser desorption/ionization time-of-flight mass spectrometer. The specific activity of eSS was determined using previously described assay conditions (4). The dependence of velocity on the concentration of Mg²⁺/H₁₁₀₀₁ was investigated using saturating substrate concentrations (300 μM ATP, 65 μM CAIR, and 7.5 mM L-aspartate), with concentrations of free Mg²⁺ ranging from 90 to 7000 μM.

**Crystallization**—Crystals were grown by the method of hanging-drop vapor diffusion in VDX plates (Hampton Research). Two μl of protein solution (15 mg/ml protein, 15 mM Tris-HCl, 25 mM KCl, 55 mM MgCl₂, 50 mM ADP, 5 mM dithiothreitol, and 5 mM EDTA, pH 8.0) were mixed with 2 μl of well solution (3.4–3.8 M sodium formate and 50 mM Tris-HCl, pH 8.5) and allowed to equilibrate against 0.5 ml of well solution. Crystallization experiments for the ADP complex and CAIR·ADP complex employed selenomethionine-substituted and native proteins, respectively.

**Data Collection**—For the ADP complex, crystals were transferred to a cryoprotectant solution containing 4 M sodium formate, 50 mM Tris-HCl (pH 8.5), 25 mM MgCl₂, 25 mM ADP, and 10% (w/v) sucrose. This buffer was supplemented with 1 mM CAIR and 10 mM L-aspartate for the CAIR·ADP complex. After ~30 s of equilibration, crystals were plunged into liquid nitrogen.

For the ADP complex, MAD data were collected on Beamline 4.2.2 of the Advanced Light Source, Lawrence Berkeley Laboratory. Complete anomalous sets were taken at wavelengths of peak absorbance, the inflection point, and remote from the absorption edge of selenium. Data were indexed, integrated, scaled, and merged using d*trek (19). Intensities were converted to structure factors using the CCP4 (20) program TRUNCATE.

Data from the CAIR·ADP complex were collected at Iowa State University from a single crystal (temperature, 115 K) on a Rigaku R-AXIS IV+ rotating anode/image plate system using CuKα radiation from an Osmonic confocal optics system. Data were processed and reduced using the program package CrystalClear provided with the instrument. Intensities were converted to structure factors using the CCP4 program TRUNCATE.

**Structure Determination and Refinement**—Structure determination for the selenomethionine-replaced protein was accomplished using the SOLVE/RESOLVE software package (21, 22). Electron density was modeled as polyalanine by SOLVE, followed by manual fitting using XTALVIEW (23). Refinement was performed against the structure factors from the remote wavelength using CNS (24). Non-crystallographic restraints were not used during refinement. Refinement began with a cycle of simulated annealing (starting temperature of 3500 K) with slow cooling in increments of 25 K to a final temperature of 300 K, followed by

**FIGURE 1. Structures of SAICAR synthetases.** Left, the active site of eSS is a deep cleft that extends without interruption between subunits of the dimer. Bold lines and filled circles represent bound ADP-Mg²⁺ and CAIR-Mg²⁺. Center, the relative position and orientation of helix α5 of tSS (dark gray) differs significantly from that of helix α5 of eSS. Right, four sequence inserts (dark gray) described under “Results” are mapped onto the ySS monomer. Parts of this figure were drawn with MOLSCRIPT (42).
100 steps of conjugate gradient energy minimization. Subsequent cycles had lower initial starting temperatures (as low as 500 K). Individual thermal parameters were refined after each cycle of simulated annealing and subject to the following restraints: bonded main chain atoms, 1.5 Å²; angle main chain atoms, 2.0 Å²; bonded side chain atoms, 2.0 Å²; and angle side chain atoms, 2.5 Å². Water molecules were automatically added using CNS if a peak greater than 3.0 was present in Fourier maps with coefficients $\left( F_{\text{obs}} - F_{\text{calc}} \right)^2$. Refined water sites were eliminated if they were further than 3.2 Å from a hydrogen-bonding partner or if their thermal parameters exceeded 50 Å². The contribution of the bulk solvent to structure factors was determined using the default parameters of CNS. Constants of force and geometry for the protein came from Engh and Huber (25) and those for ADP from CNS resource files with appropriate modification of dihedral angles of the ribosyl moiety to maintain a $\beta$-endo ring conformation.

