Aminimidazole-4-carboxamide ribonucleotide (AICAR) transformylase/IMP cyclohydrolase (ATIC) is a bifunctional enzyme with folate-dependent AICAR transformylase and IMP cyclohydrolase activities that catalyzes the last two steps of purine biosynthesis. The AICAR transformylase inhibitors BW1540 and BW2315 are sulfamido-bridged 5,8-dideazafolate analogs with remarkably potent $K_i$ values of 8 and 6 nM, respectively, compared with most other antifolates. Crystal structures of ATIC at 2.55 and 2.60 Å with each inhibitor, in the presence of substrate AICAR, revealed that the sulfonyl groups dominate inhibitor binding and orientation through interaction with the proposed oxanion hole. These agents then appear to mimic the anionic transition state and now implicate Asn431 in the reaction mechanism along with previously identified key catalytic residues Lys256 and His267. Potent and selective inhibition of the AICAR transformylase active site, compared with other folate-dependent enzymes, should therefore be pursued by further design of sulfonyl-containing antifolates.

Cancer is responsible for 25% of all deaths within the United States, making it one of the leading causes of mortality second only to heart disease (1). As multiple metabolic pathways are implicated in disease initiation and progression, substantial efforts have been focused on inhibition of pathways that would either limit the spread of or completely eradicate tumors. In particular two enzymes, glycaminidase ribonucleotide (GAR)1 transformylase (Tfase) and aminimidazole-4-carboxamide ribonucleotide (AICAR) Tfase/inosine monophosphate (IMP) cyclohydrolase (ATIC), in the de novo purine biosynthesis pathway have been prime targets for chemotherapeutic development for two main reasons: 1) rapidly dividing tumors rely on purine de novo synthesis for production of adenine and guanine, whereas normal cells prefer the salvage pathway (2), and 2) both proteins are folate-dependent enzymes that can utilize the same cellular folate transport systems that facilitate entry of natural folates into the cell.

ATIC is a bifunctional enzyme that catalyzes the final two steps of the de novo purine biosynthesis pathway (3). The AICAR transformylase domain (residues 199–592) catalyzes the transfer of the one-carbon formyl group from the co-factor N$_5$-(10-formyl-tetrahydrofolate) (10-f-THF) to the substrate AICAR to produce 5-formyl-AICAR and tetrahydrofolate (see Fig. 1A). The cyclohydrolase domain (residues 1–198) then enhances the intramolecular cyclization of 5-formyl-ATIC to the final product of the pathway, IMP, via elimination of a water molecule. The individual AICAR transformylase and IMP cyclohydrolase domains can be expressed separately and are active (4).

Previous crystal structures of avian and human ATIC revealed an extensively intertwined homodimer (5–7) (see Fig. 1B) in which the transformylase and cyclohydrolase active sites were separated by ~50 Å. However, no evidence of channeling or tunneling of 5-formyl-ATIC between the two domains has been forthcoming (5, 8), although strong electropositive patches that connect the two active sites may sequester 5-formyl-ATIC to the vicinity of the binding sites.

Cleland-type kinetic inhibition experiments indicated that the AICAR Tfase reaction is ordered and sequential with folate co-factor binding first to the AICAR Tfase active site (3). The formyl group is transferred directly from 10-f-THF to AICAR without any formylated enzyme-bound intermediate (9), as evidenced from crystal structures of avian ATIC in complexes with AICAR (Protein Data Bank entry 1Imn) (6) and a multisubstrate adduct inhibitor (MSA) β-DADF (10) (7). From these structures, His267 was proposed to increase the nucleophilicity of the 5-amino group of AICAR and then act as a catalytic base to deprotonate the AICAR amino group concomitantly; ATIC, AICAR transformylase/IMP cyclohydrolase; 10-f-THF, N$_5$-formyl-tetrahydrofolate; MSA, multisubstrate adduct inhibitor; BW2315, BW2315U89UC; BW1540, BW1540U88UD; XMP, xanthosine 5’-monophosphate; β-DADF, 2-[(2-amino-4-oxo-3,4-dihydropyridin-6-imidin-6-ylmethyl)-3-(3,4-dihydro-5-phosphonooxymethyltetrahydrofuran-2-yl) 3H-imidazol-4-yl]-acryloyl]amino/benzoylarnino/pentanedioic acid.
ariant with nucleophilic attack of the substrate formyl group on 10-f-THF (6). Lys<sup>266</sup> is then poised to stabilize the oxyanion transition state and shuttle protons to the N<sub>10</sub> of tetrahydrofolate. The key role of Lys<sup>266</sup> and His<sup>267</sup> in this catalytic mechanism is further supported by kinetic studies of site-directed mutant enzymes (10, 11), where mutation of Lys<sup>266</sup> and His<sup>267</sup> to alanine results in the complete loss of activity without significant change of the substrate.

