An analogue of isoleucyl-adenylate (Ile-AMS) potently inhibits the isoleucyl-tRNA synthetases (IleRSs) from the three primary kingdoms, whereas the antibiotic mupirocin inhibits only the eubacterial and archaeal IleRSs, but not the eukaryotic enzymes, and therefore is clinically used against methicillin-resistant Staphylococcus aureus. We determined the crystal structures of the IleRS from the thermophilic eubacterium, Thermus thermophilus, in complexes with Ile-AMS and mupirocin at 3.0- and 2.5-Å resolutions, respectively. A structural comparison of the IleRS-Ile-AMS complex with the adenylate complexes of other aminoacyl-tRNA synthetases revealed the common recognition mode of aminoacyl-adenylate by the class I aminoacyl-tRNA synthetases. The Ile-AMS and mupirocin, which have significantly different chemical structures, are recognized by many of the same amino acid residues of the IleRS, suggesting that the antibiotic inhibits the enzymatic activity by blocking the binding site of the high energy intermediate, Ile-AMP. In contrast, the two amino acid residues that concomitantly recognize Ile-AMS and mupirocin are different between the eubacterial/archaeal IleRSs and the eukaryotic IleRSs. Mutagenic analyses revealed that the replacement of the two residues significantly changed the sensitivity to mupirocin.

Aminoacyl-tRNA synthetases (aaRSs)\(^1\) esterify the cognate amino acids with their specific tRNAs via the following two-step reactions: the formation of aminoacyl-adenylate (aa-AMP), an active intermediate, from an amino acid and ATP; and the transfer of the aminoacyl moiety to the 3’-terminal adenosine (A\(^3\)) of tRNA. The aaRSs can be divided into two classes, class I and class II, comprising 10 members each, which have distinct catalytic domain architectures with exclusive signature motifs for the ATP binding (1). The class I aaRSs have a catalytic domain constructed with the Rossmann fold, and display two signature amino acid motifs “HIGHT” and “KMSKS.” The Rossmann fold has a \(\beta_{6}\alpha_{6}\) topology and is apparently divided into two symmetrical halves (\(\beta_{3}\alpha_{3}\) topology each), which are believed to have evolved by a genetic event such as gene duplication. The class I aaRSs are further divided into three subclasses (class Ia, Ib, and Ic) on the basis of the sequence homology and the domain architectures (2). The class Ia aaRSs consist of the isoleucyl-, methionyl-, valyl-, leucyl-, cysteinyl-, and arginyltRNA synthetases (IleRS, MetRS, ValRS, LeuRS, CysRS, and ArgRS, respectively); the class Ib aaRSs include the glutamyl- and glutaminyl-tRNA synthetases GluRS and GlnRS, respectively; and the class Ic aaRSs are the tyrosyl- and tryptophanyl-tRNA synthetases (TyrRS and TrpRS, respectively).

In general, an aa-AMP analogue potently inhibits the corresponding aaRSs from eubacteria, archaea, and eukaryotes. In contrast, some antibiotics inhibit only the eubacterial (and archaeal) aaRSs, but not the eukaryotic enzymes. The best known example is mupirocin, which targets bacterial and archaeal IleRSs (6, 7). Mupirocin (the chemical structure is shown in Fig. 1) is produced by Pseudomonas fluorescens NCIB 10586 (4) and has been clinically used as an antibiotic against methicillin-resistant Staphylococcus aureus (5). The amino acid sequences of the eubacterial IleRSs are apparently different from those of the eukaryotic and archaeal IleRSs in their C-terminal domains (8). On the other hand, no significant difference in the amino acid sequence has been shown in the catalytic domains of the IleRSs from the three primary kingdoms. Therefore, it is suggested that mupirocin may discriminate the fine structural differences between the substrate-binding sites of the bacterial/archaeal IleRSs and the eukaryotic IleRSs. Although the peptide protection from trypsin digestion suggested that mupirocin acts as an analogue of isoleucyl-adenylate (Ile-AMP, its chemical structure is shown in Fig. 1) (13), the chemical structure of mupirocin is significantly different from that of Ile-AMP (Fig. 1) (14). Therefore, the precise mechanism by which mupirocin acts against IleRS remains to be elucidated. Furthermore, a highly mupirocin-resistant, methicillin-resistant S. aureus was found, which harbors a new plasmid-borne, eukaryotic-type IleRS (type-II IleRS) (8–12). Therefore, it is clinically important to design new drugs for the resistant bacteria.