For the native CAIR-ADP complex, molecular replacement was performed using AMORE with the ADP complex as the starting model. Refinement was performed as for the ADP complex. Routines in the CCP4 suite of programs were used in the calculation of surface areas and in the superposition of structures.

RESULTS

Protein Preparation, Data Collection, and Structure Determination—Selenium-modified and native eSS were pure on the basis of SDS-PAGE. The specific activity of the selenomethionine-substituted protein was 15 ± 1 units/mg, comparable with that of the native protein (4). Mass spectrometry of native and selenomethionine-substituted proteins indicated 8.5 (relative to a maximum of 10) selenium atoms per monomer. SOLVE initially located 17 selenium sites, generating a phase set with a figure-of-merit of 0.37. Iterations of density modification by RESOLVE increased the figure-of-merit to 0.67. Statistics of data collection and refinement are in Tables 1 and 2.

Overview of eSS Structure (Protein Data Bank Identifiers 2GQR and 2GQS)—An eSS homodimer occupies the crystallographic asymmetric unit. The subunits of the dimer are virtually identical with a superposition of all Cα atoms yielding a root mean square deviation of 0.34 Å for both nucleotide complexes. No electron density is present for the polyhistidyl tag. Observable electron density begins with Met1 and continues to the C terminus (Asp237). Electron density is weak only for residues 35–39 of the ADP complex but strong for the same segment in the ADP-CAIR complex.
Domain 1 of the eSS fold (Fig. 1) consists of a β-sheet (strands β1-β3, β6, and β7) with its inter-strand connections (helix α1 and an anti-parallel loop β4–β5). Domain 2 consists of a β-sheet (strands β8–β13) and associated helices α2–α6. The β-sheet of domain 1 curls (like the fingers of a right hand relative to its palm) over domain 2, creating a cleft, half of which is filled by ADP-Mg^{2+} and the other half by CAIR. The subunits come together with 2-fold symmetry forming a dimer that buries ~2400 Å² of surface at the interface.

The major structural difference between the ADP and ADP-CAIR complexes is the aforementioned levels of electron density associated with residues 35–39. Superposition of all Cα carbons of subunit A from the ADP and ADP-CAIR complexes gives a root mean square difference of 0.18 Å and a maximum displacement of 0.67 Å. The former value is comparable with the coordinate uncertainty of 0.25 Å determined by the CCP4 program SFCHECK. The high level of agreement occurs despite the difference in ligation, and infers selenomethionine substitution in the ADP complex causes little perturbation to the structure. The largest Ca displacements (0.7 Å) are in the loop (residues 124–130) that coordinates metal ions associated with CAIR and for residues in the vicinity of the 5’-phosphoryl group of CAIR. The conformation of the adenine nucleotide and its interactions with the protein are identical (within coordinate uncertainty) in the ADP and ADP-CAIR complexes.

Comparison of eSS to tSS—eSS (237 residues) and tSS (PDB identifier 1KUT, 230 residues) share 39% sequence identity. tSS, like eSS, is a dimer (Fig. 1). Cα atoms of the eSS and tSS subunits superimpose with a root mean square deviation of ~1.2 Å, using the sequence alignment of Fig. 2; however, the polypeptide fold associated with segment 204–221 of eSS, which includes strand β13 and helix α5, differs strikingly from that of tSS (Figs. 1 and 3). The alternative fold of tSS exposes six hydrophobic residues and increases the solvent-accessible surface area of each subunit by ~1000 Å² (from 1220 Å² in eSS to 2200 Å² in tSS).

