A Unique Hydrophobic Cluster Near the Active Site Contributes to Differences in Borrelidin Inhibition among Threonyl-tRNA Synthetases*

Benfang Ruan†, Michael L. Bovee‡, Meik Sacher‡, Constantinos Stathopoulos§, Karl Poralla‡, Christopher S. Francklyn§§, and Dieter Söll‡ ‡‡§§

From the †Departments of Molecular Biophysics and Biochemistry and §§Chemistry, Yale University, New Haven, Connecticut 06520-8114, ‡Department of Biochemistry, The University of Vermont Health Sciences Complex, Burlington, Vermont 05405-0068, and ‡‡Institut für Mikrobiologie, Eberhardt-Karls-Universität Tübingen, Auf der Morgenstelle 28, D-72076 Tübingen, Germany

Borrelidin, a compound with anti-microbial and anti-angiogenic properties, is a known inhibitor of bacterial and eukaryal threonyl-tRNA synthetase (ThrRS). The inhibition mechanism of borrelidin is not well understood. Archaea contain archaeal and bacterial genre ThrRS enzymes that can be distinguished by their sequence. We explored species-specific inhibition of ThrRSs. The activity of ThrRS from Sulfolobus solfataricus and Halobacterium sp. NRC-1 was inhibited by borrelidin, whereas ThrRS enzymes from Methanocaldococcus jannaschii and Archaeoglobus fulgidus were not. In Escherichia coli ThrRS, borrelidin binding induced a conformational change, and threonine was not activated as shown by ATP-PP exchange and a transient kinetic assay measuring intrinsic tryptophan fluorescence changes. These assays further showed that borrelidin is a noncompetitive tight binding inhibitor of E. coli ThrRS with respect to threonine and ATP. Genetic selection of borrelidin-resistant mutants showed that borrelidin binds to a hydrophobic region (Thr-307, His-309, Cys-334, Pro-335, Leu-489, Leu-493) proximal to the zinc ion at the active site of the E. coli ThrRS. Mutating residue Leu-489 → Trp reduced the space of the hydrophobic cluster and resulted in a 1500-fold increase of the $K_i$ value from 4 nM to 6 µM. An alignment of ThrRS sequences showed that this cluster is conserved in most organisms except for some Archaea (e.g. M. jannaschii, A. fulgidus) and some pathogens (e.g. Helicobacter pylori). This study illustrates how one class of natural product inhibitors affects aminoacyl-tRNA synthetase function, providing potentially useful information for structure-based inhibitor design.

Aminoacyl-tRNA synthetases (aaRSs) catalyze the acylation of transfer RNAs with their cognate amino acids and are therefore essential enzymes for protein synthesis in all organisms (1). They display exquisite specificity in discriminating between similar amino acid or tRNA substrates. Even though the core architectures of the active sites of individual aaRSs are relatively conserved, protein sequence alignments showed significant divergence among the three domains of life. However, finely tuned structural differences between aaRS orthologs provide ample opportunities for the evolution of species-specific inhibitors of any given tRNA synthetase. For example, mupirocin, an antibiotic produced by Pseudomonas fluorescens, selectively inhibits the prokaryotic isoleucyl-tRNA synthetases but has little or no effect on the eukaryotic enzymes (2, 3). Indolmycin, a secondary metabolite of Streptomyces griseus and analogue of tryptophan, inhibits only one of the two tryptophanyl-tRNA synthetases in Streptomyces coelicolor (4).

ThrRS is a class II aaRS containing an N-terminal editing domain, a C-terminal tRNA binding domain, and a zinc-binding catalytic domain with the class II conserved motifs 1, 2, and 3 that provide critical ATP binding determinants (5). ThrRSs from Chinese hamster ovary cells, Saccharomyces cerevisiae and Escherichia coli (reviewed in Ref. 6) are specifically inhibited by borrelidin, an 18-membered macrolide-polyketide (Fig. 1) produced by Streptomyces spp (7–9). Borrelidin was shown to have anti-malarial activity (10). Furthermore, borrelidin was found to interfere with capillary tube formation, possibly through anti-angiogenesis effects that are mediated through the ThrRS inhibition and the caspase activation pathways (11). Borrelidin also inhibited a S. cerevisiae cyclin-dependent kinase (Cdc28/Cln2), an indication of anti-tumor activity (12). Gene expression profiling of S. cerevisiae revealed that borrelidin up-regulated GCN4 leucine zipper mRNA synthesis (13), and this in turn induced the expression of amino acid biosynthetic enzymes. Because of its intriguing biological activities, the chemical synthesis (14, 15) and biosynthesis (16) of borrelidin have been explored.