We present the crystal structures of the Thermus thermophilus IleRS in complexes with Ile-AMS (Fig. 1), an analogue of Ile-AMP, and with mupirocin (Fig. 1). The present structure provides a structural basis for the mechanisms of the IleRS recognition of Ile-AMP and mupirocin and the inhibition by the antibiotic.
Structure of IleRS Complexed with Ile-AMS and Mupirocin

EXPERIMENTAL PROCEDURES

Crystallization—The recombinant T. thermophilus IleRS was purified as described by Nureki et al. (3). Crystals of the IleRS-Ile-AMS complex were prepared by soaking the IleRS crystals in a harvesting buffer containing 0.1 M Ile-AMS. Crystals of the IleRS-mupirocin complex were obtained by co-crystallization of 20 mg/ml Ile-AMS and 1 mM mupirocin under the conditions reported previously (3).

Data Collection—Diffraction data of crystals of the IleRS-Ile-AMS complex and the IleRS-mupirocin complex were collected at room temperature to 3.0- and 2.5-A resolutions, respectively, with a Weissenberg camera for macromolecules installed on the beam line 6A2 at the Photon Factory in KEK (Tsukuba, Japan). All data were processed with the DENZO and SCALEPACK programs (15).

Structure Solution, Model Building, and Refinement—The initial phases were calculated by molecular replacement with the program AMORE (16) using the 2.5-A resolution structure of IleRS (3) as a search model. Atomic models were built with the program O (17). The Ile-AMS and mupirocin molecules were unambiguously identified in the F̃_o − F̃ omit maps. Crystallographic positional and slow-cooling refinements were carried out with the Crystallography & NMR System (18). The stereochemical parameters of the final model were analyzed by the program PROCHECK (19). The data collection and the refinement statistics are summarized in Table I.

Measurement of the Mupirocin Kᵢ—The mutant IleRS genes were constructed by cassette mutagenesis, and the recombinant mutant IleRSs were purified as described previously for the wild type enzyme (3). T. thermophilus tRNA<sup>Leu</sup> was prepared by in vitro transcription with T7 RNA polymerase. The aminoacylation was carried out at 65 °C in a reaction containing 100 mM Tris-HCl (pH 8.0), 5 mM Mg(OAc)<sub>2</sub>, 2 mM ATP, 10 mM KCl, 100 µM t-L-isoleucine, 5 µM tRNA<sup>Leu</sup>, and 30 nM wild type IleRS or 300 nM mutant IleRS. The inhibition constant Kᵢ of mupirocin with respect to isoleucine, and the Kᵢ values for isoleucine were obtained from Dixon plots and Lineweaver-Burk plots, respectively. For the kinetic analyses, the concentration ranges of L-isoleucine and mupirocin were 60–480 µM and 0–4 µM, respectively.

RESULTS AND DISCUSSION

Recognition of Ile-AMS by IleRS—Ile-AMS (5'-N-[L-(t-isoleucyl)sulfooamido]adenosine) is a non-hydrolysable analogue of Ile-AMP, in which the phosphoester bond between the isoleucyl and adenosine moieties is replaced with a sulfoamide bond (Fig. 1). Ile-AMS strongly inhibits all of the IleRSs from eubacteria, archaebacteria, and eukaryotes (data not shown). The IC<sub>50</sub> value against the T. thermophilus IleRS was 5.6 nM.

We prepared the crystals of the complex of IleRS and Ile-AMS by a soaking experiment. However, we could only obtain the crystals with a low concentration of Ile-AMS (0.1 mM), which led to the low occupancy of Ile-AMS (50%). Nevertheless, we could unambiguously identify the Ile-AMS molecule in the F̃_o − F̃ omit map (Fig. 2a).