Unlike the alternative fold of tSS, the eSS fold has an extensive network of hydrogen bonds. Interacting residues fall in two clusters: Asp202, Arg231, and Thr205 and Arg39, Asp175, Arg199, Asp210, Lys211, Asp212, Arg213, and Arg215. The latter more extensive cluster apparently anchors helix α5 with respect to domains 1 and 2, while positioning hydrophilic side chains in the active site cleft of eSS. In contrast, helix α5 in tSS is displaced relative to that of eSS (Fig. 3), taking residues corresponding to Arg199, Lys211, and Arg215 away from the active site. Most of the residues in segment 204–221 of eSS are conserved among microbial SAICAR synthetases; for instance, Asp175, Arg199, Asp210, Lys211, Asp212, and Arg215 are conserved and present in tSS.

Comparison of eSS to ySS—SAICAR synthetase from S. cerevisae (ySS) has 69 more amino acids than eSS, appearing pri-
marily as insertions before residues 1, 77, 105, and 221 of the E. coli synthetase (Figs. 1 and 2). Neglecting insertions, eSS and ySS are 27% identical in sequence, and superimpose with a root mean square deviation of 3.3 Å. The first and second sequence insertions come together in ySS (PDB identifiers 1OBD, 1OBG, and 1A4B), where they define a putative binding site for AMP. (AMP appears in good electron density only at a lattice contact in 1OBG. Hence, the functional significance of the first two insertions in the ySS sequence remains unclear.) The third insertion occurs at the subunit interface of the eSS dimer and probably blocks the dimerization of ySS subunits. The fourth insertion extends the helix corresponding to /H9251 of eSS and the connecting segments at the N- and C-terminal ends of that helix. The fourth segment replaces residues 204–221 in eSS but, nonetheless, retains a functional active site.

**Adenine Nucleotide Interactions**—ADP-Mg²⁺ binds to eSS in an anti-conformation (Fig. 4). Val¹⁵, Leu²⁴, Leu²⁶, and Val⁸¹ are in contact with one side of the adenine base, whereas Met⁸⁶ packs against the other. Atom N-1 of ADP binds to the backbone amide group of Leu⁸⁴, and atom N-6 binds to the backbone carbonyl group of Lys⁸² and the side chain of Gln⁶⁹ (Table 3). No side chain interaction between atom N-6 and the protein was reported for ySS (PDB identifiers 1OBG and 1OBD); however, His⁷² of the ySS structurally corresponds to Gln⁶⁹ of eSS and is in a position to interact with the adenine nucleotide. This position is conserved as glutamine or histidine in microbial systems.

The ribosyl moiety is C₂-endo, as observed for the adenine nucleotides in ySS structures. Atom O-2 of the ribose binds to Glu¹⁷⁹, corresponding to an equivalent interaction with Glu²¹⁹ in ySS.

The polyphosphoryl group of the adenine nucleotide interacts with strands β₁ and β₂, which together constitute a P-loop motif (26, 27). The α-phosphoryl group interacts with backbone amide groups of Lys¹¹, Ala¹², and Lys¹³, with atom NZ of Lys¹³, and with Mg²⁺ (hereafter, Mg²⁺ site 1). The β-phosphoryl group interacts with the backbone amide group and side chain of Lys¹¹, the amino group of Lys¹², and Mg²⁺ site 1. Four water molecules complete the octahedral coordination sphere of the Mg²⁺ site 1 (Table 4). Lys¹³, Glu¹⁷⁹, Lys¹⁷⁷, and Asp¹⁹¹ form additional hydrogen bonds with the hydrated magnesium.

