Idiosyncratic Helix-Turn-Helix Motif in Methanosarcina barkeri Seryl-tRNA Synthetase Has a Critical Architectural Role

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All seryl-tRNA synthetases (SerRSs) are functional homodimers with a C-terminal active site domain typical for class II aminoacyl-tRNA synthetases and an N-terminal domain involved in tRNA binding. The recently solved three-dimensional structure of Methanosarcina barkeri SerRS revealed the idiosyncratic features of methanogenic-type SerRSs; that is, an active site zinc ion, a unique tRNA binding domain, and an insertion of ~30 residues in the catalytic domain, which adopt a helix-turn-helix (HTH) fold. Here, we present biochemical evidence for multiple roles of the HTH motif; it is important for dimerization of the enzyme, contributes to the overall stability, and is critical for the proper positioning of the tRNA binding domain relative to the catalytic domain. The changes in intrinsic fluorescence during denaturation of the wild-type M. barkeri SerRS and of the mutated variant lacking the HTH motif combined with cross-linking and gel analysis of protein subunits during various stages of the unfolding process revealed significantly reduced stability of the mutant dimers. In vitro kinetic analysis of enzymes, mutated in one of the N-terminal helices and the HTH motif, shows impaired tRNA binding and aminoacylation and emphasizes the importance of this domain for the overall architecture of the enzyme. The role of the idiosyncratic HTH motif in dimer stabilization and association between the catalytic and tRNA binding domain has been additionally confirmed by a yeast two-hybrid approach. Furthermore, we provide experimental evidence that tRNA binds across the dimer.

The fidelity of protein synthesis relies upon the interpretation of the genetic code by aminoacyl-tRNA synthetases (aaRSs). Each enzyme from this family catalyzes covalent attachment of a cognate amino acid to a specific set of tRNA isoacceptors in the first step of protein biosynthesis (1). Extensive studies of the structure and function of these enzymes have shown that the family is divided into two classes based on the topology of the adenylate binding sites and the modes of tRNA binding (2, 3). The class I aaRSs share a canonical dinucleotide binding fold (Rossmann-fold) with two regions of conserved sequences, named HIGH and KMSKS motifs. The class II synthetases are built around an antiparallel β-sheet partly capped by helices, thus creating a unique fold first identified in seryl-tRNA synthetase. Most of the class II synthetases are homodimers characterized by three conserved signature motifs. Although motif 1 is involved in dimerization, motifs 2 and 3 are crucial components of the enzymatic active site. aaRSs are modular enzymes that, in addition to the active site domain, possess a tRNA binding domain responsible for selection and binding of the cognate tRNA. Sometimes, additional domains such as an editing domain are added to the two-domain core of synthetase (4).

The seryl-tRNA synthetases catalyze the aminoacylation of several tRNAs isoacceptors as well as tRNAsec, with serine in a two-step reaction (5). Interestingly, two distinct types of SerRS enzymes have occurred; the methanogenic-type SerRS exists only in methanogenic Archaea, whereas the other, bacterial-type SerRS functions in a variety of archaeal, bacterial, and eukaryotic organisms (6). In the bacterial-type SerRS, as revealed by studies on the synthetases of Escherichia coli and Thermus thermophilus, each subunit possesses a C-terminal active site domain typical for class II aaRSs, which comprises the three class II conserved signature motifs, whereas the first 100 N-terminal residues form an antiparallel α-helical coiled-coil involved in binding of tRNA (5, 7, 8) and positioning of the 3′-end of tRNA in the active site of the C-terminal domain. Thus, the tRNA molecule interacts with the N-terminal domain of one subunit and the C-terminal domain of the other subunit in the dimer (9, 10).

The x-ray structure of Methanosarcina barkeri SerRS (mMbSerRS) (Fig. 1) (11) revealed that although the overall structure of the catalytic module is built from antiparallel strands surrounded by α-helices as for the bacterial-type SerRS, the mode of amino acid substrate binding is quite different and involves a zinc ion located in a deep cleft in the active site of the methanogenic-type enzyme (11). The fold of the mMbSerRS N-terminal domain, made of a six-stranded antiparallel β-sheet capped by a helical bundle on one side, is very different from the coiled-coil tRNA binding domain in bacterial-type SerRS (3).
Because the crystal structure of mMbSerRS in the complex with tRNA has not been obtained yet, as it has been for the bacterial system (10), the role of the N-terminal domain in the tRNA binding and aminoacylation is still unclear.