Presumably as a consequence of borrelidin inhibition of human ThrRS, the compound may also have undesired cytotoxic effects. To develop borrelidin into an important anti-microbial, anti-angiogenesis, and anti-tumor drug candidate, a fuller understanding of the mechanism of borrelidin inhibition of ThrRS is required. Previous reports showed that borrelidin is a non-competitive inhibitor with respect to threonine (17) and inhib...
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**FIG. 1. Chemical structure of borrelidin.**

its the transfer of activated threonine to tRNA\(^{Thr}\) (18). Characterization of borrelidin resistant mutants showed that resistance resulted from ThrRS overexpression in vivo (19) or from a mutation affecting the \(K_m\) of threonine (17). However, the binding site is not known. Here we report the difference in borrelidin inhibition of the recombinant ThrRSs from *Methanocaldococcus jannaschii*, *Sulfolobus solfataricus*, *Halobacterium spp.*, and *Archaeoglobus fulgidus* as well as the identification of the binding site of ThrRS.

**EXPERIMENTAL PROCEDURES**

*General—*\(^{[1]}\)H\)threonine, \(^{[4]}\)C\)threonine, and \(^{[32]}\)P\)pyrophosphate were from Amersham Bioscience. *Escherichia coli* total RNA was from Sigma. The TOPO-TA cloning kit was from Invitrogen. The QuikChange system for site-directed mutagenesis and Mutatize kit for error-prone PCR were from Stratagene (La Jolla, CA). Oligonucleotide synthesis and DNA sequencing were performed by the Keck Foundation Research Biotechnology Resource Laboratory at Yale University (New Haven, CT). Nickel-nitrilotriacetic acid-agarose for His\(_{10}\)-tagged protein purification was from Qiagen (Valencia, CA). Protein concentrations were determined by the Bio-Rad protein assay using bovine serum albumin as a standard. *A. fulgidus* genomic DNA was a gift from Patricia Hartzell (Moscow, Idaho). The *E. coli* wild-type strain W3110 and ThrRS temperature sensitive strain W3110 was induced with 1 mM isopropyl-1-thio-\(\beta\)-galactoside for 2 h. Cells were harvested and lysed, and the wild-type and mutant His\(_{10}\)-ThrRS proteins were prepared from the cell extracts by nickel-nitrilotriacetic acid-agarose chromatography to >95% purity as judged by SDS-PAGE after Coomassie Brilliant Blue staining. Active site titration (21) was performed for ThrRS preparations by incubating each enzyme in 100 mM Tris-HCl, pH 7.5, 10 mM MgCl\(_2\), 10 mM KCl, 5 mM dithiothreitol, 4 mM ATP (pH 7), 1 mM \(^{[1]}\)C\)threonine (200 pmol), and 1 unit inorganic pyrophosphatase at a total volume of 0.1 ml at 37 °C for 15–20 min. The wild-type ThrRS enzymes were 80–100% active, whereas the activity of the mutant enzymes varied from 0.1–100% active. These values were used to determine enzyme concentrations for calculation of \(K_m\). The enzymes were stored in 40% glycerol buffer (60 mM Tris-HCl, pH 7.5, 10 mM MgCl\(_2\), 30 mM KCl, 5 mM dithiothreitol) at −20 °C.