ATIC is highly conserved from <i>Escherichia coli</i> to human but has no sequence homology with other folate-dependent enzymes, such as GAR Tfase. Thus, folate-based inhibitors of GAR Tfase do not usually inhibit ATIC because of differential interactions within the two active sites (7). For example, 6R-dideazatetrahydrofolate (Lometrexol), potently inhibits GAR Tfase (nM) but not ATIC (μM) (13, 14).

Compared with the number of relatively potent inhibitors of GAR Tfase (15–17, 19–21), specific inhibitors of ATIC have been scarce. However, Burroughs Wellcome (Research Triangle Park, NC) has designed and synthesized two antifolates that are specific (nM) for human ATIC, as compared with other folate-dependent enzymes, GAR Tfase, dihydrofolate reductase, and thymidylate synthase. These compounds are both sulfamido-bridged 5,8-dideazafolate analogs identified as BW1540 and BW2315 (22). BW1540 and BW2315 have approximate <i>K<sub>i</sub></i> values against human ATIC of 8 and 6 nM, respectively, whereas the <i>K<sub>i</i></sub> values against GAR Tfase, dihydrofolate reductase and thymidylate synthase are within the micromolar range, except for BW1540, which showed low nanomolar inhibition against dihydrofolate reductase.<sup>2</sup>

Cytotoxicity assays against human colon cell lines yielded an approximate IC<sub>50</sub> of 0.7–3 μM for BW1540 and 1–5 μM for BW2315. To elucidate their mechanism of inhibition, BW1540 and BW2315 were co-crystallized with human ATIC in the presence of substrate AICAR. These first human ATIC ternary complexes with independently bound substrate and folate moieties not only advance the ATIC mechanistic studies but provide insights into the future design of antifolates selective against ATIC.

**EXPERIMENTAL PROCEDURES**

**Materials**—Luria broth and agar were obtained from Invitrogen. All common buffers and reagents were purchased from Sigma-Aldrich. The folate-based inhibitors BW1540US8UD and BW2315US89UC were kind gifts from Dr. Robert Ferrone (Burroughs Wellcome).

**Protein Preparation and Purification**—Human ATIC was prepared as previously reported (22). Inhibitors BW1540 and BW2315 and substrate AICAR were added in a 10-fold molar excess to human ATIC protein (0.1–0.4 mg/mL), heated in a 37° C water bath for 30 min to prevent precipitation, and then incubated overnight at 4° C. The protein solution was concentrated to 10 mg/mL using Millipore Ultrafree-15 filters (molecular mass of 10,000 Da) and stored at 4°C for crystallization experiments.

**Crystallization and Data Collection**—Crystals of human ATIC in complex with AICAR and BW1540 were grown at 4°C by sitting drop vapor diffusion by mixing equal volumes of human ATIC (10 mg/mL) and a reservoir solution consisting of 0.1 M Tris, pH 7.5, 10% glycerol, 6 m diethiothreitol, and 0.1 M NaCl. Streak seeding (23) with apo human ATIC crystals that were produced in similar conditions facilitated growth of needle-shaped crystals. The data were collected to 2.5 Å resolution on a single, flash-cooled crystal at 83 K in a cryoprotectant consisting of mother liquor and 20% glycerol on Beaml ine 11.1 at the Stanford Synchrotron Radiation Laboratory (Menlo Park, CA). The data were processed to 2.55 Å with HKL2000 (24) in orthorhombic space group P<sub>2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> (4, 74, 15). BW1540 and BW2315U89UC (BW2315) and BW1540U88UD (BW1540) (see Table I) that differ only in the disposition of the imido and sulfamido-bridged 5,8-dideazafolate analogs identified as an endogenously bound xanthosine 5'-monophosphate (XMP), within the AICAR Tfase and IMP cyclohydrolase active sites, respectively. Difference electron density maps clearly identified inhibitors BW1540 and BW2315, as well as an endogenously bound xanthosine 5'-monophosphate (XMP), within the AICAR Tfase and IMP cyclohydrolase active sites, respectively. Approximately 2-fold noncrystallographic symmetry restraints were applied during the initial round of refinements and then released later to accommodate differences within the individual monomers. Water molecules were automatically positioned by CNS using a 3° cut-off in F<sub>o</sub>−F<sub>c</sub> maps and manually inspected. For the BW1540 structure, the final R<sub>cryst</sub> and R<sub>free</sub> are 18.9 and 25.7%, respectively. For the BW2315 structure, the final R<sub>cryst</sub> and R<sub>free</sub> are 21.3 and 27.5%, respectively (Table II).