In the present structure, the Ile-AMS molecule is tightly bound to the bottom of the catalytic cleft of the Rossmann fold domain of T. thermophilus IleRS (Fig. 2b). The side chain of the isoleucyl moiety is recognized by a hydrophobic pocket consisting of Pro-46, Trp-518, and Trp-558 through van der Waals interactions (Fig. 2c). The amino group of the isoleucyl moiety hydrogen-bonds to the main chain carbonyl group of Pro-46 (Fig. 2, c and d). The Nε of the side chain of Gln-554 hydrogen-bonds to the carbonyl group of the isoleucyl moiety (Fig. 2, c and d). This recognition manner of the isoleucyl moiety of Ile-AMS is the same as that of L-isoleucine (3), except that the amino group hydrogen-bonds to the Oδ of Asp-85 in place of the main chain carbonyl group of Pro-46 in the IleRS-I-iso- leucine complex (3). The Nε of His-57, the second His of the class I-specific signature motif, 54^HIGH57, hydrogen-bonds to one of the oxygens of the sulfamoyl group (Fig. 2, c and d). The imidazole ring of His-558 provides van der Waals interactions with the ribose moiety (Fig. 2c). The 2'-hydroxyl group of the ribose moiety hydrogen-bonds to the main chain amide of Gly-551 (Fig. 2, c and d). Furthermore, the Oδ of Asp-553 and the Oε of Glu-550 hydrogen bond to the 2'- and 3'-hydroxyl groups, respectively (Fig. 2, c and d). The N3 group of the adenine moiety is recognized by the Nε of His-551 through hydrogen bonding (Fig. 2, c and d). The adenine is further recognized by the side chain of Leu-553 through a van der Waals interaction (Fig. 2, c and d).

Comparison of the Recognition Manners of aa-AMP by Class I aaRSs—To date, in the class I aARs, the crystal structures of T. thermophilus LeuRS (20), Bacillus steatorrhophilus TyrRS (Fig. 3d) (21, 22), and B. stearothermophilus TrpRS (23) have already been reported, as both free forms and complexes with their cognate aminoacyl-adenylates. Furthermore, the crystal structures of T. thermophilus ValRS (Fig. 3b) (25) and Escherichia coli GlnRS (Fig. 3c) (26) were reported in complexes with aminoacyl-adenylates. To elucidate the general recognition mechanism of the aminoacyl-adenylate, we compared the present structure of the IleRS-Ile-AMS complex with these adenylate complexes of the other class I aARs. The result is summarized in Fig. 3e.

Amino Group of the Aminoacyl Moiety—In the class Ia and Ib aaRSs, the amino group of the aminoacyl moiety hydrogen-bonds to the main chain carbonyl group of the residue (Pro-46 in T. thermophilus IleRS) located at the C terminus of the first β-strand of the Rossmann fold (Fig. 3, a–e). Furthermore, in ValRS, LeuRS (20), and GlnRS, the amino group of the aminoacyl moiety hydrogen-bonds to the Oδ of the aspartate residue corresponding to Asp-85 of T. thermophilus IleRS, which resides at the C terminus of the second β-strand of the Rossmann fold and is highly conserved in most of the class Ia and Ib aaRSs (Fig. 3, b and c, and e and f). In contrast, whereas the Oδ of Asp-85 hydrogen-bonds to the amino group in the complex with Ile-AMS. Both of the two residues are located in the first symmetrical half of the Rossmann fold. On the other hand, in the class Ie aaRSs, the side chain of the aminoacyl moiety rotates by almost 180° around the C–C<sub>α</sub> bond as compared with the class Ia and Ib aaRSs, and the amino group hydrogen bonds to the side chains of the
residues located in the second symmetrical half of the Rossmann fold (Fig. 4d).