Aminoacylation of tRNA—Thr-tRNA formation was measured by acid-precipitable radioactivity (21) in 0.1 ml of reaction mixtures containing 60 mM Tris-HCl, pH 7.5, 10 mM MgCl\(_2\), 30 mM KCl, 5 mM dithiothreitol, 0.02–10 mM ATP, 0.01–1 mM \(^{[3]}\)H\)threonine, 4 mg/ml total tRNA (E. coli tRNA for *E. coli* ThrRSs and *M. jannaschii* tRNA for other ThrRSs), 0–6000 nm borrelidin, and 0.1–100 nm enzyme. Reactions were carried out in triplicate at 37 °C for *E. coli* and *Halobacterium* ThrRSs, at 55 °C for *S. solfataricus* ThrRSs, and 65 °C for *M. jannaschii* and *A. fulgidus* ThrRSs. For steady state kinetic constants \(K_m\) and \(K_{cat}\), measurement, the enzymes were diluted to obtain linear initial velocities. \(K_m\) and \(K_{cat}\) were calculated in KaleidaGraph 3.0 using a Michaelis-Menten equation fit. For \(K_m\), measurements, saturating amounts of substrates (0.5 mM threonine, 1 mM ATP, 4 mg/ml tRNA) were mixed with various amounts of borrelidin (0–6 μM). The reaction mixture (ATP, tRNA, ThrRS enzyme) was preincubated in the presence of borrelidin at 37 °C for 3 min, and then threonine was added to initiate the reaction. Each reaction was repeated at least twice. The initial velocities obtained were plotted against borrelidin concentration to obtain \(K_m\) values. The apparent \(K^{(app)}\) values were calculated based on the simplified equation \(K^{(app)} = IC_{50}/E/2\) derived (22) for noncompetitive tight binding inhibition. ATP-PP, Exchange Assay—ThrRS activity was measured by quantitation of \(^{[\gamma-\text{32P}]}\)ATP retained on activated carbon (13) in 0.2 ml of reaction mixtures containing 60 mM Tris-HCl, pH 7.5, 10 mM MgCl\(_2\), 30 mM KCl, 5 mM dithiothreitol, 2 mM ATP, 0.01–1 mM threonine, 1 mM KF, 2 mM \(^{[3]}\)P\)pyrophosphate (4 cpm/pmol), 0–2400 nm borrelidin, and 1–100 nm enzyme. The kinetic constants were obtained as described above for the aminoclaylation reactions.

**RESULTS AND DISCUSSION**

*Borrelidin Inhibition of Archaeal and Bacterial ThrRS Enzymes—*Phylogenetic analysis of ThrRS sequences (24, 25) showed the existence of markedly different bacterial/eukaryal and archaeal versions of ThrRS. There are large deletions or additions in the N-terminal region of these enzymes; archaeal ThrRS has a shorter N-terminal region, an insertion domain at motif 3 (26), and many conserved differences in the catalytic domain. In this work we investigated the *S. solfataricus* and *Halobacterium sp. NRC-1* ThrRS, which are more closely related to the bacterial genre enzyme, and the archaeal genre enzymes from *A. fulgidus* and *M. jannaschii*. The influence of borrelidin on the enzyme activity was measured in the aminoclaylation assay. As shown in Fig. 2, ThrRS from *M. jannaschii* and *A. fulgidus* were not inhibited at 1 μM borrelidin, whereas those of *S. solfataricus* and *Halobacterium* were inhibited. To compare these enzymes under similar conditions, the ATP-PP exchange reaction was used for steady-state kinetic measurements. As shown in Table I, the tested ThrRSs had similar \(K_m\) values for threonine, but the \(K_{cat}\) values of ThrRSs from *M. jannaschii* and *A. fulgidus* were lower than those of the *S. solfataricus* and *E. coli* enzymes. The inhibition constants \(K_i\) for *M. jannaschii* and *A. fulgidus* ThrRSs were at least 1500 times higher than that of *S. solfataricus* ThrRS, indicating that the *M. jannaschii* and *A. fulgidus* enzymes may not bind borrelidin.