Although adenine nucleotides in ySS and eSS are in proximity to corresponding residues, significant differences are evident. Structural superpositions using the β-sheet of domain 2 reveal displacements in domain 1 by as much as 4 Å, with the

**TABLE 3**

Selected polar contacts involving ligands

| Ligand atom | Contact partner | Distance Å |
|-------------|----------------|------------|
| ADP N-1     | Leu⁴⁸ N        | 3.06       |
| N-6         | Gln⁶⁹ OE1      | 2.62       |
| N-7         | Lys⁷⁵ O        | 2.98       |
| O-2'        | Asp⁵⁶ N        | 3.03       |
| O-1A        | Lys¹¹ N        | 3.03       |
| O-1B        | Ala¹² N        | 2.70       |
| O-2A        | Lys¹³ NZ       | 2.73       |
| O-1B        | Lys¹³ NZ       | 2.75       |
| O-2B        | Lys¹³ NZ       | 2.68       |
| CAIR O-3A   | Ser¹⁰⁰ OG      | 2.64       |
| O-2A        | Arg⁶⁴ NH2      | 2.61       |
| O-3'        | Asp⁷⁶ OD1      | 2.62       |

**TABLE 4**

Coordination distances and coordinating atoms of Mg²⁺ at sites 1–3

| Mg²⁺ site 1 | Mg²⁺ site 2 | Mg²⁺ site 3 |
|-------------|-------------|-------------|
| ADP O-2A    | 2.21 Asp¹²⁹ OD2 | 2.12 Asp¹²⁹ OD2 | 2.16 |
| ADP O-3B    | 2.09 Glu⁰⁰ OE1 | 2.06 Glu⁰⁰ OE1 | 2.17 |
| Formate O1  | 2.02 C⁴⁴ R-8  | 2.07 C⁴⁴ R-8  | 2.12 |
| Wat¹        | 2.10 C⁴⁴ R-3  | 2.16 Wat¹¹ | 2.19 |
| Wat²        | 2.22 Wat¹   | 2.13 Wat¹² | 2.01 |
| Wat³        | 2.25 Wat¹⁰  | 2.01 Formate O2 | 2.25 |

**FIGURE 5. Enzyme-bound CAIR.** Left and center, stereoview of CAIR in which dotted lines represent donor-acceptor interactions. Asp²¹² is shown but not labeled. Parts of this figure were drawn with MOLSCRIPT (42). Right, omit electron density (contour level of 1 σ with a cutoff radius of 1 Å) covering formate, hydrated Mg²⁺, and CAIR. Filled circles are Mg²⁺, and crosses are water molecules. Parts of this figure were drawn with XTALVIEW (23).
Structure of SAICAR Synthetase

eSS structure being more tightly closed about its adenine nucleotide relative to the ySS structures. In 1OBD of ySS (ATP-Mg\(^{2+}\) introduced by soaking), a lattice neighbor hydrogen bonds with the P-loop and is in proximity to bound ATP-Mg\(^{2+}\). In 1OBG (ATP-Mg\(^{2+}\) introduced by co-crystallization), the intrusive lattice contact is gone, but the active site has AMP and a sulfate anion. The \(\alpha\)-phosphoryl group of ADP-Mg\(^{2+}\) in eSS, a sulfate anion in 1OBG, and a water molecule in 1OBD occupy corresponding sites; whereas, the \(\beta\)-phosphoryl group of ADP-Mg\(^{2+}\) in eSS and the \(\gamma\)-phosphoryl group of ATP-Mg\(^{2+}\) in 1OBD occupy equivalent sites.

Interactions of CAIR—The CAIR molecule and its two associated Mg\(^{2+}\) atoms are covered by strong electron density (Fig. 5). The 5’-phosphoryl group of CAIR interacts with the side chains of Arg\(^{294}\), Ser\(^{100}\), and Arg\(^{199}\) as well as the backbone amide group of Ser\(^{100}\). These interactions resemble those of the sulfate anion in ySS. The phosphoryl group is near the N-terminal end of helix \(\alpha_2\), a structural element often observed in the binding of phosphoryl groups (28). Hydrogen bonds between the 5’-phosphoryl group of CAIR and the protein involve only two of its terminal oxygen atoms; the third hydrogen bonds with a water molecule that in turn interacts with a hydrated Mg\(^{2+}\) associated with CAIR (hereafter, Mg\(^{2+}\) site 2).