Carbonyl Group of the Aminoacyl Moiety—In the class Ia and Ib aaRSs, the carbonyl group of the aminoacyl moiety is directed toward the second half of the Rossmann fold (Fig. 3, a–c). In LeuRS, the carbonyl oxygen is recognized with a hydrogen bond by the Nε of the invariant His residue (corresponding to Gln-554 of T. thermophilus IleRS) located in the fourth α-helix of the Rossmann fold (20). In contrast, in ValRS and GlnRS, the corresponding residues are well conserved hydrophobic residues, which cannot hydrogen-bond to the carbonyl oxygen (Fig. 3, b, c, and f). In these cases, the carbonyl oxygen of aa-AMP is not recognized by any residue of the aaRSs. In TrpRS, the carbonyl group of the aminoacyl moiety is directed toward the second half of the Rossmann fold as well, but is not recognized by any residue.

Phosphate Moiety—Except for IleRS, the main chain amide of the residue located on the loop following the first β-strand of the Rossmann fold (Thr-48 of T. thermophilus IleRS) hydrogen-bonds to one of the oxygens of the phosphate group (Fig. 3, b–f). In IleRS and ValRS, the phosphate oxygen is further recognized through hydrogen bonding by the Nε of the second His (His-57 of T. thermophilus IleRS) of the class I-specific signature motif, HIGH (Fig. 3, a, b, and f). On the other hand, in GlnRS and TrpRS (23, 24), the phosphate oxygen hydrogen-bonds to the Nε of the second Lys (Lys-594 of T. thermophilus IleRS) of the class I-specific signature motif, KMSKS (Fig. 3, c and f).

Ribose Moiety—The 2'-hydroxyl group of the ribose moiety is recognized through hydrogen bonding by the main chain amide of Gly (Gly-551 of T. thermophilus IleRS), which is located at the C terminus of the fourth β-strand of the Rossmann fold and is conserved in most of the class I aaRSs. Furthermore, the carboxyl group of the acidic residue (Asp-553 of T. thermophilus IleRS), located at the N terminus of the fourth α-helix of the Rossmann fold, hydrogen-bonds to the 2'-hydroxy group (Fig. 3, a–d). In contrast, in GlnRS, the residue corresponding to Asp-553 of T. thermophilus IleRS is not involved in the interaction with the ribose (Fig. 3, c and f), but instead, the 2'-hydroxyl group hydrogen-bonds to the side chain of the invariant Thr, which corresponds to Gly-551 of T. thermophilus (Fig. 3, c and f).

In IleRS and ValRS, the side chain of the residue corresponding to Glu-550 of T. thermophilus IleRS, on the fourth β-strand
of the Rossmann fold, is involved in hydrogen-bonding to the 3'-hydroxyl group of the ribose (Fig. 3, a, b, and f). In contrast, in LeuRS, GlnRS, TyrRS, and TrpRS (23, 24), the residue corresponding to Glu-550 of \textit{T. thermophilus} IleRS is replaced by a hydrophobic residue (Fig. 3, c and d). Consequently, in these aaRSs, the 3'-hydroxyl group is not recognized by any residue (Fig. 4, c and d).

In the class Ic aaRSs, the O-4' of the ribose moiety hydrogen-bonds to the second His (His-57 of \textit{T. thermophilus} IleRS) of the class I-specific signature motif, HIGH (Fig. 3, d and f). However, in the class Ia and Ib aaRSs, the O-4' of the ribose moiety is not recognized by any residue of the aaRSs (Fig. 3, a–c).

Adenine Moiety—Except for IleRS and TyrRS, the main chain amide of the residue corresponding to Ile-584 of \textit{T. thermophilus} IleRS (Swiss-Prot accession number P56690), \textit{T. thermophilus} ValRS (P96142), \textit{T. thermophilus} LeuRS (20), \textit{E. coli} GlnRS (P00962), and \textit{B. stearothermophilus} TyrRS (P00952), and \textit{B. stearothermophilus} TrpRS (P00953). The α-helices, β-strands, and loops are shown by green bars, blue arrows, and black lines, respectively. The amino acid residues that recognize the aa-AMP analogue with the main chain and the side chain are colored in purple and red, respectively.