**The N-terminal Editing Domain Does Not Contribute to Borrelidin Binding—**No sequence homology of the N-terminal...
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**Fig. 2.** Borrelidin inhibition of aminoacylation catalyzed by ThrRS enzymes. The initial velocity curves were generated in the absence (●) or presence (第三次) of borrelidin (1 μM).

**Fig. 3.** Borrelidin inhibition of N-terminal truncated ThrRS enzymes. The initial velocity curves were generated in the absence (●) or presence (第三次) of borrelidin (1 μM).

**Fig. 4.** Intrinsic fluorescence of ThrRS in the presence or absence of borrelidin. A, titration of 1 μM ThrRS with various borrelidin concentrations in the stopped flow at 30 °C. All data sets were best described by a single exponential curve fit. The final borrelidin concentrations were 0, 1.5, 3, 5, and 8 μM shown by the curves. B, apparent first order rate constants from Fig. 4A versus borrelidin concentration. Error bars indicate the 95% confidence limit. The slope and y-intercept represent estimates of $k_{\text{on}}$ and $k_{\text{off}}$, respectively.

**TABLE I**

Steady-state kinetic constants of ThrRSs from various organisms

| Source of ThrRS | $K_m$ (Thr) ($\mu$M) | $k_{\text{cat}}$ (s$^{-1}$) | $K_d$ (nM) |
|-----------------|---------------------|-----------------------------|------------|
| E. coli         | 10.0 ± 5.0          | 33 ± 2.0                    | 3.7 ± 0.2  |
| S. solfataricus | 100 ± 5             | 16 ± 1.0                    | 4.5 ± 0.4  |
| A. fulgidus     | 90 ± 5              | 3.8 ± 0.3                   | >6000      |
| M. jannaschii   | 100 ± 5             | 1.2 ± 0.1                   | >6000      |

*To compare the enzymes under the same experiment conditions, kinetic data were determined by ATP-PPi exchange reactions.

**Borrelidin Is a Slow but Tight Binding Inhibitor of E. coli ThrRS**—The $K^{(app)}$ value (3.7 nM) for borrelidin inhibition of E. coli ThrRS was very close to the enzyme concentration (1–2 nM) used, and a linear dose-response of $IC_{50}$ to the enzyme concentration at a fixed substrate concentration was also observed (data not shown). These results suggested that borrelidin binding is tight and appears to be stoichiometric with dimeric ThrRS. Estimates of $k_{\text{on}}$ and $k_{\text{off}}$ rates as well as apparent $K_d$ values for borrelidin were determined from stopped flow fluorescence experiments. As shown in Fig. 4A, borrelidin binding quenched ThrRS fluorescence; this is clear evidence that the method of inhibition cannot be competitive. Single exponential curve fits were used to measure the apparent first order rate constants at a variety of borrelidin concentrations. The apparent rate constants were plotted with respect to borrelidin concentration as shown in Fig. 4B. Rates varied linearly with increasing borrelidin, suggesting a single borrelidin binding step. Estimates of $k_{\text{on}}$ and $k_{\text{off}}$ can be read directly from the slope and y-intercept, respectively. From this experiment, $k_{\text{on}}$ was estimated to be about $5 \times 10^6$ M$^{-1}$ s$^{-1}$. The y-intercept could not be accurately determined because of a requirement for a relatively high enzyme and borrelidin concentrations, but a conservative upper bound of 0.1 s$^{-1}$ fixes the $K_d$ of $k_{\text{off}}$ at no more than 20 nM; reasonably close to the 4 nM inhibitory constant. Therefore, borrelidin is a tight binding inhibitor of ThrRS. Because of the small off rate, overexpression of thrS might be expected to confer apparent borrelidin resistance to E. coli by virtue of titration of the drug with the excess enzyme (19).