Another idiosyncratic feature of mMbSerRS is a unique ~30-residue-long insertion positioned between motifs 1 and 2 in the catalytic domains of all methanogenic-type SerRSs (11). The structure reveals that these residues adopt a helix-turn-helix (HTH) fold. Two protomers in the mMbSerRS dimer interact in a side-by-side fashion with their HTH motifs swapped. The amphipathic helices of the HTH fold bury their hydrophobic surface against the catalytic core of the other protomer and use charged side chains to make a series of salt bridges with the helix of the tRNA binding domain (11). Thus, structural data suggest that the HTH motif contributes to the interactions between the protomers and to the positioning of the tRNA binding domain. The aim of this study is to provide functional evidences on the role of the HTH motif in dimer stabilization and in interdomain interactions.

**EXPERIMENTAL PROCEDURES**

**tRNA Preparation and Aminoacylation Assays**—Production of *in vivo* expressed *M. barkeri* tRNA<sup>Ser</sup><sub>GGAG</sub> aminoacylation assays, and active site titration were carried out as previously described (12).

**Site-directed Mutagenesis, Construction of Deletion Fragments of *M. barkeri* SerRS, and Purification of the Proteins**—The seryl-tRNA synthetase expression vector (pET15b-mMbSerRS), which contained the wild-type sequence of *M. barkeri* SerRS inserted between the NdeI and XhoI restriction sites (13), was used as the template for construction of different mutants and deletion fragments (Table 1) by using the QuikChange mutagenesis kit (Stratagene). All mutations were confirmed by DNA sequencing. The importance of the HTH motif was investigated by excision of the HTH sequence from the *serS* gene, generating the pET15b-mMbSerRSΔHTH mutant.

The recombinant vectors pET28b-mMbSerRS-NTD and pET15b-mMbSerRS-CTD were used to overexpress the individual synthetase domains (N-terminal domain (NTD); C-terminal domain (CTD)). *E. coli* BL21 cells were co-transformed with both plasmids for concomitant overexpression of two mMbSerRS domains. 1 mM ZnCl<sub>2</sub> was added to the medium during the expression of mutated proteins. They were purified as reported previously for the wild-type enzyme (11). The tRNA binding and catalytic domains of SerRS were purified by the same general protocol, employing affinity chromatography on nickel-nitrilotriacetic acid resin and ion-exchange and size-exclusion chromatography. No degradation of protein fragments was observed on SDS-gel (supplemental Fig. 1). The concentrations of the full-length protein, different single point mutants, and deletion mutants were determined by the absorption at A<sub>280</sub> using theoretical extinction coefficients. Western blot analysis with *E. coli* anti-SerRS antibodies excluded the presence of contaminating endogenous SerRS in all purified mMbSerRS variants. The overall structures of the wild-type and mutated SerRS were examined by circular dichroism spectroscopy as previously described (12).

**Native PAGE and Gel Retardation Assay**—The gel retardation assay was performed under conditions described previously (12) unless stated differently.

**Irreversible Thermal Denaturation**—SerRS and its mutant derivatives (50 μl of 50 nm wild-type and 200 nm mutants in 50 mM HEPES/KOH (pH 7.6), 4 mM 1,4-dithiothreitol, 10 mM MgCl<sub>2</sub>, 50 mM KCl) were incubated simultaneously in a PCR thermocycler for 60 min at increasing temperatures. At the end of irreversible heat denaturation, the temperature was lowered to 4 °C. Residual aminoacylation activity was measured at 37 °C. sigmoidal curves were fitted to the datasets by nonlinear regression, and the temperature at which 50% of activity was lost under given conditions was calculated (T<sub>50</sub>). The experiments were repeated at least three times.

**Preparation of Heterodimers**—For overexpression of the heterodimeric form of mMbSerRS, *E. coli* BL21 cells were co-transformed with pET20b-mMbSerRS and pET28b-mMbSerRS-CTD (or pET28b-mMbSerRS-CTDΔHTH) plasmids. pET20b-mMbSerRS allows the expression of the whole SerRS polypeptide without N-terminal His tag, whereas the C-terminal domain (expressed from the construct with or without HTH) carries the affinity tag at its N terminus.