Fig. 3. a–d, comparison of the molecular recognition of the cognate aa-AMP analogues by \textit{T. thermophilus} IleRS (a); \textit{T. thermophilus} ValRS (Ref. 25; Protein Data Bank code 1GAX) (b); \textit{E. coli} GlnRS (Ref. 26, Protein Data Bank code: 1QTQ) (c); and \textit{B. stearothermophilus} TyrRS (Ref. 22; Protein Data Bank code 3TS1) (d). The bound aa-AMP analogues are shown in green. The amino acid residues that recognize the aa-AMP analogues are indicated by ball-and-stick models. e, schematic drawing of the consensus recognition model of an aa-AMP by the class I aaRSs. The color of each moiety follows that in Fig. 2d, f, amino acid sequence alignment of the region near the HIGH sequence (top) and the region near the KMSKS sequence (bottom) of \textit{T. thermophilus} IleRS (Swiss-Prot accession number P56690), \textit{T. thermophilus} ValRS (P96142), \textit{T. thermophilus} LeuRS (20), \textit{E. coli} GlnRS (P00962), \textit{B. stearothermophilus} TyrRS (P00952), and \textit{B. stearothermophilus} TrpRS (P00953). The α-helices, β-strands, and loops are shown by green bars, blue arrows, and black lines, respectively. The amino acid residues that recognize the aa-AMP analogue with the main chain and the side chain are colored in purple and red, respectively.
nature motif, KMSKS (Fig. 3, b, c, e, and f). In contrast, in TyrRS, there is no hydrogen bonding interaction between the N-6 group and the main chain carbonyl oxygen of any residue (Fig. 3d). In the present crystal structure of the IleRS-Ile-AMS complex, the distance between the N-6 atom and the carbonyl oxygen of Met-592 is too far to allow hydrogen bond formation (6.05 Å). Therefore, movement of the loop bearing the KMSKS motif is required for the interaction, as discussed below. In IleRS, ValRS, and LeuRS (20), the side chain of the His residue (His-581 of T. thermophilus IleRS), at the C terminus of the last β-sheet, hydrogen-bonds to the N-3 atom of adenine (Fig. 3, a, b, and f). In GlnRS, the N-7 of adenine hydrogen-bonds to the Nη of the Arg corresponding to Leu-583 of the T. thermophilus IleRS (Fig. 3, c and f). In TyrRS, the adenine moiety is not recognized by a hydrogen bonding interaction with the enzyme (Fig. 3d).

Catalytic Loop Movement upon Adenylate Binding—In LeuRS and TrpRS, structural comparisons of the free forms and the complexes with the aminoacyl-adenylate revealed that the loop bearing the KMSKS motif moves toward the inside of the catalytic cleft of the Rossmann fold (Fig. 3, b and c). Consequently, in these four aaRSs, the KMSKS motif can interact with the adenine or the phosphate group of their cognate aa-AMP (Fig. 3, b and c). This “induced fit” may reconfigure the residues involved in the ATP recognition and stabilize the transition state for amino acid activation (27).

In contrast, in the structures of IleRS and TyrRS complexed with the aminoacyl-adenylate, the KMSKS loop still adopts the open conformation (Fig. 4, a and d). In these two aaRSs, the catalytic loop itself is involved in the crystal packing. Consequently, the signature loop may hardly move toward the amino acid-binding site, in keeping with the crystal packing. Actually, for the T. thermophilus IleRS, a high concentration of Ile-AMS led to cracking of the crystals, which can be ascribed to the movement of the signature loop. On the other hand, a low concentration of Ile-AMS may not cause the movement of the signature loop, thus resulting in the low occupancy of Ile-AMS in the active site. Therefore, further structural studies on the cocrystals of IleRS or TyrRS and aa-AMP are required.