The models were analyzed with PROCHECK (30), WHATCHECK (31), CNS (29), and CCP4 (32). S<sub>Crime</sub>-co-efficient and buried surface areas were calculated with SC (32) and MS (33) using 1.7 and 1.4 Å probes, respectively. Analysis with PROCHECK (30) revealed that 88.6 and 88.0% of the residues are located in the most favorable regions in the Ramachandran plot for the BW1540 and BW2315 complexes, respectively. In both structures, a surface Asp<sup>502</sup> lies in the disallowed region of the Ramachandran plot, located near the generously allowed region.

**Table I**

<table>
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<tr>
<th></th>
<th>BW1540US8UD complex</th>
<th>BW2315US89UC complex</th>
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<td>Space group</td>
<td>P&lt;sub&gt;2&lt;sub&gt;1&lt;/sub&gt;&lt;/sub&gt;2&lt;sub&gt;1&lt;/sub&gt;2&lt;sub&gt;1&lt;/sub&gt;</td>
<td>P&lt;sub&gt;2&lt;sub&gt;1&lt;/sub&gt;&lt;/sub&gt;</td>
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<tr>
<td>Unit cell dimensions (Å)</td>
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<td>a = 77.12, b = 92.97, c = 178.49, β = 91.2</td>
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<td>2</td>
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<td>11.0 (48.9)</td>
<td>12.4 (69.5)</td>
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<sup>a</sup> Numbers in parentheses refer to the highest resolution shell.

<sup>b</sup> R<sub>cryst</sub> = Σ[|I<sub>o</sub> − |I<sub>c</sub>|]/Σ|I<sub>o</sub> × 100, where |I<sub>o</sub>| and |I<sub>c</sub>| are the intensities of individual and mean structure factors, respectively.

<sup>2</sup> R. Ferrone, personal communication.


TABLE II

<table>
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<tr>
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<td>Protein Data Bank code</td>
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\[a\] \(R_{	ext{cryst}} = \frac{\sum |F_o| - k|F_c|/\sum |F_o| \times 100,\) where \(F_o\) and \(F_c\) are the observed and calculated structure factors, respectively. \(R_{	ext{free}}\) is computed as described for \(R_{	ext{cryst}},\) but with the test set of reflections only. ref. 6.

\[b\] Cross-validated Luzzatti coordinate error.

but has well ordered electron density. Figs. 1B–6 were created with PyMol (34). The coordinates and structure factors have been deposited in the Protein Data Bank (35) with accession entries 1p4r (BW1540U88UD) and 1p10 (BW2315U89UC).

RESULTS

The crystal structures of human ATIC in complex with antifolates BW1540U88UD and BW2315U89UC provide the first view of how the substrate AICAR and folate-based co-factor moieties simultaneously occupy the AICAR Tfase active site. The AICAR Tfase active site is located in a long, narrow cleft at the dimer interface where AICAR interacts primarily with one subunit and folate with the opposing subunit (7).

The refined human ATIC BW1540 structure consists of residues 4–592 in monomer A and residues 5–592 in monomer B with 399 water molecules, two potassium ions, two BW1540 inhibitors within the AICAR Tfase domains, but only one XMP in the IMP cytidylylase active site of monomer A (Fig. 1B). XMP is carried through protein purification and crystallization and also selectively binds to only one of the two IMP cytidylylase active sites in the homodimer in apo avian and human ATIC structures (5, 22, 36).

The final model for the BW2315 complex consists of two dimers (i.e. monomers A (4–592) and B (4–592) compose one dimer, and monomers C (5–592) and D (4–592) compose the second dimer) and 388 water molecules. No main chain density was observed between residues 482 and 485 (B), 481–485 (C), and 477–486 (D). Each monomer has a bound potassium ion within the AICAR Tfase domain with only one XMP per dimer (monomers A and C). Interpretable density for the substrate AICAR was found only in monomers B, C, and D, whereas interpretable BW2315 density was found only in monomers B and D. The AICAR Tfase domain contains three subdomains (domains 2–4) as previously described (5–7). For each complex, domain 3 has higher \(B\) values (41–47 Å²) compared with the overall protein \(B\) values (27–30 Å²) as previously found (6, 7).