**Borrelidin Inhibits E. coli ThrRS at the Step of Threonine Activation**—ThrRS catalyzes Thr-tRNA$^{Thr}$ formation in two steps (6); first it activates threonine to form the adenylate Thr-AMP, and then it transfers the activated amino acid onto tRNA$^{Thr}$ to generate Thr-tRNA$^{Thr}$. Borrelidin was reported to

main was observed between the borrelidin-inhibited ThrRS and the uninhibited type. Also, S. solfataricus contains two thrS related genes (25, 27); the archaeal genre ThrRS lacks the putative catalytic domain, and the bacterial genre ThrRS lacks most of the entire N-terminal editing domain. The bacterial genre S. solfataricus ThrRS can be inhibited by borrelidin. To determine whether the N-terminal region contributes to borrelidin binding, we aminoacylated E. coli tRNA with wild-type E. coli ThrRS and M. jannaschii tRNA with the A. fulgidus enzyme in the presence or absence of borrelidin. A similar experiment was carried out with the corresponding N-terminal domain-deleted enzymes. As shown in Fig. 3, the E. coli N-terminal-deleted ThrRS was inhibited by borrelidin to the same extent as the wild-type enzyme; the A. fulgidus N-terminal-deleted ThrRS remained uninhibited. Therefore, the N-terminal domain does not contain a borrelidin binding site.
inhibit the second step, the transfer of activated threonine to tRNA^{Thr} (18). However, because borrelidin is a tight binding inhibitor, the IC_{50} values could vary remarkably depending on the enzyme concentration. Therefore, we measured the IC_{50} value of the same amount of E. coli ThrRS enzyme determined by both ATP-PP_i exchange and aminoacylation. ATP-PP_i exchange reaction measures the kinetic parameter of the first step, threonine activation; aminoacylation determines the overall formation of Thr-tRNA^{Thr} formation. Fig. 5, A and B show that the IC_{50} values were similar in both reactions; the calculated K_i\textsuperscript{app} for E. coli ThrRS in the ATP-PP_i exchange reaction (4.2 nM) is very similar to the K_i\textsuperscript{app} of 3.7 nM in aminoacylation, indicating that borrelidin inhibits E. coli ThrRS at the first step of threonine activation and thus the overall aminoacylation. To confirm this, presteady-state kinetic analysis of the ThrRS-catalyzed adenylation reaction was carried out in the presence of borrelidin. Fig. 5C shows transient changes in the intrinsic tryptophan fluorescence of ThrRS in the presence or absence of substrates and inhibitor. All data traces were well described by fits to a single exponential function. In the presence of threonine and ATP, the enzyme fluorescence is quenched by 10%, with half the quench amplitude occurring within the dead time of the instrument (bottom fitted trace). This instantaneous quench reflects the enzyme response to the rapid binding of the substrates, whereas the fitted transient corresponds to an isomerization that precedes the chemical step of adenylation (23). In the absence of substrates the fluorescence change is negligible (top trace). The addition of various concentrations of borrelidin to ThrRS prior to rapid mixing with threonine and ATP abolishes the adenylation- associated quench but not that associated with substrate bind- ing, as expected for a noncompetitive inhibitor. At the highest borrelidin concentration, the fluorescence quench includes a small contribution from the prebound borrelidin in addition to the rapid binding of threonine and ATP. Taken together, both steady-state and presteady-state kinetic data showed that borrelidin inhibits ThrRS at the activation step rather than at the transfer step (18).

**Borrelidin Is a Noncompetitive Inhibitor of E. coli ThrRS with Respect to Threonine and ATP**—All the data above indicated that borrelidin may bind near or at the active site. To examine whether borrelidin has overlapping binding sites with the other enzyme substrates, we evaluated the inhibition mechanism with respect to threonine and ATP. The Michaelis-Menten derived double-reciprocal plot is commonly used to distinguish the inhibition pattern of reversible inhibitors with the assumption that the free inhibitor concentration could approximate the total concentration of the added inhibitor. However, borrelidin is a tight binding inhibitor that inhibits ThrRS at a very low concentration that is close to the enzyme concentration. Under these conditions the concentration of the enzyme-inhibitor complex is not negligible compared with the inhibitor concentration, so we can not use the double-reciprocal plot method to determine the inhibition patterns of borrelidin.