Co-expression of two SerRS polypeptides (whole-length and truncated, comprising only the C-terminal domain) resulted in three types of dimers (one hetero- and two homodimers). As only the C-terminal domain contains the affinity tag, heterodimer and homodimer of C-terminal domains were separated by nickel-nitrilotriacetic acid chromatography from the

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**TABLE 1**

| Name                  | Description of alteration |
|-----------------------|---------------------------|
| mMbSerRS-NTD          | STOP codon introduced at position 164, which generated the N-terminal domain |
| mMbSerRS-CTD          | START codon introduced at position 167, which generated C-terminal domain |
| mMbSerRS-ΔHTH         | Deleted HTH motif |
| mMbSerRS-CTDΔHTH      | START codon introduced at position 167 of mMbSerRS-ΔHTH, generating the C-terminal domain without HTH |
| mMbSerRS-R147A        | R147A replacement in helix 4 located in the N-terminal domain |
| mMbSerRS-E273A        | E273A replacement in helix 9 located in the C-terminal domain |
| mMbSerRS-E273A/K280A  | Triple replacement (E273A, D277A, K280A) in helix 9 |
| mMbSerRS-K146A        | K146A replacement in the linker peptide |
wild-type homodimer. The separation of heterodimer from truncated homodimer (homodimer consisting of C-terminal domains) was performed by electrophoresis on 0.7% native agarose gel in 90 mM Tris borate buffer, pH 8.4, at 80 mA for 4 h. The bands were visualized by transferring the proteins onto nitrocellulose membrane and staining with Ponceau S (supplementary Fig. 2). Bands corresponding to the heterodimer were cut out, and the protein was electroeluted from gel slices in 3 ml of electrophoresis buffer. The sample was dialyzed at 4 °C overnight against 30 mM HEPES/KOH (pH 7.6), 5 mM 2-mercaptoethanol, 10 mM MgCl₂, 200 mM KCl buffer. All procedures were performed at 4 °C.

4-(2-Pyridylazo)-resorcinol-p-(hydroxymercuri)phenylsulfonate (PMPs) Assay—Wild-type and different C-terminal domains of SerRS were dialyzed against 25 mM Tris/HCl, pH 7.5, 5 mM MgCl₂, 200 mM KCl. Three buffer exchanges ensured complete removal of 2-mercaptoethanol. 3.2 mM dialyzed protein with 4-(2-pyridylazo)-resorcinol (PAR) added to a final concentration of 0.1 mM was titrated at room temperature with 0.5 mM stock solution of PMPs. Absorbance at 500 nm was recorded 5 min after each PMPs addition to allow for a stable reading. The absorbance change was converted into moles of zinc by using the reported extinction coefficient for the Zn(II)PAR₃ complex (ε = 6.6 × 10⁴ M⁻¹ cm⁻¹).

Intrinsic Fluorescence Experiments—Samples for spectroscopic measurements were diluted at least 100-fold into solutions containing 50 mM Tris/HCl, pH 7.5, 200 mM KCl, 1 mM dithiothreitol and the indicated concentration of urea. Corresponding blanks were prepared using enzyme storage buffer. Sample solutions were prepared at least 8 h before measurements, as preliminary experiments revealed that a time-dependent change of fluorescence intensity after preparation of the sample is ended within the corresponding time. Fluorescence measurements were performed on a Varian Cary Eclipse spectrofluorometer at room temperature using quartz cuvettes (1 cm). Under the experimental conditions the fluorescence intensities of studied species were proportional to their concentrations. The samples were excited at 280 and 295 nm, and the signal of emission was monitored at 337 and 340 nm for wild type and mutant, respectively. The fluorescence spectrum of the protein was corrected by subtraction of the fluorescence spectrum of the buffer alone with urea added in a corresponding concentration. Fluorescence intensities could be reproduced to within ±2% for each sample in independent experiments.

Cross-linking—0.2 μM protein samples at different urea concentrations were prepared as for fluorescence spectroscopy experiments. After denaturation in urea, samples were cross-linked with 0.5% glutaraldehyde for 30 min at room temperature. Cross-linking reactions were stopped with 100 mM NaBH₄ for 3 min at room temperature and analyzed on 9% denaturing SDS-PAGE.

Yeast Two-hybrid Analysis—The N-terminal domain was recloned from the pET15b-mMbSerRS-NTD vector as an Ncol/Xhol cassette in-frame to the 3’-end of the coding sequence of the transcription factor Gal4 activation domain of the pACT2 prey vector (Clontech, Mountain View, CA). C-terminal domains with or without the HTH motif were recloned from pET28b-mMbSerRS-CTD and pET28b-mMbSerRS-CTDΔHTH plasmids in-frame to the 3’-end of the coding sequence of the transcription factor LexA DNA binding domain (LexA BD) of bait plasmid pAB151 (14) as Ncol/Smal cassettes. Further, C-terminal domains were recloned from pET28bmMbSerRS-CTD and pET28b-mMbSerRSCTDΔHTH plasmids as Ncol/Xhol cassettes to the 3’-end of the coding sequence of the transcription factor Gal4 activation domain of pACT2 prey vector (Clontech, Mountain View, CA). The resultant fusion proteins were tested for expression in Western blot with anti-hemagglutinin antibodies (Roche Applied Science) or anti-LexA antibodies (Invitrogen) (data not shown).

