The C-terminal Extension of Yeast Seryl-tRNA Synthetase Affects Stability of the Enzyme and Its Substrate Affinity*

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Saccharomyces cerevisiae seryl-tRNA synthetase (SerRS) contains a 20-amino acid C-terminal extension, which is not found in prokaryotic SerRS enzymes. A truncated yeast SES1 gene, lacking the 60 base pairs that encode this C-terminal domain, is able to complement a yeast SES1 null allele strain; thus, the C-terminal extension in SerRS is dispensable for the viability of the cell. However, the removal of the C-terminal peptide affects both stability of the enzyme and its affinity for the substrates. The truncation mutant binds tRNA with 3.6-fold higher affinity, while the K_m for serine is 4-fold increased relative to the wild-type SerRS. This indicates the importance of the C-terminal extension in maintaining the overall structure of SerRS.

Aminoacyl-tRNA synthetases are essential enzymes that catalyze the esterification of an amino acid to its cognate tRNA with exquisite specificity. The combination of biophysical, biochemical, and genetic techniques have significantly deepened our understanding of the structure and function of these enzymes (1-3). The amino acid sequences of seryl-tRNA synthetases from Escherichia coli (4), Thermus thermophilus (5), Bacillus subtilis (6), Coxiella burnetii (7), Saccharomyces cerevisiae (8), Chinese hamster (partial) (9), and human (human) contain a 20-amino acid C-terminal extension, which is typical of class II synthetases (10). The crystal structures of two prokaryotic enzymes isolated from E. coli (11) and T. thermophilus (12) are quite similar, as is their mode of interaction with tRNA^Ser^ (13). We have been working with yeast SerRS, which shows only moderate similarity (about 30%) with prokaryotic seryl-tRNA synthetases on the level of the primary structure (5), but still recognizes bacterial tRNA^Ser^ and can functionally substitute for the bacterial enzyme in vivo (13). The expression of the yeast SES1 gene in E. coli generated high amounts of functional enzyme (13), although with somewhat lower specific activity and slightly different electrophoretic mobility compared to the enzyme isolated from yeast. This raises the possibility that the yeast enzyme may not be modified or folded correctly in the bacterial host. In this paper we describe the purification of yeast SerRS from an overproducing strain of S. cerevisiae. The alignment of the primary sequences of all SerRS proteins reveals that the enzymes from yeast (8), Chinese hamster (9), and human (1) contain C-terminal extension between 20 and 40 amino acids long not found in prokaryotic synthetases. We speculated whether this peptide was important for maintaining the structure of the eukaryotic enzymes or if it had another function. Thus, we deleted the part of the S. cerevisiae SES1 gene encoding the short C-terminal domain and analyzed the expressed truncated protein.

MATERIALS AND METHODS

General Procedures—[14C]Serine (180 Ci/mmol) and [3H]ATP (40 Ci/mmol) were from Amersham Corp.YPD medium contained 1% yeast extract, 2% peptone, and 2% glucose (14). Selection for yeast auxotrophic markers was done in a medium of 0.67% nitrogen base and 2% glucose lacking amino acids, supplemented as needed with adenine (20 μg/ml), uracil (20 μg/ml), and amino acids (20-30 μg/ml). Sporulation medium contained 1% potassium acetate and amino acids as needed for auxotrophic diploids. For induction of the GAL promoter, glucose was replaced by galactose (2%) in all liquid media and agar plates. Transformation of yeast was performed by the lithium acetate procedure or by electroporation (14).

Strains and Plasmids—These are described in Table I. SES1 was used for various purposes, and thus several different constructs were made. Plasmid pBRSE51, used for disrupting the SES1 gene (see scheme in Fig. 2) contained the genomic SES1 yeast DNA fragment inserted in the opposite orientation compared to pBRSE51 (15). For the construction of the centromeric plasmid pUN70SES1, the SalI/BamHI fragment of pBRSE51 was used. The truncation of the SES1 gene was carried out (see below) in pJTU-1035SES1, where the BamHI cassette containing SES1 was inserted behind the ADH (alcohol dehydrogenase) promoter. The resulting gene, lacking the 60-bp encoding amino acids 443-462 of SerRS, was named SES1C20. For complementation of the null allele strain, a 2.1-kb SphI fragment containing SES1 or SES1C20 behind the ADH promoter was cloned into YEp351 (16) to generate YEp351SES1 and YEp351SES1C20, respectively. For overexpression, the BamHI cassettes with SES1 or SES1C20 were cloned behind the GAL promoter in pCJ11 (17) to generate the plasmids pCJ11SES1 and pCJ11SES1C20, respectively.