Mupirocin Blocks the Binding Site of Ile-AMP—Our kinetic analysis revealed that a mupirocin moderately inhibits the T. thermophilus IleRS. a, omit electron density, contoured at 3.5σ, for the mupirocin molecule bound to the T. thermophilus IleRS. b, crystal structure of the complex of T. thermophilus IleRS with mupirocin. The bound mupirocin molecule is shown in red. c, mupirocin molecule bound to the catalytic site of the IleRS. The mupirocin molecule is shown in green. The amino acid residues that recognize mupirocin are indicated by ball-and-stick models. d, schematic drawing of the recognition of the mupirocin by the IleRS. The color of each corresponding moiety follows that in Fig. 2d, e, amino acid sequence alignment of the region near the HIGH sequence (top) and that near the KMSKS sequence (bottom) of six IleRSs, from T. thermophilus (Swiss-Prot accession number P566890), S. aureus type II (P41368), E. coli (P00956), S. aureus (P41972), Methanothermococcus jannaschii (Q58357), and S. cerevisiae (P09436). The amino acid residues corresponding to His-581 and Leu-583 of the T. thermophilus IleRS are shown in pink and violet for eukaryotic and eu- and archaeal IleRSs, respectively. The amino acid residues that recognize the side chain of the isoleucine moiety, the amide group of the isoleucine moiety, the sulfamoyl group, and the ribose group of Ile-AMS are shown in red, yellow, green, and blue, respectively.

Mupirocin binds to the active site of IleRS, blocking the binding of Ile-AMP. The binding of mupirocin is thermodynamically favorable, as indicated by the binding free energy of −6.0 kcal/mol. Interestingly, the recognition of Ile-AMP by IleRS is sensitive to the concentration of Ile-AMS, with a high concentration of Ile-AMS leading to cracking of the crystals. This observation suggests that the movement of the KMSKS motif is crucial for the binding of Ile-AMP.
thermophilus IleRS, with an IC\textsubscript{50} value of 0.9 μM. In the present structure, the mupirocin molecule snugly binds to the catalytic cleft of the Rossmann fold domain of the \textit{T. thermophilus} IleRS, like the Ile-AMS molecule (Fig. 4, a and b).

The moiety that morphologically resembles the hydrophobic side chain of l-isoleucine (C-12 to C-14 and C-17) is recognized by the isoleucine-specific pocket, consisting of Pro-46, Trp-518, and Trp-558, through van der Waals interactions. However, the direction of the moiety is different from that of the side chain of Ile-AMS or l-isoleucine (Fig. 4c). This difference may come from the hydroxyl group attached to C-13, which is specific to mupirocin, and from the structural difference between the short epoxy group of mupirocin and the long sulfamoyl group of Ile-AMS (Figs. 2c and 4c).

The pyran hexa-ring stacks with the imidazole ring of His-581 (Fig. 4c). The main chain amide group of Gly-551 hydrogen-bonds to the hydroxyl groups attached to C-6 (Fig. 4, c and d). Furthermore, the C-6-attached hydroxyl group in turn hydrogen-bonds to the Oε of Asp-553, and the C-7-attached hydroxyl group hydrogen-bonds to the Oε of Glu-550 (Fig. 4, c and d). Therefore, the hydroxyl groups of the pyran ring are recognized in the same way as those of the ribose moiety of Ile-AMS (Figs. 2c and 4c).

The conjugated system of C-1 to C-3 interacts with the side chain of Leu-583 through van der Waals interactions (Fig. 4c). The main chain amide of Ile-584 hydrogen-bonds to the carbonyl oxygen attached to C-1 (Fig. 4, c and d). This interaction mimics the interaction between the main chain amide of Ile-584 and the N-1 of the adenine moiety of Ile-AMS. Therefore, the conjugated system at C-1 to C-3 of mupirocin functionally mimics the adenine ring of Ile-AMP (Figs. 2c and 4c). Consequently, we can conclude here that mupirocin blocks the binding site of Ile-AMP, the high energy intermediate of the aminoacylation reaction.