The inhibition pattern was then determined according to the equations derived for tight binding inhibitors (22, 28, 29). Several graphic approaches are available, and the most direct one is to determine the IC_{50} values for the inhibitor at a fixed enzyme concentration but at different substrate concentrations. For the competitive type, IC_{50} values increase linearly with increasing substrate concentration; that of the uncompetitive type is linear with the reciprocal value of the substrate concentration, i.e., IC_{50} values decrease sharply with the increasing substrate concentration. For the noncompetitive type, the relationship between IC_{50} values and substrate concentration varies depending on the dissociation constant of the inhibitor-enzyme-substrate complex (\(aK_i\)) and the inhibitor-enzyme complex (\(K_i\)). As shown in Fig. 6A, when \(K_i\) equals \(aK_{i}\), the equation for a tight binding competitive inhibitor reduces to IC_{50} = \(K_i + [E]/2\), indicating that substrate binding does not influence the binding of inhibitor to ThrRS enzyme. If \(aK_i < K_i\), the inhibitor-enzyme-substrate complex formation is favored over the enzyme-inhibitor complex, indicating that the substrate has a positive influence on inhibitor binding to the enzyme. If \(aK_i > K_i\), the substrate must have a negative influence on inhibitor binding to the enzyme.

Each \(IC_{50}\) value shown in Fig. 6, B and C was the average of \(IC_{50}\) values determined by dose-response curves of ThrRS activity to borrelidin concentration from repeated triplicate experiments with a less than 10% error. The IC_{50} values we obtained at different ATP or threonine concentrations showed slightly higher values at low ATP or threonine concentrations but essentially the same at higher substrate concentrations, indicating a noncompetitive pattern. Further, we curve fitted data points of IC_{50} values against substrate concentrations using the equation for a tight binding noncompetitive inhibitor. The plot of IC_{50} against threonine concentration gave a K_i value of 4.3 ± 0.2 nM and a \(aK_i\) value of 3.1 ± 0.1 nM. The plot for ATP gave a K_i value of 4.6 ± 0.2 nM and a \(aK_i\) value of 3.2 ± 0.1 nM. The smaller \(aK_i\) compared with \(K_i\) for both ATP and

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**Fig. 5. Borrelidin inhibits threonine activation by E. coli ThrRS.** A, plot of the dose-response curve showing the percentage of initial velocity reduced by various amounts of borrelidin as measured by aminoacylation reaction. The percentage of inhibition was calculated from the ratio of initial velocities of the aminoacylation reaction in the presence of borrelidin to that of in the absence of borrelidin. K_i\textsuperscript{app} determined is 3.7 nm. B, plot of the dose-response curve measured by ATP-PP_i exchange reaction; the K_i\textsuperscript{app} determined is 4.2 nm. C, intrinsic fluorescence of the ThrRS-borrelidin complex in the presence or absence of threonine and ATP at 30 °C. Equal volumes from the two syringes were rapidly mixed, and the final enzyme concentration in all reactions was 1.0 μM. Threonine and ATP were 0.5 mM and 1.5 mM, respectively. Final borrelidin concentrations in the presence of threonine and ATP were 0, 0.25, 0.5, and 1.0 μM, as marked beside the individual curves.
threonine indicated that the conformational changes in \textit{E. coli} ThrRS upon ATP or threonine binding (23) have some positive influence on borrelidin binding to ThrRS enzymes, although the positive effect is too small to be an uncompetitive pattern. Because borrelidin is a noncompetitive inhibitor with respect to threonine and ATP, borrelidin is not likely to have an overlapping binding site with that of ATP and threonine.

**Screening of ThrRS Mutants Indicates Possible Binding Sites for Borrelidin**—Possible binding sites at the catalytic domain were located by screening a library of randomly mutagenized \textit{E. coli} \textit{thrS} for borrelidin-resistant alleles. Eight of ten resistant colonies contained the same \textit{Leu489Met thrS} allele; another resistant allele is \textit{thrS} Pro296Ser. Both mutants were subcloned for overexpression as His-tagged proteins, and the purified recombinant enzymes were characterized \textit{in vitro}.