SES1 Gene Disruption—The experimental strategy (18) is presented in Fig. 2. In order to eliminate a HindIII site in the vector, pBRSE51 was digested with SalI and the larger fragment religated. The 0.4-kb HindIII fragment of the SES1 gene was excised and replaced with a LYS2 cartridge (19). This is a 4.5-kb HindIII fragment with the LYS2 structural gene behind the CYC1 promoter. A 5-kb SpsI fragment, in which the LYS2 gene was flanked by 137 and 379 bp of SES1 gene sequence, was then transformed into Saccharomyces cerevisiae diploid strain BR2727. Transformants were selected, sporulated, and tetrads dissected. Total DNA was also isolated from Lys^+ transformants and used for Southern blot analysis.

In Vitro Mutagenesis—The SES1 gene was truncated by loop-out in vitro mutagenesis, with a 1:1 molar ratio of phosphorylated dinucleotide to U-containing single-stranded pJTU-1035SES1 DNA. The structure of the synthetic 37-mer was as follows: 5'-CATTCCATGATATC-TATATTTTAAAATAATTTTCTGGTC-3'. After mutagenesis, DNA was...
TABLE I
Strains and plasmids

| Strain                  | Genotype, description, source, and/or reference | Strain                  | Genotype, description, source, and/or reference |
|------------------------|------------------------------------------------|------------------------|------------------------------------------------|
| S. cerevisiae          |                                                | BR2727                 | MATα/a ade2-1 arg4-9 his4 leu2-3,112 lys2 trp1 ura3-1 (gift from B. Rockmill, Yale University, New Haven, CT) |
| BR2727ΔSES1            | Same as above, but one type allele disrupted with SES1:LYS2 (this work) | S2088                  | MATα ura3-52 trp1 lys2-801 leu2Δ1 his3-l200 pep4::HIS3 prbΔ1.6R can1 GAL (gift from B. Rockmill, Yale University, New Haven, CT) |
| Yeast plasmids         |                                                | pVTU-103               | Multiplcy, ADH promoter, URA3 selectable marker (43) |
| pUN70                  | Centromeric, URA3 selectable marker (44)       | pCJ351                 | Episomal, LEU2 selectable marker (16) |
| pCJ 11                 | Multiplcy, GAL promoter, LEU2 selectable marker (17) | pVTU-103SES1           | 1.4-kb BamHI fragment, containing the yeast SES1 structural gene (13), cloned into pVTU-103 distal to the ADH promoter (this work) |
| pVTU-103SES1C20        | Same as pVTU-103SES1, but SES1 carries a deletion of 60 bp, coding for 20 C-terminal amino acids of SerRS (this work) | pUN70SES1              | pUN70 containing ~3-kb SalI/BamHI fragment (13), with the entire SES1 structural gene and its promoter region (this work) |
| YEp351SES1             | YEp351 containing 2.1-kb SphI fragment from pVTU-103SES1, which includes SES1 cloned between ADH promoter and its transcription terminator (this work) | pCJ 11SES1             | 1.4-kb BamHI fragment, containing SES1 structural gene, cloned distal to the GAL promoter of pCJ 11 (this work) |
| YEp351SES1C20          | Same as above but contains SES1C20 (this work) | pCJ 11SES1C20          | Same as above, except SES1 carries a 60-bp deletion (this work) |

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Transformation of yeast SerRS and SerRSC20 was 2–5 nM. The SerRS active site concentration was determined by the formation of the enzyme bound intermediate seryl-AMP according to Boré et al. (22).

Southern and Northern Blot Hybridizations—Total yeast DNA was isolated from transformed and non-transformed yeast cells (14). After digestion with BamHI, the fragments were separated on 0.7% agarose gel, transferred to nitrocellulose membrane and probed with 1.4-kb BamHI fragment (Table I) containing the entire SES1 coding region. For Northern analysis, total yeast RNA was isolated by the guani-

Results

Rationale—We wanted to test the functional and structural importance of the C-terminal extension of yeast SerRS, comprising 20 mostly basic and hydrophilic amino acids. Since this peptide is not a part of three highly conserved motifs characterizing class II synthetases (23), and is not present in four prokaryotic SerRS enzymes (5, 7, 6, 24) (Fig. 1), a non-catalytic role was proposed. We therefore investigated charging by the truncated protein, both in vivo and in vitro, as well as its stability.