**AICAR Interactions for Both the BW1540- and BW2315-bound Human ATIC Structures**—Clear \(F_o-F_c\) electron density for the AICAR substrate was present in the AICAR Tfase active sites of both complexes, except for monomer A of the BW2315-bound complex structure, which had weak density that could not be unequivocally interpreted. AICAR binds to both complex structures in a similar conformation with a C₈'-endo sugar pucker (Fig. 2). The 4-carboxamide amino group of AICAR points toward the aromatic ring of Phe⁵⁴¹ with an approximate distance of 3.6 Å to the center of the phenyl ring (Figs. 3A and 4A) as previously described (6, 7). This \(\pi\)-electron hydrogen bond interaction stabilizes the preferred orientation of the 4-carboxamide in which intramolecular hydrogen bonds are formed between the 4-carboxamide amino and the lone pair of N3 electrons, as well as between the 4-carboxamide oxygen and 5-amino group of AICAR. The main chain carbonyl oxygen of Phe⁵⁴¹ and the side chain guanido nitrogen atoms of Arg⁴⁵⁸ residues 1 XMP2 XMP 2 AICAR 3 AICAR 4 B. The sulfonyl group of BW1540 is located at the N terminus of AICAR and folate moieties (7). Similarly, Ser⁴⁵⁰ was proposed to hydrogen bond to the reduced N-5 of the natural co-factor (7).

The imidazole group of AICAR and the p-aminobenzoyl ring of BW1540 stack at a distance between the rings of 4.1 Å in an interaction also not observed in the \(\beta\)-DADF-bound structure again because of constrained covalent interaction between the AICAR and folate moieties (7). The phenyl rings of Phe⁵⁴⁰ and Phe⁴⁵⁵ also provide perpendicular \(\pi\)-stacking (3.7 Å) with the AICAR imidazole and p-aminobenzoyl ring of BW1540. The sulfonyl group of BW1540 is located at the N terminus of α-helix 17 (residues 450–468), suggesting that the helix dipole contributes to stabilizing the partial negative charge of one of...
Fig. 1. ATIC reaction, co-factors, inhibitors, and crystal structure of AICAR and antifolate-bound human ATIC. A, formyl transfer and IMP cyclohydrolase reactions catalyzed by ATIC and the structures of the 10-THF co-factor and ATIC-specific sulfamido-bridged 5,8-dideazafolate analogs BW1540U88UD (K<sub>i</sub> = 8 nM) and BW2315U89UC (K<sub>i</sub> = 6 nM). B, two rotated views of the homodimeric human ATIC in complex with BW1540 (monomer A colored in rose and monomer B colored in blue). The bound AICAR, BW1540 antifolate and XMP molecules are depicted in a cpk representation. Yellow, carbon; red, oxygen; blue, nitrogen; orange, sulfur; purple, phosphate. Bound potassium ions are represented as purple balls.
the sulfonyl oxygens. A similar interaction was noted with the
exocyclic 4-carbonyl of the 8-deazapterin ring of β-DADF in the
avian MSA structure (7). The other sulfonyl oxygen hydrogen
bonds to the main chain carbonyl of Gln449 and the side chains
of Asn431 and Lys266 (Figs. 3A and 4A), similar to the hydrogen
bonding network seen for the 10-formyl group in the
β-DADF complex, although the Asn431 interaction was not observed
previously. These interactions assist in orienting the Lys266
side chain and may also facilitate in destabilization of the
oxyanion transition state (7).

The BW1540 glutamate tail orients toward the ATIC surface
with two hydrogen bonds from the α-glutamate to the side
chain of Ser565 and the amide backbone of Ala566. The γ-gluta-
mate forms no protein hydrogen bonds but does orient sur-
face water molecules (Fig. 4A). Extensive electropositive sur-
face surrounding the active site presumably accounts for
binding to the negatively charged γ-glutamate tail (7), which
not only increases cellular retention (37), but also increases
affinity for ATIC (38, 39).

**BW2315 Interaction within the AICAR Tfase Active Site**—
Interactions of BW2315 within the AICAR Tfase active site
(monomers B and D) are very similar to those observed in the
BW1540 complex structure, except for the complete disorder of
the glutamate tail (Fig. 2B). Although some positive density for
BW2315 was observed within the AICAR Tfase active sites of
monomers A and C, the inhibitor could not be confidently
interpreted. Approximately 87% of the BW2315 surface is bur-
ried (41 Å² of exposed surface) within the active site, which is
slightly larger than the corresponding BW1540 buried surface
only because of the inability to observe its solvent-exposed

![Fig. 2. Stereo views of substrate and antifolate electron density in the AICAR Tfase active site. A, AICAR and BW1540. B, AICAR and BW2315. Final refined structures are shown superimposed on their Fo−Fc simulated annealing omit maps (blue) contoured at 2.5 σ.](image-url)
glutamate. Strikingly, superposition of the BW1540 and BW2315 structures reveals that their sulfonyl groups occupy essentially the same location. As a consequence, slight translational and rotational differences of the 5,8-dideazapterin rings are observed within their respective AICAR Tfase active site (Fig. 5A). The dideazapterin rings are rotated by $-15^\circ$ relative to each other with a maximum translation of 1.4 Å (as calculated for C-6) (Fig. 5A). However, the translational differences diminish toward the 2-amino and 4-carboxamide ring substituents to 1.0 and 0.4 Å, respectively, where the BW2315 ring contours to maximize possible ring substituent interactions, as in BW1540 (Fig. 4). Similarly, the benzoyl rings of the