The ribosyl moiety of CAIR is C2’-endo. Its 3’-hydroxyl group hydrogen bonds with Asp\(^{175}\) and its 2’-hydroxyl group interacts with Arg\(^{215}\) and the backbone carbonyl of Asp\(^{196}\).

The base moiety of CAIR interacts extensively with the active site by way of octahedrally coordinated Mg\(^{2+}\) at sites 2 and 3 (Fig. 6). The side chain of Glu\(^{90}\) bridges between the two metal sites, as do single oxygen atoms from the 4-carboxyl group of CAIR and the carboxyl side chain of Asp\(^{129}\). Atoms N-3 of CAIR coordinates to Mg\(^{2+}\) site 2, whereas a formate molecule bridges Mg\(^{2+}\) sites 1 and 3. Water molecules occupy all other coordination positions of the metals.

Water molecules associated with metals at sites 2 and 3 hydrogen bond with Asp\(^{36}\) and Asp\(^{125}\). In fact, the appearance of strong electron density for residues 35–39 in the ADP-CAIR complex may be due to interactions of Asp\(^{36}\) with one water molecule in each of the inner coordination spheres of the metals (Fig. 7). Asp\(^{36}\) is in a loop that probably binds \(\lambda\)-aspartate. The interactions of Asp\(^{36}\) appear in concert with several new hydrogen bonds between the backbone elements of Gly\(^{35}\), Gly\(^{37}\), Ala\(^{38}\), and Arg\(^{29}\) and the side chain of Ser\(^{33}\).

The Mg\(^{2+}\) requirement observed here for substrate recognition is consistent with findings from kinetics. Plots of reciprocal velocity versus 1/[Mg\(^{2+}\)] and 1/[Mg\(^{2+}\)]\(^2\) are nonlinear; however, the plot of reciprocal velocity versus 1/[Mg\(^{2+}\)]\(^3\) is linear with a regression \(R\) value of 0.99 (data not shown).

DISCUSSION

Nucleotide complexes presented here are probably the closest representations of a productive substrate-enzyme complex for a SAICAR synthetase to date. The number of direct hydrogen bonds between ADP-Mg\(^{2+}\) and protein in the eSS structure (a total of 12) exceeds that for the ySS structures (8 for 1OBD and 7 for 1OBG). Additional nucleotide-protein interactions correlate with the more closed active site in the eSS relative to ySS. Moreover, lattice contacts in 1OBD of ySS could prevent the relaxation of its P-loop in the presence of ATP-Mg\(^{2+}\), and sulfate could well interfere with the recognition of the adenine nucleotide in all complexes of ySS. The recognition of the adenine nucleotide as observed in eSS may facilitate the binding of CAIR. The ADP-CAIR complex provides the first instance of an enzyme-bound CAIR molecule covered by strong electron density.

The reaction catalyzed by SAICAR synthetase could resemble that of adenylosuccinate synthetase, an enzyme involved in the first committed step in \(de novo\) AMP biosynthesis (29–31). Adenylosuccinate synthetase putatively transfers the \(\gamma\)-phosphoryl group of GTP to atom O-6 of IMP. The \(\alpha\)-amino group of \(\lambda\)-aspartate then attacks the resulting phosphoryl intermediate (6-phosphoryl-IMP), forming adenylosuccinate. 6-Phosphoryl-IMP appears in crystal structures of adenylosuccinate synthetases from several sources (32–34). Kinetic experiments using positional isotope exchange (35) and isotope exchange at equilibrium (36) support this mechanism; however, no experiment has proven that 6-phosphoryl-IMP lies on the reaction pathway. Markham and Reed (37) have suggested an alternative mechanism in which \(\lambda\)-aspartate first reacts with IMP. The resulting intermediate has a nucleophilic 6-oxyanion that attacks the \(\gamma\)-phosphoryl group of GTP, forming a tetrahedral intermediate identical to that created by the reaction of \(\lambda\)-aspartate with 6-phosphoryl-IMP.