LexA BD domain and Gal4 activation domain fusion constructs were introduced by lithium acetate transformation into L40 Saccharomyces cerevisiae strain (MATa his3-D200 trp1-leu2-3, 112 ade2 LYS2::(lexAop)₃-HIS3 URA3::(lexAop)_₉-lacZ) (14). Double transformants were grown in selective -Trp-Leu media until late log phase (A₆₀₀ of 0.7–1.0), and serial dilutions were spotted as 4-μl aliquots on selective -Trp-Leu or -Trp-Leu-His synthetic dropout (SD) agar plates supplemented with 1–100 mM 3-amino-1,2,4-triazole. Furthermore, transformants were tested for β-galactosidase activity using X-gal for colony-lift filter assay or β-nitrophenyl-β-d-galactoside (15).

RESULTS

The Role of HTH-fold in Building the Dimerization Interface—Most class II synthetases are dimers, except for AlaRS, which is a tetramer, and PheRS, which is either monomeric or tetrameric (1). Glycyl-tRNA synthetase is one of the few synthetases which exhibit different oligomeric structures in different organisms (α₆-dimeric or α₂β₂ tetrameric in some prokaryotic species) (16). In the dimeric class II synthetases, motif 1 contributes to the formation of the dimer interface. A similar role has also been observed in the α₂β₂ PheRS (17). In tetrameric AlaRS, motif 1 is not involved in any intermolecular interactions, as the C-terminal domain is responsible for enzyme oligomerization (18). In mMbSerRS, the axes of the motif 1 interface helices are 15 Å apart (11), too far for their side chains to interact directly unlike E. coli SerRS where the helix axis separation is about 10 Å (3). Nevertheless, other regions of motifs 1 contribute to the stabilization of the dimer interface (Fig. 2). In
addition, as revealed by the crystal structure, the HTH motif of one protomer is also responsible for extensive interactions with the catalytic domain of the other protomer, significantly increasing the buried surface area of the dimer interface (Fig. 2).

To understand the role of the HTH motif in dimerization, we created a mutated enzyme mMbSerRSΔHTH in which the HTH motif has been removed and neighboring loops connected. As mMbSerRS protein is rich in aromatic amino acid residues, we used the intrinsic fluorescence to monitor protein conformational changes and stability during unfolding in urea. The difference in the stability of the two proteins was obtained by dividing the fluorescence intensity at 320 nm by the intensity at 365 nm ($I_{320}/I_{365}$) (Fig. 3). The fluorescence change in the unfolding experiments was measured over a range of protein concentrations (0.1–10.4 mM), and no concentration dependence was observed, although at concentrations lower than 0.06 mM some protein loss is noticeable, probably due to adsorption to glass.

To confirm that the observed differences in intrinsic fluorescence during the denaturation of the mutant and wild-type mMbSerRS are due to differences in dimer to monomer dissociation, cross-linking of protein subunits with glutaraldehyde was performed at the same protein concentration as used for the fluorescence study followed by the SDS-PAGE. In the absence of urea we detected a species with a mobility expected for the dimer (Fig. 3). At higher concentrations of urea (2.5 M), mMbSERSΔHTH mutant migrated to a position expected for the monomer. The transition from dimer to monomer for the wild-type enzyme was detected at even higher denaturant concentrations. The contribution of the HTH fold to dimer stability was additionally documented by a yeast two-hybrid approach (see below). Such stabilization could be of biological significance, considering that many methanogenic Archaea are thermophilic organisms and/or live in special environments.