**Fig. 3.** The AICAR Tfase active site with BW antifolates bound. A, electrostatic and hydrogen bonding residues in the AICAR Tfase active site that interact with antifolate BW1540. BW1540 and ATIC residues are represented in ball-and-stick with BW1540 atoms colored as in Fig. 1B. Carbon atoms for the active site residues are colored blue for subunit A and purple for subunit B of the dimer with structural water molecules represented as red spheres. B, corresponding view of hydrogen bonding residues within the AICAR Tfase active site that interact with AICAR and antifolate BW2315. BW2315 and ATIC residues are represented in ball-and-stick with BW2315 atoms colored as in Fig. 1B. Carbon atoms for the active site residues are colored blue for subunit C and purple for subunit D with structural water molecules represented as red spheres. C, stereo view of the AICAR Tfase active site bound with BW1540 depicts the hydrophobic residues within the folate-binding region. BW1540 and ATIC residues are labeled and colored according to A.
two antifolates are displaced from one another by 1.2 Å but with no rotational variation, consistent with the approximate 1.5 Å difference in the disposition of the two sulfonyle groups relative to the C-6 of the dideazapterin (40). Because both sulfonyle groups of BW1540 and BW2315 sit atop the N terminus of α-helix 17, the stabilization of the partial negative charge of the sulfonyle oxygen by the helix dipole appears to be the driving force that defines the binding and orientation of the
Two additional hydrogen bonds are observed with the BW2315 5,8-dideazapterin ring as a result of the relative placement of the 5,8-dideazapterin ring. Asn 489 provides hydrogen bonds not only to the exocyclic 2-amino group of the 5,8-dideazapterin group, as observed in the BW1540 structure, but also to the reduced N1 atom (Figs. 3B and 4B). Similarly, the Ile 452 backbone amide provides an additional hydrogen bond (2.8 Å) to the exocyclic 4-carbonyl of the 5,8-dideazapterin ring. The only unique hydrogen bond found in the BW1540 complex arises from interaction of the Gln 449 main chain carbonyl with one of the sulfonyl oxygens. The sulfonyl oxygen of BW2315 is displaced 1.1 Å toward the AICAR 5-amino relative to the equivalent BW1540 oxygen. Despite these slight differences in antifolate positioning, the AICAR Tfase side chains responsible for electrostatic interactions (Asn 489, Asp 546, and Asn 547) and those that establish the hydrophobic pocket bottom (Met 312, Ile 452, and Ser 450) do not substantially change conformation. However, the phenyl rings of Phe 544 and Phe 315 rotate to optimize perpendicular π-stacking interactions with the 5,8-dideazapterin in both complexes.

**DISCUSSION**

Both BW1540 and BW2315 ATIC structures not only reinforce previous mechanistic proposals based on the crystal structures of avian ATIC in complex with AICAR (6) and β-DADF (7) but also provide new information on how the AICAR and folate moieties are oriented in the ternary complex. The most surprising observation is the domination of the active site oxyanion hole in determining the location and orientation of these bound sulfonyl-based antifolates.

**Conformational Changes in the AICAR Tfase Active Site**—Superposition of the AICAR/BW1540- and AICAR/BW2315-bound human ATIC structures with the apo human ATIC structure (1pkx) (22) (main chain root mean square deviation values of 0.92 and 0.54 Å, respectively) reveal that the AICAR Tfase active site itself is relatively rigid. Nevertheless, some local conformational rearrangements of some side chains, as well as the abutting main chain, tend to constrict the AICAR Tfase active site around the bound inhibitor (Fig. 5B). The side chains of Arg 207, Tyr 208, and Phe 541 slightly reorient so as to interact optimally with the substrate AICAR (Fig. 5B), whereas the side chains of Asn 489, Asp 546, Asn 547, and Ser 565 reposition toward the pterin ring of the folate inhibitors. Main chain movements are observed in two of the α-helices within the AICAR Tfase active site. The α-helix 17 (residues 450–468) that anchors the sulfonyl oxygens has an average displacement of 1.2 Å, whereas α-helix 23 that interacts with the α-glutamate is displaced by an average of 1.5 Å (Fig. 5B).