\textbf{Function of the Unique Nonanoic Acid Moiety of Mupirocin—}

The 9-hydroxyxnonanoic acid encompassing C-1’ to C-9’ of mupirocin lies along the catalytic loop bearing the class I signature motif, KMSKS\textsuperscript{595} and interacts with the side chain and the main chain of Met-592 (Fig. 4c). The present complex structure is consistent with the previous biochemical experiment, which revealed that mupirocin protects the KMSKS catalytic loop of \textit{E. coli} IleRS from tryptophin digestion (13). In the crystal structure of \textit{S. aureus} IleRS complexed with \textit{E. coli} tRNA\textsuperscript{Ile} and mupirocin, the carboxylate tail of mupirocin binds to the carbonyl oxygen of Ser-597, the first Ser of the class I-specific signature motif, KMSKS, corresponding to Ser-593 of \textit{T. thermophilus} IleRS (28). This difference in the binding manner of mupirocin may be ascribed to the fact that the KMSKS loop of the \textit{S. aureus} IleRS adopts a closed conformation, in contrast to the open conformation in the \textit{T. thermophilus} IleRS complex.

The nonanoic acid moiety of mupirocin is characteristic of the antibiotic, in contrast to the other moieties that mimic Ile-AMP. It has already been reported that when the conjugated system and the nonanoic acid moiety are replaced by proper chemical groups that maintain both the electrostatic potential and the unoccupied molecular orbital features, the inhibitory activities do not change (29). The present crystal structure of the IleRS-mupirocin complex suggests that the hydrogen bond between the carbonyl oxygen attached to C-1 and the main chain amide group of Ile-584 mainly contribute to the electrostatic potential and that the interaction between the C-1 to C-3-conjugated system and the side chains of Leu-583 mainly contribute to the unoccupied molecular orbital. Therefore, it can be postulated that the long chain of the nonanoic acid moiety is not likely to be essential for the inhibitory activity.

On the other hand, as already discussed, the binding of ATP or an aminoacetyl-adenylate may induce the movement of the KMSKS signature loop to the inside of the catalytic cleft of the Rossmann fold. However, when mupirocin binds to the IleRS, the nonanoic acid moiety is located beside the signature loop, where it would fix its conformation to the open form and prevent the signature loop from accessing the active site. In contrast to the IleRS-Ile-AMS complex, where the signature loop has an open conformation by the crystal packing force, the nonanoic acid moiety of mupirocin might substantially stabilize the open conformation of the loop by the molecular interaction. Therefore, this may be an additional inhibitory mechanism of mupirocin.

\textbf{Differences in the Active Site Structures between the Bacterial/Archaeal and Eukaryotic IleRSs—} As described, the bacterial IleRSs apparently differ from the archaeal and eukaryotic IleRSs in their C-terminal domain and the C-terminal zinc finger structure (8). In contrast, the Rossmann fold domain, to which binds mupirocin and Ile-AMS, plays a crucial role in the aminoacylation reaction, and therefore, the amino acid sequences in the Rossmann fold domain are highly conserved throughout the IleRSs from the three primary kingdoms. Nevertheless, mupirocin inhibits only bacterial and archaeal IleRSs but not eukaryotic IleRSs (7). This suggests that some amino acid residues that recognize mupirocin, but are not crucial for the Ile-AMP recognition, should differ between the bacterial/archaeal and eukaryotic IleRSs. Considering that the IC\textsubscript{50} value of mupirocin against the \textit{T. thermophilus} IleRS is between those against the bacterial and eukaryotic IleRSs, the active site of the \textit{T. thermophilus} IleRS is composed of bacteriarchaeal type residues and eukaryotic type residues. By combining the present structure and the sequence alignment, we could identify two amino acid residues that directly recognize mupirocin but are not conserved between the IleRSs from bacteria/archaea and eukaryotes.