The Role of the HTH Fold in Positioning the N-terminal tRNA Binding Domain Relative to the Catalytic Domain—Besides its role in dimer stabilization, structural data suggested that a portion of HTH motif is involved in the positioning of the N-terminal domain with respect to the catalytic domain (11). Despite structural differences between the two types of SerRSs, the N-terminal domain of mMbSerRS is also proposed to be the main tRNA binding region (11), and its mispositioning would certainly affect the second step of aminoacylation reaction. We intended to test the ability of mMbSerRSΔHTH to properly position the 3’-end of tRNA in the enzyme active site by the aminoacylation reaction. However, the deletion mutant was inactive in tRNA seryltransferase and, surprisingly, in serine activation reactions (supplemental Fig. 4). Possible changes in the structure and folding of mMbSerRSΔHTH were evaluated by CD spectroscopy. As expected, CD data revealed a lower helical content in the mutant enzyme but a slightly higher ratio of sheets and unstructured elements in comparison with the wild-type synthetase (not shown, see supplemental Fig. 4). However, mMbSerRSΔHTH retained the tRNA binding capacity, as revealed by a gel shift assay (not shown, see supplemental Fig. 4), arguing against overall protein misfolding. Together, these data exhibit a subtle structural change in the overall fold of mMbSerRSΔHTH.

The HTH motif is positioned above the catalytic domain and interacts with the latter through numerous hydrophobic interactions. Removal of HTH motif exposes these hydrophobic surfaces to the hydrophilic environment. In sequence space, zinc ligand Cys-261 is only 10 residues apart from the HTH motif, and conformational changes in this motif could affect its positioning and zinc binding capabilities. Therefore, the zinc ion content in the wild-type and mMbSerRSΔHTH mutant was determined using the PMPS test (not shown, see supplemental Fig. 5). It revealed that the removal of the HTH motif indeed diminishes the metal binding ability of the active site. Loss of activity of mMbSerRSΔHTH mutant prevented its use in seryltransferase assays. Therefore, to investigate the role of HTH motif in positioning of the N-terminal domain toward the catalytic domain and, consequently, the tRNA in the active site, we mutated several residues in helix 4 (the N-terminal domain) and helix 9 (the HTH motif) that contribute to the interactions between tRNA binding and the catalytic domain to alanines. Mutated proteins R147A, E273A, and E273A/D277A/K280A (positions are highlighted in Fig. 1) were purified under identical conditions as the wild-type mMbSerRS; gel filtration chromatography confirmed that they also form homodimers (data not shown). The steady-state kinetic parameters for wild-type and mutant SerRSs were determined in aminoacylation reactions with in vivo overexpressed M. barkeri tRNA<sup>ser</sup><sub>GGA</sub> (12). Mutant R147A shows a 3-fold decrease in $k_{cat}$ and almost 6-fold increase in the Michaelis-Menten constant (Table 2) in comparison with the wild-type enzyme, whereas E273A displays...
The Role of mMbSerRS HTH Motif

TABLE 2
Kinetic parameters for *M. barkeri* tRNA<sup>Ser</sup> aminoaoylation with wild-type and mutant mMbSerRS enzymes

| mMbSerRS | \(K_m\) \(10^{-3}\) M | \(k_{cat}\) \(s^{-1}\) | \(k_{cat}/K_m\) | \(K_m\) (rel) |
|----------|-----------------|---------|---------|---------|
| WT       | 2.6 ± 0.5       | 0.45 ± 0.04 | 173     | 1       |
| R147A    | 14.6 ± 0.7      | 0.15 ± 0.01 | 10.6    | 0.06    |
| E273A    | 4.2 ± 0.6       | 0.55 ± 0.10 | 130     | 0.75    |
| E273A/D277A/K280A | 4.7 ± 0.6 | 1.4 ± 0.2 | 290 | 1.68 |
| K164A    | 2.4 ± 0.4       | 0.0087 ± 0.0009 | 3.6     | 0.02    |

only modest reduction in the specificity constant, caused by slightly increased \(K_m\) value accompanied with a small increase in \(k_{cat}\). E273A/D277A/K280A mutant, having disabled several interactions between the two domains, showed an \(~2\)-fold increase in \(K_m\) and, interestingly, 3-fold elevated \(k_{cat}\). Table 2 reveals that this role may involve the direct interaction with the tRNA substrate (Fig. 1).

Higher flexibility of the N-terminal domain and weaker interactions with the catalytic domain could cause destabilization of dimeric SerRS. To compare the stabilities of mMbSerRS and its variants bearing alterations in helix 9, irreversible thermal denaturation was performed followed by the determination of residual aminoaoylation activity. Mutated enzymes with multiple preserved interactions between the two domains displayed higher stability, whereas the replacement of several helix 9 residues (mutant E273A/D277A/K280A) presumably results in additional conformational freedom between N- and C-terminal domains and consequently reduces the stability of the protein (supplemental Fig. 6).