**Comparison of Human Antifolate Complexes with MSA-bound Avian ATIC**—The overall structures of human ATIC in complex with AICAR and antifolates BW1540 and BW2315 are similar to the avian ATIC structure bound with the MSA.
β-DADF (1o20) (7) (main chain root mean square deviations of only 1.1 and 1.0 Å, respectively). However, the substrate and folate ligand positions vary somewhat, mainly because of the binding of independent AICAR and folate moieties in the BW1540 and BW2315 human structures compared with the equivalent moieties that are covalently tethered in the MSA in the avian structure. The imidazole ring of the AICAR moiety of β-DADF is slightly rotated and translated away from the folate ring by ~1 Å with respect to the untethered AICAR molecules in the human complexes. Similarly, the tethered pterin ring of β-DADF is rotated by 40° and translated out of the folate-binding region by about 2 Å compared with the 5,8-dideazapterin of the BW compounds (Fig. 6A) that reduces electrostatic interactions of the pterin ring with Asn489, Asp546, and Asn547. The more limited interactions of β-DADF within the active site may explain why main chain conformational changes were not observed as with the BW compounds. However, despite these fluctuations in the folate ring position, the 10-carbonyl of β-DADF (equivalent to the N10 formyl group) superimposes remarkably closely with the sulfonyl oxygen atoms of both BW inhibitors within the oxyanion hole so that similar interactions are made with the catalytic Lys266.

The BW inhibitors and β-DADF all have an aromatic 5,8-dideazapterin ring substituted for the naturally reduced pterin of 10-f-THF that results in loss potential hydrogen bond interactions with active site residues, such as the potential N-8 interaction of the natural co-factor with the main chain carbonyl of Met312 and the Ser450 hydroxyl, as well as with a main chain amide interaction of Arg451 with N-5. Further, the aromatic dideazapterin adopts a rigid ring conformation compared with the reduced pterin, which is more flexible and can mold better to the active site to optimize electrophilic interactions.

Role of Asn431 in Catalysis—In previous avian ATIC structures, the Asn431 side chain pointed away from the bound substrates with an active site water molecule positioned near the 5-amino group of AICAR. The AICAR and BW1540 inhibitor of the BW1540-bound human structure are colored as in Fig. 1B with Lys266, His267 and Asn431 of the human BW1540-bound (cyan carbons, 1p4r), apo human (forest green carbons, 1pxk), avian AICAR/XMP-bound (wheat carbons, 1m9n), and avian β-DADF-bound (grey carbons, 1o20) depicted.

**Fig. 6.** BW1540, BW2315, and β-DADF and active site catalytic residue superposition for all known ATIC structures. A, two rotated views of the overlay of β-DADF and the BW antifolates reveals slight variations in the positioning of the MSA because of the trans-double bond linker between the AICAR and folate moieties. The BW1540-bound human structure is colored as in Fig. 1B, with the BW2315-bound ATIC carbons colored teal and the β-DADF-bound carbons colored salmon. B, determination of the antifolate-bound human ATIC structures reveals Asn431 positions both the AICAR 5-amino and 10-formyl group of folate for optimal transformylation. Previous structures show Asn431 pointing away from the bound substrates with an active site water molecule positioned near the 5-amino group of AICAR. The AICAR and BW1540 inhibitor of the BW1540-bound human structure are colored as in Fig. 1B with Lys266, His267 and Asn431 of the human BW1540-bound (cyan carbons, 1p4r), apo human (forest green carbons, 1pxk), avian AICAR/XMP-bound (wheat carbons, 1m9n), and avian β-DADF-bound (grey carbons, 1o20) depicted.
Human ATIC Sulfonil-Antifolate Complexes

Human ATIC has a catalytic triad composed of Asn\textsubscript{106}, His\textsubscript{108}, and Asp\textsuperscript{144}, where Asn\textsubscript{106} is involved in orienting GAR and 10-f-THF, as well as stabilizing the transition state (41). Similarly, cellobiose dehydrogenase, an extracellular flavocytochrome, has a catalytic His\textsubscript{268} and Asn\textsuperscript{702}, where His\textsuperscript{702} acts as a general base and Asn\textsubscript{702} optimally positions the cellobiose anomerlic β-hydroxyl for catalysis (42, 43). The class III anaerobic ribonucleotide reductase from bacteriophage T4 has two asparagine residues within its active site that are proposed to position the substrate formate to orient it toward the proposed thyl radical of the catalytic residues Cys\textsuperscript{79} (44). Therefore, the likely role of Asn\textsuperscript{431} in the AICAR Tfase active site is to position and orient the AICAR 5-amino and formyl group of 10-f-THF for nucleophilic attack as well possibly stabilizing the transition state along with Lys\textsuperscript{266}.