The two mechanisms as they pertain to the SAICAR synthetase reaction appear in Fig. 8. Unlike adenylosuccinate synthetase...
tase, no information is available regarding the intermediate generated in the active site of SAICAR synthetase. The electron withdrawing effects of Mg$^{2+}$/H$_{11001}$ sites 2 and 3 should enhance the electrophilic properties of the carbon atom of the 4-carboxyl group. Conceivably then, L-aspartate could react with CAIR and form a dioxyanion intermediate, which in turn is phosphorylated by ATP. L-Aspartate, however, is present in the crystallization experiment, and yet no electron density appears for L-aspartate or the L-aspartate adduct of CAIR, suggesting the phosphorylation step precedes the nucleophilic attack of L-aspartate.

The ADP$^{3-}$/H$_{18528}$ CAIR structure is a reasonable starting point for modeling the transition state in the formation of a carbonyl phosphate intermediate (Fig. 9). The bridging oxygen atom between the $\beta$- and $\gamma$-phosphoryl groups of ATP coordinates the Mg$^{2+}$ at site 1 and is in line with the proximal oxygen atom of the 4-carboxyl group of CAIR. Terminal oxygen atoms of the $\gamma$-phosphoryl group of ATP hydrogen bond with Lys$^{11}$, Lys$^{123}$, Lys$^{177}$, and metal ions at sites 1 and 3. The reaction coordinate is the movement of the $\gamma$-phosphorus atom of ATP through the plane defined by its terminal oxygen atoms.

Nelson et al. (4) suggested a catalytic abstraction of a proton from the 5-amino group of CAIR analogous to the abstraction of a proton from atom N-1 of IMP by an aspartyl side chain in adenylosuccinate synthetase (31, 38). No protein side chain of eSS, however, interacts or could be in a position to interact with the 5-amino group of CAIR. Furthermore, 4-carboxyimidazole ribonucleotide (CAIR without the 5-amino group) is a substrate for yeast SAICAR synthetase (14), again supporting the absence of an essential role for the 5-amino group of CAIR.

Other observations, however, caution against the complete dismissal of the 5-amino group of CAIR in the chemical mechanism. The 5-amino group of enzyme-bound CAIR is in a cluster of water molecules and probably has an environment similar to that of CAIR in solution. Even in solution, the 5-imino form of CAIR may be dominant. NMR resonances of atom H-4 and atom C-4 of AIR (CAIR without a carboxyl group) come at unusually high field strengths, consistent with the imino form (39). Slow chemical exchange of atom H-4 of AIR with solvent deuterium further supports the imino form (39). Enhanced charge density at atom C-4 would retard spontaneous decarboxylation of CAIR. Indeed, transition metals decrease decarboxylation rates probably by stabilizing the imino form of CAIR (40, 41). Hence, Mg$^{2+}$/H$_{11001}$ sites 2 and 3 could stabilize the imino form of CAIR as suggested by Nelson et al. (4), would also increase the dianionic form of the 4-carboxyl group and thereby enhance its nucleophilic properties.

Another mechanism by which the 5-amino group of CAIR could participate in the SAICAR synthetase reaction is by hydrogen bonding with L-aspartate. In this respect, differences in the active sites of the E. coli and yeast SAICAR synthetases are possible as malate is a substrate for the yeast (14) but not the E. coli enzyme (4).