The kinetic data in Table II show that the selected mutant enzymes had a similar \(K_m\) value for threonine, an up to 2-fold higher \(K_m\) value for ATP, and an up to 2-fold higher \(K_i\) value for borrelidin compared with those of the wild type. Therefore, residues at position 489 and 296 are relevant to borrelidin inhibition.

**Point Mutagenesis of ThrRS Highlights a Hydrophobic Cluster Associated with a Putative Binding Site**—To determine the binding pocket in \textit{E. coli} ThrRS, we performed \textit{in silico} prediction using the CASTP-pocket/cavity predicting program (30).

Leu-489 belongs to two neighboring cavities (31): cluster A contains Thr-307, His-309, Cys-334, Pro-335, Leu-489, and Leu-493, and cluster B contains Cys-334, Val-338, Leu-489, Leu-493, and Met-509. Pro-296 belongs to cluster C containing Gly-295, Pro-296, Met-299, Lys-330, Asn-333, Gly-336, His-337, Ile-340, and Glu-357.

Further, \textit{thrS} alleles, containing mutations of the predicted contact residues and insertion or deletion constructs of the unique motif three sequence (EKG) of the archaean genre ThrRS, were made by PCR mutagenesis (the mutant changes are described in Fig. 7). After overexpression and purification, the \(K_i\)\textsubscript{app} for these enzymes was determined by aminoacylation under the same experimental conditions. Fig. 7 shows the \(K_i\)\textsubscript{app} for 15 ThrRS mutants at fixed enzyme concentration (2 nM). Deleting the N-terminal 167 amino acids as well as a deletion of Motif 3 sequence (Δ485–492) in \textit{A. fulgidus} ThrRS did not affect enzyme activity, and these enzymes were not inhibited by borrelidin. However, insertion of the three amino acid archaean motif three sequence (26) into \textit{E. coli} ThrRS greatly reduced enzyme activity, and the ThrRSHis309Ala enzyme showed very weak aminoacylation activity. The other mutant enzymes had \textit{good in vitro} activity. \textit{E. coli} mutant ThrRS enzymes with single amino acid changes (residues 335, 337, 489, 307, and 309) showed 2–1500-fold higher \(K_i\)\textsubscript{app} values; mutations in other residues had no effect on borrelidin inhibition. The most sensitive residue is leucine 489, located in the middle of the hydrophobic cluster A. Replacement of this Leu with Trp increased the \(K_i\) value 1500-fold.

These \textit{in vitro} results were confirmed by \textit{in vivo} tests of ThrRS activity and borrelidin inhibition. ThrRS activity was tested by complementation of a temperature-sensitive \textit{E. coli} \textit{thrS} strain, whereas borrelidin resistance was checked by growth on minimal medium containing 2 mM borrelidin (Fig. 8). With the exception of the \textit{E. coli} \textit{thrS} 531GEKG strain all mutant \textit{thrS} strains grew at the non-permissive temperature (Fig. 8, left panel). N-terminal-deleted \textit{thrS} and wild-type \textit{thrS} were borrelidin sensitive; however, \textit{thrSLeu489Met, thrSLeu489Trp, thrSHis337Ala, and thrSHis309Ala} gave rise to borrelidin-resistant transformants (Fig. 8, right panel). These data support our conclusion that cluster A is the borrelidin binding site.

A representation (Fig. 9) of cluster A was generated in the PyMOL program (32) using the crystallographic coordinates of \textit{E. coli} ThrRS (Protein Data Bank accession code 1QF6). This region is located at the “back” of the active site. Cys-334 of cluster A directly contacts the zinc ion, which is essential for ThrRS activity. Borrelidin binding to cluster A may cause relocation of Cys-334, resulting in further distortion of the zinc ion coordination and disruption of ThrRS activity (31). As cluster A is very close to the binding sites for threonine, ATP, and the terminal A residue of tRNAThr, substrate binding may also influence cluster A conformation. The fact that other aaRSs do not have such a hydrophobic core in this part of their active site may explain why borrelidin only inhibits ThrRS but not any other aaRSs.