The presence of Asn\textsuperscript{431} within hydrogen bonding distance of His\textsuperscript{267} may also perturb the pK\textsubscript{a} of His\textsuperscript{267}. His\textsuperscript{267} and Lys\textsuperscript{266} were previously proposed to suppress the pK\textsubscript{a} of His\textsuperscript{267} for deprotonation of the AICAR 5-amino concomitant with nucleophilic attack (6). Mutation of Asn\textsuperscript{431} to an aspartate should determine whether a negative charge would reduce the pH optimum of the reactions through destabilization of His\textsuperscript{267} as a proton acceptor at neutral pH. Likewise, mutation of Asn\textsuperscript{431} to an alanine or glutamine should determine what role it plays in AICAR and folate binding.

AICAR Tfase Mechanistic Considerations—Based on the previously determined avian ATIC structures, His\textsuperscript{267} was proposed to act as a catalytic base and abstract the proton from the AICAR 5-amino with Lys\textsuperscript{266} poised to protonate the N\textsubscript{10} leaving group of the tetrahydrofolate (6, 7). One of the sulfonil oxygens in both BW1540 and BW2315 superimpose with the 10-formyl oxygen of β-DADF and are within hydrogen bonding distance to Lys\textsuperscript{266} in all three structures (Fig. 6A), reinforcing the proposal that the transition state oxyanion is stabilized by the positively charged side chain of Lys\textsuperscript{266}. The side chain amino nitrogen of Lys\textsuperscript{266} is further positioned by interactions with the main chain carbonyl oxygens of Phe\textsuperscript{515}, Ser\textsuperscript{313}, and Gin\textsuperscript{497}, with distances of 2.8, 2.8, and 2.9 Å in the BW1540 complex, respectively.

Role of the Oxyanion Hole in Positioning the 10-Formyl Group of Natural Co-factor—Superposition of the BW inhibitor sulfonil oxygens and the β-DADF 10-formyl oxygen also suggests that one of the primary determinants for the inhibitor positioning within the active site stems from the interaction with the oxyanion hole composed of main chain amides from the N terminus of the adjacent α-helix (residues 450–468) (note that BW1540 and β-DADF oxygens are separated by only 0.9 Å, the BW1540 and BW2315 oxygens are separated by 1.1 Å, and BW2315 and β-DADF oxygens are separated by 0.8 Å). Oxyanion holes that primarily consist of two main chain amides are a common active site feature for stabilizing transition state intermediates in various enzymes, including all known α/l hydrolyase family members (18, 45). The sulfonil oxygen of BW1540 is situated closer to the main chain nitrogen atoms of Ser\textsuperscript{450} and Arg\textsuperscript{451} compared with BW2315, in which the nitrogen (at position 9) is inserted between the sulfonil and the 5,8-dideazapterin ring (Figs. 1A and 5A). Nevertheless, the dideazapterin ring substituents of these two BW inhibitors maintain similar hydrogen bonds and bond lengths with active site residues.

On the other hand, the reduced folate ring of the natural co-factor is able to provide additional functional groups for hydrogen bonding within the active site that may then further influence co-factor binding as compared with the dominant interaction of the sulfonil compounds with the oxyanion hole. Distances between the AICAR 5-amino nitrogen and the closest sulfonil oxygens of the BW1540 and BW2315 structures are 3.5 and 2.6 Å, respectively, which are too far for nucleophilic attack if the BW1540 sulfonil oxygen is considered to be functionally equivalent to the less electrophilic 10-formyl carbon of 10-f-THF (Fig. 1A). Similarly, in the β-DADF-bound structure, despite the covalent bond between the 10-formyl group and the AICAR 5-methenyl (equivalent to the 5-amino of 10-f-THF) that includes an additional 1.5 Å spacer because of the extra double bond linker (7), the formyl carbonyl position of β-DADF is maintained within the oxyanion hole. However, in the natural co-factor, the additional potential hydrogen bond interactions with the AICAR Tfase active site, the weaker electron withdrawing effects of the 10-formyl oxygen, the ability of the Lys\textsuperscript{266} side chain to stabilize the oxyanion transition state without steric hindrance from other active site residues (6, 7), and the presence of Asn\textsuperscript{431} to fine-tune the distance between the two groups could facilitate motion of the 10-formyl group of 10-f-THF away from the oxyanion hole toward the AICAR 5-amino for covalent bond formation.