The absolute requirement of the HTH motif for domain-domain interaction was additionally confirmed by yeast two-hybrid assays. As shown in Fig. 4, the separated C-terminal domain with the HTH motif interacts with the N-terminal domain, whereas the catalytic domain lacking HTH does not. Furthermore, the experiment confirms that the HTH fold contributes significantly to the stability of mMbSerRS dimer (Fig. 4).

**N-terminal Domain Affects Folding of the Catalytic Domain in Trans**—The short linker peptide that connects the N- and C-terminal domains is highly flexible and makes no interactions with the domains as observed in the crystal structure (11). This emphasizes the importance of the HTH motif as a main scaffold in maintaining the connectivity between two functional regions of the synthetase. Therefore, we decided to test whether the HTH motif is capable of positioning and orienting the N-terminal domain relative to the catalytic core even when produced separately i.e. in the absence of covalent connection.

Based on the crystal structures, the enzyme was dissected into the N- and C-terminal fragments that contained residues 1–164 and 168–502, respectively. Both domains were highly expressed separately in *E. coli* and purified to homogeneity as judged by SDS-PAGE (supplemental Fig. 1). Size exclusion chromatography confirmed that the N-terminal domain is a monomer, as expected, as two N-terminal regions do not contribute to the dimerization interface of mMbSerRS. In contrast, the C-terminal domain formed higher oligomeric species (not shown). Besides, the PMS assay revealed the absence of zinc ion in the purified C-terminal domain. These data demonstrate that the domain was not correctly folded. However, when both mMbSerRS domains were simultaneously co-expressed in *E. coli*, purified catalytic domain retained its zinc binding ability (supplemental Fig. 5) and the enzymatic activity, both in the seryl-adenylate (supplemental Fig. 3) and tRNA<sup>Ser</sup> synthesis (Fig. 5). Therefore, the N-terminal domain assisted proper folding of the catalytic domain in trans. To check the ability of non-covalently connected fragments to seryl tRNA<sup>Ser</sup>, the mixtures of purified domains were incubated at different molar ratios and assayed for aminoaoylation. Although there was no covalent connectivity between the two domains, they were
capable of catalyzing seryl-tRNA synthesis but less efficiently than the wild-type enzyme (supplemental Fig. 7). This demonstrates communication between the domains, presumably through the interactions between the HTH motif in the catalytic core and helix 4 in the N-terminal region (Fig. 5). The aminoclaylation reaction was inhibited upon the addition of higher amounts of N-terminal domain (27 μM). This result is consistent with our finding that the free-standing N-domain of methanogenic-type SerRS is a nonspecific tRNA binding domain (see later). To ensure that aminoclaylation was not due to the contaminating activity of the endogenous SerRS, the N-terminal domain alone has been assayed for catalytic activity. No serylation was observed (Fig. 5). The complex formation between tRNA and two non-covalently linked domains was confirmed by the gel shift assay, which also reveals the ability of the isolated N-terminal region to non-specifically bind tRNA (Fig. 6). Although this assay did not detect non-covalent association of two domains (not shown), the interactions between two domains was confirmed by a yeast two-hybrid method that allows detection of less stable interactions.

The Role of the Linker Peptide in the Positioning of the tRNA Acceptor End into the mMbSerRS Active Site—The alignment of methanogenic-type SerRSs shows a conservation of Lys-164 (in Methanothermobacter thermautotrophicus it is replaced with a functionally equivalent Arg), suggesting a certain level of functional importance. We hypothesized, therefore, that the linker peptide might have a more complex role than just bridging the domains. To test the functional role of this residue, we performed site-specific mutagenesis. The point mutation K164A decreased the catalytic activity of mMbSerRS in tRNA charging by 51-fold (Table 2). Because of the structural flexibility of the linker peptide in mMbSerRS apo structure and in the complex with small substrates (11), it is unlikely that Lys-164 has a structural role or that it would interact with other amino acid residues of mMbSerRS even when complexed with the tRNA. Thus, significantly affected catalytic parameters most probably reflect the interaction between Lys-164 and tRNA Ser.