**Comparison of the Putative Borrelidin Binding Region Among ThrRSs**—Alignment of a selection of archaean, bacterial, and eukaryotic ThrRS protein sequences was performed to reveal the extent of sequence conservation of the \textit{E. coli} ThrRS cluster A residues (Thr-307, His-309, Cys-334, Pro-335, His-337, Leu-489, and Leu-493) position. The Cys-334 residue is conserved in all ThrRS enzymes. The above mentioned cluster A residues (except for Thr-307) are found in the corresponding positions of the human and \textit{S. cerevisiae} enzyme, both of which are inhibited by borrelidin. Furthermore, \textit{Plasmodium} ThrRS has the same residues, which may explain its observed antimalarial activity (10). Some bacteria, including some pathogens (e.g. \textit{Helicobacter pylori} and \textit{Mycobacteria}), have amino acid replacements in some of the conserved residues in cluster A. The influence of these variations on borrelidin resistance by the corresponding ThrRS enzymes remains to be investigated.

However, the archaean genre ThrRS, found in \textit{M. jannaschii},...
Borrelidin Binding Site of ThrRS Enzymes

| ThrRS enzymes | Threonine | ATP | Borrelidin |
|---------------|-----------|-----|------------|
|               | $k_{\text{cat}}$ | $K_m$ | $k_{\text{cat}}$ | $K_m$ | $K_{\text{app}}$ |
| Wild type*    | 0.44 ± 0.04 | 24.4 ± 2 | 0.40 ± 0.04 | 94 ± 9 | 3.7 ± 0.3 |
| L489M         | 0.76 ± 0.04 | 27.4 ± 3 | 0.78 ± 0.08 | 163 ± 9 | 7.8 ± 0.2 |
| P296S         | 0.17 ± 0.01 | 25.8 ± 2 | 0.17 ± 0.01 | 187 ± 12 | 4.5 ± 0.2 |

* Literature values are $K_m$ for ATP 0.1—0.6 mM; $K_m$ for Thr 0.012—0.025 mM; $K_m$ for tRNA$^{\text{Thr}}$ 0.037—0.06 μM (6).

Fig. 7. In vitro borrelidin inhibition test of ThrRS mutants. The IC$_{50}$ values were determined by monitoring the aminoacylation reaction in the presence of 2 nm ThrRS and saturating concentrations of substrates while varying the borrelidin concentration. The apparent $K_{\text{app}}$ values were calculated based on the simplified equation ($K_{\text{app}}$ = IC$_{50}$/[E]/2). The x axis describes the 15 tested ThrRS mutants; the y axis shows the $K_{\text{app}}$ values in log scale. E. coli enzymes are presented in gray bars; ΔL1–210 is the E. coli ThrRS with the N-terminal 210 amino acids deleted, G531GEKG is the E. coli ThrRS with a EKG loop inserted after position 531, and the others contain point mutations.

Fig. 9. The active site structure of E. coli ThrRS with substrates. The α carbon backbone of the ThrRS active site is rendered as a red ribbon. The bound AMP and tRNA are sticks rendered in green and blue, respectively. The coordinated zinc atom is denoted as a yellow sphere. The putative borrelidin binding cluster A (Leu-493, His-307, and Pro-335) is rendered in space filling mode as an orange sphere; Leu-489 is highlighted in green, and Cys-334 is highlighted in purple. ThrRS is colored as red ribbon. The putative hydrophobic cluster B is (Met-509 and Val-338) is rendered as blue spheres.
Fig. 10. Protein sequence alignment of the borrelidin binding region in ThrRS. The ▼ indicates the residues of the borrelidin binding cluster A. Strictly conserved residues are indicated by dark shading. ThrRS enzymes are from S. cerevisiae, Homo sapiens, Halobacterium spp., E. coli, S. solfataricus, M. jannaschii, and A. fulgidus. The last two enzymes are not inhibited by borrelidin.

Jesse Rinehart, Jeffrey Sabina, and Juan Salazar for critical reading of the manuscript.

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J. Biol. Chem. 2005, 280:571-577.
doi: 10.1074/jbc.M411039200 originally published online October 26, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M411039200

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