The different folds for tSS and eSS present an intriguing issue. Does the solvent-exposed fold of tSS represent a functionally relevant state of microbial SAICAR synthetases? Side chain atoms in the eSS ADP complex move no further than 0.8 Å upon CAIR binding; whereas, in tSS they are up to 14 Å away from comparable positions. T. maritima is a thermophile, and elevated temperatures generally enhance hydrophobic and weaken electrostatic interactions. High temperatures, then, would increase the thermodynamic penalty associated with a fold that exposes hydrophobic residues (as observed in the tSS crystal structure) as well as reduce the importance of hydrogen bonds that evidently stabilize the eSS fold but are lacking in tSS. These factors might shift tSS toward an eSS-like fold at high temperatures but favor the observed tSS fold at low temperatures. Unfortunately, the specific activity for tSS at any temperature has not been reported (9).

The tSS structure could also represent a ligand-free conformation shared by most, if not all, microbial SAICAR synthetases. No information is available regarding the intermediate generated in the active site of SAICAR synthetase.
Structure of SAICAR Synthetase

Adenine nucleotide binding could organize the active site; but once organized, the enzyme would be metastable, returning to its less compact conformation on a time scale slow in comparison to catalytic events. The kinetic mechanism is rapid equilibrium random (4), but progress curves under specific conditions exhibit a significant lag phase.3 The lag is consistent with a slow conformational transition from a catalytically non-functional to a functional state.

Vertebrate SAICAR synthetases differ fundamentally from their bacterial homologs in subunit organization (multimeric systems of perhaps eight subunits) and function (the vertebrate subunit combines SAICAR synthetase and AIR carboxylase activities). Hence, the alternative folding phenomenon observed here for microbial systems may only be a remote possibility for vertebrate systems. Stabilization of this putative non-functional state of the bacterial system may be an effective strategy in the development of agents that selectively inhibit de novo purine biosynthesis in bacteria.

Acknowledgments—We thank Dr. Jay Nix, who assisted with data acquisition and processing at Beamline 4.2.2 of the Advanced Light Source, Lawrence Berkeley Laboratory, and Professor S. Ramaswamy, Dept. of Biochemistry, University of Iowa, for providing synchrotron resources of the Molecular Biology Consortium.