The Role of mMbSerRS HTH Motif—Our docking model (11) of mMbSerRS-tRNA complex implies that the N-terminal domain of one protomer in the dimer will bind the variable arm of the tRNA Ser, whereas the 3′-end of the same tRNA will enter the active site of the other protomer (Fig. 1). Nevertheless, it might be possible, although less likely, that tRNA binds to one protomer only. To distinguish between these two possibilities we constructed two different heterodimers (see supplemental Fig. 8) named WT/CTD and WT/CTDΔHTH and compared their serylation efficiency. Both variants contain one wild-type protomer (WT), whereas the other protomer is truncated to the CTD, which either possesses (WT/CTD) or lacks (WT/CTDΔHTH) the HTH motif. Heterodimers WT/CTD and WT/CTDΔHTH have been produced in bacterial cells and separated from homodimeric enzymes as described under “Experimental Procedures.” Because the HTH motif of one mMbSerRS subunit packs above the catalytic center of the other protomer (Fig. 1 and Ref. 11) and because the homodimeric mMbSerRS without the HTH motif is catalytically inactive (see above), we would expect the catalytic domain of the WT protomer in the WT/CTDΔHTH heterodimer to be catalytically inactive because it is not stabilized by the HTH motif of the other pro-
The Role of mMbSerRS HTH Motif

The local conformation of the zinc-dependent active site in methanogenic-type SerRS seems to be altered in the isolated catalytic domain, which is then devoid of any activity. However, zinc binding ability (supplemental Fig. 5) and the propensity to catalyze seryl-adenylate synthesis (supplemental Fig. 3) is retained when mMbSerRS-CTD is purified from bacterial cells co-expressing both separated domains. Although the catalytic domain of methanogenic SerRS is not capable of tRNA binding on its own, the mixture of separated mMbSerRS-CTD and mMbSerRS-NTD fragments can moderately serylitate cognate tRNA<sub>Ser</sub> even without a covalent connection provided in the wild-type enzyme by a short linker peptide (Fig. 5).

Our experimental data show that the freestanding N-domain exhibits less specific tRNA recognition in comparison to whole mMbSerRS (Fig. 6). Therefore, the catalytic and N-terminal domains of SerRS have to act synergistically to provide a high and specific binding affinity for their cognate tRNA. It is likely that N-terminal domains of different SerRSs recognize the sugar-phosphate backbone of many tRNAs, whereas specific interactions cannot be achieved without the C-terminal domain. In support, no specific interactions between the N-terminal domain and cognate tRNA were observed in the structure of the T. thermophilus SerRS<sub>Ser</sub>tRNA<sub>Ser</sub> complex (24). Here we show that the freestanding N-terminal domain exhibits less specific tRNA recognition in comparison with whole mMbSerRS (Fig. 6). These results are in agreement with the work of Tworowski and Safro (25, 26), which suggested that nonspecific electrostatic interactions are the driving forces for primary association of aaRSs and tRNA and that this step is followed by precise binding and specific recognition of the reactants.

Among other class II dimeric synthetases, the non-catalytic domain and the catalytic domain of histidyl-tRNA synthetase (HisRS) have been purified separately (27). Interestingly, isolated catalytic domain of HisRS possesses intrinsic aminoacylation activity. Position 73 in tRNA<sub>His</sub> is the principal recognition determinant for the full-length cognate enzyme (28) and is, thus, sufficient for detecting the activity of isolated domains. However, efficient aminoacylation of tRNA<sub>Ser</sub> does not depend primarily on the acceptor stem but on the long variable arms of tRNA<sub>Ser</sub> (10, 13, 21), which interact with the non-catalytic N-terminal domains of homologous SerRSs (11, 24). Therefore, the catalytic and N-terminal domains of SerRS have to act synergistically to provide a high and specific binding affinity for two distant regions of cognate tRNA. This finding additionally supports the observation that the fusion of one or more accessory regions to a primordial catalytic domain may have been a critical evolutionary event by which aminoacyl-tRNA synthetases acquired improved catalytic properties (27, 29–31).

Although tRNA binding domains in the two SerRS types are non-homologous and evolutionarily unrelated (11), the requirement for a closing movement of the N-terminal domain upon tRNA binding has been observed in the T. thermophilus SerRS<sub>Ser</sub>tRNA co-crystal structure (24) and predicted by our docking model (11) and (Fig. 1). The different relative orientations of the tRNA binding and catalytic domains were also shown to be associated with tRNA binding in yeast (32) and E. coli AspRS (33). Importantly, methanogenic-type SerRS uses its idiosyncratic HTH motif to precisely orient the N-terminal arm of bacterial-type SerRS reduces dramatically its aminoacylation activity as well as specificity for cognate tRNA (21). However, the truncated E. coli enzyme can still catalyze seryl-adenylate formation (21). In contrast, we show that the catalytic domain of mMbSerRS is not an autonomous folding unit and needs the N-terminal domain to adopt a functional structure.