ATIC-specific Antifolate Inhibitor Design—The BW1540 and BW2315 ATIC complex structures indicate that the oxyanion hole appears to be a major factor determining the positioning of the sulfonil-containing antifolates within the AICAR Tfase active site. Thus, further ATIC antifolate design should focus on exploiting this oxyanion hole because GAR Tfase, thymidylate synthase, and dihydrofolate reductase do not contain such a feature within their active sites. Although the sulfonil groups should be conserved in the next generation of inhibitors, they should be positioned as true mimics of the 10-formyl group between the pterin and benzyl rings to emulate the actual transition state. Then the aromatic 5,8-dideazapterin ring should be substituted with the natural, reduced pterin to exploit additional active site interactions that probably require some modest reconfiguration of the folate-binding site. Substitution for the reduced pterin ring may also aid in reduced folate carrier and folicpoly-γ-glutamate synthetase recognition, thereby facilitating active transport across the cell membrane and polyglutamation, respectively. Strong electronegative substituents, such as a nitrile functional group, should also be evaluated in place of the sulfonil. This substitution would not only be optimal for utilization of the oxyanion hole interactions but also aid in possible formation of an in vivo covalent multistate adduct inhibitor.

Active Site Conservation in Avian and Other Organisms—The overall structure of human ATIC is highly homologous to the avian ATIC structure as expected from the high sequence identity (82%). Comparison of the amino acid sequences of ATIC from seven different organisms from bacteria to human (human, accession number P31939; chicken, accession number P11335; mouse, accession number Q9CWJ9; Saccharomyces cerevisiae, accession number P38009; Schizosaccharomyces pombe, accession number 074928; E. coli, accession number P15639; and Synechocystis sp., accession number P74741) shows that all proposed catalytic residues are completely conserved including Lys\textsuperscript{266}, His\textsuperscript{108}, Asn\textsuperscript{431}, and His\textsuperscript{497}. Moreover, active site residues whose side chains are involved in binding both AICAR and folate are also conserved across these seven species including Arg\textsuperscript{207}, Tyr\textsuperscript{307}, Arg\textsuperscript{451}, Phe\textsuperscript{451}, Asp\textsuperscript{456}, Ser\textsuperscript{450}, and Arg\textsuperscript{451} (Fig. 7). Only three differences are found within the AICAR Tfase active site. Asp\textsuperscript{299} has a conservative mutation to glutamate in E. coli and Synechocystis sp. that could still maintain hydrogen bonding with the AICAR ribose O-3. However, an equivalent residue to Asn\textsuperscript{431} is not present in bacterial sequences because the entire domain 3 (residues 468–531) is deleted (Fig. 7). Otherwise, the Asn\textsuperscript{347} mutation to a glycine and serine in E. coli and Synechocystis sp., respectively, would
likely reduce interactions with the exocyclic substituents of the pterin ring.

In addition to these conserved electrophilic active site residues, the hydrophobic pockets are also well preserved. Phe³¹⁵, Pro³⁴³, and Phe⁵⁹⁰, which interact with the imidazole of AICAR, the benzoyl of folate, and the 5,8-dideazapterin ring of folate, are completely conserved across the seven sequences. Met³¹² and Ile⁴⁵² that form the bottom hydrophobic patch of the folate binding pocket are conserved in eukaryotes but mutated to threonine and valine in bacterial sequences. However, if the hydroxyl of threonine in bacterial ATIC points away from the folate binding pocket, it could still otherwise provide some hydrophobic contacts. Interestingly, a stretch of seven residues from Ser⁵₃⁸ to Phe⁵₄₄ is completely conserved across the seven organisms and contains Phe⁵₄¹, which is involved in the AICAR 4-carboxamide interaction and Pro⁵₄₃ and Phe⁵₄₄, both of which provide a hydrophobic environment for the pterin ring of folate.

Conclusion—The crystal structures of human ATIC complexed with these sulfonyl-containing Burroughs Wellcome inhibitors BW1540 and BW2315, as well as substrate AICAR, provide the first details of an AICAR Tfase ternary complex. Moreover, interaction of the sulfonyl oxygens with the oxyanion hole of the AICAR Tfase active site suggests that this is the driving force behind the high affinity for these sulfonyl-containing antifolates. The $K_m$ of 10-f-THF is 100 $\mu$M (8), whereas the $K_i$ values for BW1540 and BW2315 are 8 and 6 nM, respectively. Thus, these two antifolates bind more strongly to the AICAR Tfase active site, by at least 1000-fold, compared with the natural cofactor that is probably due to the sulfonyl groups mimicking to some extent the oxyanion of the transition state. However, substitution of the reduced pterin ring for the 5,8-
dideazapterin analog in the BW compounds suggests that design of more potent inhibitors could arise from increased interactions with the folate-binding site. Nevertheless, inhibitor interactions with the unique oxoyanion hole of ATIC, which dominates the binding of these sulfonyl antifolates, could be further exploited for the next generation of selective ATIC antifolates.

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