First, His-581, located at the C terminus of the last β-sheet of the Rossmann fold, is conserved in the bacterial/archaeal IleRSs but is replaced with an asparagine or serine in the eukaryotic IleRSs (Fig. 4e). In the present complex structure, His-581 hydrophobically interacts with the pyran ring, and the Ne of His-581 also hydrogen bonds to the N-3 group of Ile-AMS. The replacement of the His residue with Asn or Ser might considerably weaken the hydrophobic interaction with the pyran ring of mupirocin or the ribose of Ile-AMS while not affecting the interaction with the adenine base of Ile-AMS. In the \textit{S. aureus} plasmid-encoded IleRS (type-II), the His residue is replaced with Leu, and therefore, the hydrophobic interactions with the pyran ring moiety may be slightly weaker, but the hydrogen bond with the N-3 group of the adenine moiety may disappear.

Second, Leu-583 in the \textit{T. thermophilus} IleRS, which was mentioned above to be involved only in the interaction with mupirocin (13), is replaced with Phe in the bacterial/archaeal IleRSs and with Ile in the eukaryotic IleRSs (Fig. 4d). In the present structure, Leu-583 makes stacking interactions with

\begin{table}
\centering
\caption{\textit{K}_i values of mupirocin versus l-isoleucine and \textit{K}_m values of l-isoleucine}
\begin{tabular}{ccc}
\hline
Species & \textit{K}_i & \textit{K}_m \\
\hline
\textit{T. thermophilus} HB8 wild type & 0.25 & 52.8 \\
Eukaryote-type mutant & 2.73 & 56.4 \\
\textit{S. aureus} II-type mutant & 2.31 & 118 \\
\textit{E. coli} B & 2.5 × 10^{-3} & 11.1 \\
\textit{P. fluorescens} NCIB 10586 (IleS) & 2.5 & ND\textsuperscript{a} \\
\textit{S. aureus} & 60 × 10^{-3} & 1.1 \\
\textit{S. cerevisiae} & 15 & 16 \\
\hline
\end{tabular}
\end{table}

\textsuperscript{a}Not determined.
the conjugated system of mupirocin. Therefore, when the Leu residue is replaced with Phe, the stacking interaction may become stronger. In the *S. aureus* type-II IleRS, the corresponding position is occupied by His, which may extensively interact with mupirocin.

**Rationally Designed Mutations to Confer Mupirocin Resistance**—To confirm that the variations of the above-discussed amino acid residues in the Rossmann fold domain actually lead to the differences in the inhibitory activity by mupirocin, we constructed two types of mutant IleRSs. The eukaryotic type of mutant IleRS has double mutations, with substitutions of Thr-48 and His-581 for Phe and Asn, respectively. The *S. aureus* (type II) mutant IleRS has double mutations, with substitutions of His-581 and Leu-583 for Leu and His, respectively. Furthermore, in the *S. aureus* (type II) mutant IleRS has double mutations, with substitutions of His-581 and Leu-583 for Leu and His, respectively. We prepared the two types of mutants and measured the mupirocin inhibition of the IleRSs (Table II). The aminoaclylation activities of the two mutants were not substantially reduced; the *K*<sub>m</sub> values for L-isoleucine of the wild type, the eukaryotic type mutant, and the *S. aureus* (type II) mutant were 52.8, 56.4, and 118 μM, respectively. For the wild type IleRS, the *K*<sub>i</sub> value of mupirocin with respect to L-isoleucine was 0.25 μM, whereas that for the eukaryotic type mutant was 2.73 μM, which is 1 order of magnitude greater than the wild type. Furthermore, in the *S. aureus* (type-II) mutant, the *K*<sub>i</sub> of mupirocin was 2.31 μM, which is also 1 order of magnitude greater than the wild type. These results suggest that the replacements of the residues that vary between the bacterial/archaeal and eukaryotic IleRSs significantly change the sensitivity to a mupirocin. Here it should be noticed that, although the *S. aureus* type II IleRS is quite similar to the *T. thermophilus* IleRS (37.8% identity), only the exchange of the positions of the residues that vary between the bacterial/archaeal and eukaryotic type mutant. Conversely, it is now clinically critical to design a new drug that specifically inhibits the *S. aureus* type II IleRS, on the basis of the present complex structure and the biochemical information.

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