FIGURE 7. The HTH motif of mMbSerRS affects \(K_m\) and \(k_{cat}\) in tRNA<sub>Ser</sub> aminoacylation. Serylation kinetics of in vivo overexpressed tRNA<sub>Ser</sub> by the two different N-truncated enzymes: WT/CTD, WT/CTDAHTH. Reactions were carried out with 49.8 pmol of active WT/CTD heterodimer and 132.1 pmol of WT/CTDAHTH. The experimental values (symbols) were fitted to the Michaelis-Menten equation (curves). \(k_{cat}\) has been calculated assuming that 50% of WT/CTD enzyme is capable of performing serylation.

DISCUSSION

Organizing Interactions between Synthetase Domains—With the addition of the tRNA binding domains to catalytic cores, which occurred during evolution via multiple gene fusions, modern synthetases gained the capacity to specifically bind cognate tRNA molecules, thus improving the discrimination against non-cognate tRNA substrates in the cell (19).

With the exception of bovine mitochondrial SerRS (20), presumably all prokaryotic (10, 21), archaeal (12, 13, 22), and eukaryotic SerRSs (22, 23) employ their characteristic but structurally different N-terminal domains to bind the extra arm of the Ser-tRNA and to position the CCA end into the catalytic site. A deletion of two long antiparallel helices of N-terminal domain of methanogenic SerRS is not capable of tRNA binding even on its own, the mixture of separated mMbSerRS-CTD and mMbSerRS-NTD fragments can moderately serylitate cognate tRNA<sub>Ser</sub> even without a covalent connection provided in the wild-type enzyme by a short linker peptide (Fig. 5).

Thus, the WT/CTD\(\Delta\)TH heterodimer could aminoacylate tRNA only if it binds across the dimer, and no aminoacylation would occur if tRNA interacts with only one of the protomers. Our results reveal that the (WT/CTDAHTH) heterodimer is active but has less favorable kinetic constants, probably due to imprecise positioning of the N-terminal domain relative to the catalytic domain of the other protomer. As expected, the \(K_m\) value for the heterodimer of one full-length synthetase chain and one chain composed of the C-terminal domain lacking the HTH motif is much higher than the \(K_m\) for mutants with single or triple alterations in the HTH motif. As anticipated, the \(K_m\) value for tRNA for the heterodimer WT/CTD, comprising both HTH motifs but lacking one N-terminal tRNA binding domain, is comparable with the wild-type enzyme (Fig. 7). In agreement with the model that tRNA binds across the dimer, constructed heterodimers showed lower \(k_{cat}\).
domain, which is otherwise only connected via a flexible linker (Figs. 1 and 2). The critical role of this motif in the hinge-like disposition of the two functional domains was demonstrated here by the yeast two-hybrid approach (Fig. 4) and kinetic analysis of a series of mutants with partially altered HTH motif (Table 2). In conclusion, only the native dimeric enzyme exerts analysis of a series of mutants with partially altered HTH motif (Fig. 5). As established by earlier work on T. thermophilus SerRS (9) and by data presented in this paper (Fig. 7), cross-dimer tRNA binding seems to be evolutionary conserved in both SerRS types.

Importance of Covalent Connectivity — We have observed that a point mutation in the linker between the two domains significantly influences seryl-tRNA parameters of mMbSerRS (Table 2), although the linker itself does not contribute to the structural integrity of the enzyme (11). There are two mechanisms by which this linker is likely to affect the efficiency of tRNA binding. First, the conserved Lys-164 may contribute to tRNA-mMbSerRS complex formation, although our results do not allow us to conclude whether this residue interacts with the tRNA directly or indirectly. Therefore, the linker peptide and the conserved lysine may be adapted by the methanogenic-type SerRS to bind and position the 3′-end of the tRNA when it enters the active site for the aminoacylation reaction. Second, the linker will ensure the stoichiometric production of the two domains and increase the local concentration of the N-terminal domain in the vicinity of the catalytic domain. The initial capture of the tRNA is likely to be facilitated by the flexible disposition of the N-terminal domain, which will then deliver the tRNA to the active site of the enzyme via a hinge-like motion mediated by HTH interactions. The importance of synthetase covalent connectivity in domain-domain communication has also been observed in CysRS (34), ValRS (35) and GlnRS (36). In some cases covalent continuity within the tRNA is required for the communication between synthetase domains, as exemplified by MetRS (37).

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