REFERENCES

1. Lukens, L. N., and Buchanan, J. M. (1959) J. Biol. Chem. 234, 1791–1798
2. Miller, R. W., and Buchanan, J. M. (1962) J. Biol. Chem. 237, 485–490
3. Meyer, E., Leonard, N. J., Bhat, B., Stubbe, J., and Smith, J. M. (1992) Biochemistry 31, 5022–5032
4. Nelson, S. W., Binkowski, D. J., Honzatko, R. B., and Fromm, H. J. (2005) Biochemistry 44, 766–774
5. Levdikov, V. M., Grebenko, A. I., Barynin, V. V., Melik-Adamyan, W. R., Lamzin, V. S., and Wilson, K. S. (1996) Crystallogr. Rep. 41, 275–286
6. Levdikov, V. M., Barynin, V. V., Grebenko, A. I., Melik-Adamyan, W. R., Lamzin, V. S., and Wilson, K. S. (1998) Structure 6, 363–376
7. Antonyuk, S. V., Grebenko, A. I., Levdikov, V. M., Urusova, D. V., Melik-Adamyan, V. R., Lamzin, V. S., and Wilson, K. S. (2001) Crystallogr. Rep. 46, 687–691
8. Urusova, D. V., Antonyuk, S. V., Grebenko, A. I., Lamzin, V. S., and Melik-Adamyan, V. R. (2003) Crystallogr. Rep. 48, 763–767
9. Zhang, R., Skarina, T., Evdokimova, E., Edwards, A., Savchenko, A., Las-kowski, R., Cuff, M. E., and Joachimiak, A. (2006) Acta Crystallograph. Sect. F Struct. Biol. Cryst. Commun. 62, 335–339
10. Patey, C. A., and Shaw, G. (1973) Biochem. J. 135, 543–545
11. Firestone, S. M., and Davidson, V. J. (1994) Biochemistry 33, 11917–11926
12. Chen, Z. D., Dixon, J. E., and Zalkin, H. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3097–3101
13. Tyagi, A. K., and Cooney, D. A. (1980) Cancer Res. 40, 4390–4397
14. ALENIN, V. V., OSTANIN, K. V., KOSTIKOVA, T. R., DOMKIN, V. D., Zubova, V. A., and Smirnov, M. N. (1992) Biokhimiya 57, 845–855
15. BATOVA, A., Diccianni, M. B., Omura-Minamisawa, M., Yu, J., Carrera, C. J., Bridgeman, L. J., Kung, F. H., Pullen, J., Amylon, M. D., and Yu, A. L. (1999) Cancer Res. 59, 1492–1497
16. Van Duyne, G. D., Standaert, R. F., Karplus, P. A., Schreiber, S. L., and Clardy, J. (1993) J. Mol. Biol. 229, 105–124
17. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
18. Laemmli, U. K. (1970) Nature 227, 680–685
19. Pfuglath, W. J. (1999) Acta Crystallogr. Sect. D Biol. Crystallogr. 55, 1718–1725
20. Collaborative Computational Project, Number 4 (1994) Acta Crystallogr. Sect. D Biol. Crystallogr. 50, 760–763
21. Terwilliger, T. C., and Berendzen, J. (1999) Acta Crystallogr. Sect. D Biol. Crystallogr. 50, 760–763
22. Terwilliger, T. C. (2000) Acta Crystallogr. Sect. D Biol. Crystallogr. 56, 965–972
23. McMee, D. E. (1992) J. Mol. Graph. 10, 44–46
24. Brunker, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D Biol. Crystallogr. 54, 905–921
25. Eng, R. A., and Huber, R. (1991) Acta Crystallogr. Sect. A 47, 392–400
26. Dever, T. E., Glynias, M. J., and Merrick, W. C. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1814–1818
27. Saraste, M., Sibbald, P. R., and Wittinghofer, A. (1990) Trends Biochem. Sci 15, 430–434
28.Hol, W. G., van Duijnen, P. T., and Berendsen, H. J. (1978) Nature 273, 443–446
29. Lieberman, I. (1956) J. Biol. Chem. 232, 327–339
30. Fromm, H. J. (1958) Biochim. Biophys. Acta 29, 255–262
31. Honzatko, R. B., and Fromm, H. J. (1999) Arch. Biochem. Biophys. 370, 1–8
32. Poland, B. W., Bruns, C., Fromm, H. J., and Honzatko, R. B. (1997) J. Biol. Chem. 272, 15200–15205
33. Choe, J. Y., Poland, B. W., Fromm, H. J., and Honzatko, R. B. (1999) Biochemistry 38, 6953–6961
34. Iancu, C. V., Borza, T., Fromm, H. J., and Honzatko, R. B. (2002) J. Biol. Chem. 277, 26779–26787
35. Bass, M. B., Fromm, H. J., and Rudolph, F. B. (1984) J. Biol. Chem. 259, 12330–12333
36. Cooper, B. F., Fromm, H. J., and Rudolph, F. B. (1986) Biochemistry 25, 7323–7327
37. Markham, G. D., and Reed, G. H. (1978) J. Biol. Chem. 253, 6184–6189
38. Honzatko, R. B., Stayton, M. M., and Fromm, H. J. (1999) Adv. Enzymol. Relat. Areas Mol. Biol. 73, 57–102, ix–x
39. Groziak, M. P., Bhat, B., and Leonard, N. J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7174–7176
40. Litchfield, G. J., and Shaw, G. (1971) J. Chem. Soc. (B), 1474–1484
41. Groziak, M. P., Huan, Z. W., Ding, H., Meng, Z., Stevens, W. C., and Robinson, P. D. (1997) J. Med. Chem. 40, 3336–3345
42. Kraulis, P. J. (1991) J. Appl. Crystallogr. 24, 946–950

3 D. J. Binkowski and R. B. Honzatko, unpublished observations.