C-terminal Truncation of SerRS—A 60-bp fragment of the SES1 gene, coding for 20 C-terminal amino acids of SerRS, was deleted by in vitro loop-out mutagenesis using a discontinuously homologous oligonucleotide. The truncated protein SerRSC20 ends with Leu-442. It was expressed from a truncated SES1 gene, which has its natural TAA stop codon, followed by 19-bp of SES1′-flanking region up to the introduced BamHI site (see “Materials and Methods”). The removal of the unusual, positively charged C-terminal peptide (amino acids 443–462; pl = 10.8), substantially changes the ionic properties of the whole enzyme: the calculated pl is 5.6 for the full-length protein and 5.3 for the truncated form.

Construction of S. cerevisiae SES1 Null Allele Strain—In order to test activity of the SES1 truncation mutant in vivo, we constructed the S. cerevisiae strain with inactivated SES1 allele, BR2727ΔSES1 (Fig. 2). As previously shown by Southern blot analysis (8), SES1 is a single-copy gene in the haploid genome, residing on a 15-kb BamHI fragment of chromosomal DNA. The SES1 gene contains an internal 0.4-kb HindII fragment, which was replaced on the plasmid with a LYS2 cartridge, which also serves as a selectable marker. Lys’ trans-
formants were selected, and the correct LYS2 integration at the SES1 locus was verified by Southern blot analysis (Fig. 2). Total DNA from BR2727 (55) and the parental strain BR2727 was digested with BamHI, separated by agarose gel electrophoresis, and transferred to nitrocellulose membrane. Hybridization with a radioactively labeled 1.4-kb BamHI fragment containing the entire SES1 structural gene, revealed the presence of one wild-type SES1 allele, giving a 15-kb BamHI fragment (Fig. 2, lane 1) of genomic DNA, whereas the other SES1 allele has been replaced in BR2727 by the mutant allele SES1LYS2 (25). Since the LYS2 gene carries an internal BamHI site, its integration into SES1 gives rise to two new BamHI fragments of 14 and 5 kb. Seven Lys⁰ isolates were sporulated and their tetrads dissected. All viable spores were lysine auxotrophs, and in no case did more than two of four separated spores form colonies. Inactivation of SES1 renders the cell inviable, indicating that the SES1 gene is essential and therefore indispensable for cell growth. The deletion could be rescued when SES1 was supplied on a centromeric or multi-copy plasmid. Transformed diploids were sporulated and viable Lys⁰ isolates were isolated by the method of Rockmill et al. (15). Selected haploids with a disrupted SES1 gene (Lys⁺ phenotype) where SerRS function was provided in the cell. This poor level of overexpression of the truncated enzyme is not fully galactose inducible (26). In addition, the serylation activity of the extract, determined by standard aminoacylation assay with 25 \( \mu \)M serine, was only 10 times above the control (extract made from non-induced cells) (Table II) and thus undetectable on a Coomassie-stained gel (Fig. 3A, lane 4). Therefore, in order to study the effect of removal of the C-terminal extension on the activity and the stability of the enzyme, full-length SerRS and mutant SerRSC20 were purified from yeast. To facilitate the purification of substantial amounts of proteins required for in vitro studies, we were interested in developing a high level overexpression system. Only 4-fold overproduction of SerRS was determined by Western blot analysis and aminoacylation with crude protein extract prepared from yeast cells transformed with pVTU103SES1 (Table II). The expression of the truncated protein from pVTU103SES1C20 was negligible under the same experimental conditions. Induction of the GAL promoter on pCJ11SES1, transformed in strain S2088, resulted in 150-fold overproduction of full-length SerRS protein and a corresponding increase of the seryl-accepting activity measured in the crude protein extract (Fig. 3A, lanes a and b; Table II). We found this system very suitable for the purification of the wild-type enzyme. However, in the protein extract made from induced yeast cells transformed with pCJ11SES1C20, the overproduction of truncated protein, as judged by scanning of the bands on the Western blot (not shown), was only 10 times above the control (extract made from non-induced cells) (Table II) and thus undetectable on a Coomassie-stained gel (Fig. 3A, lanes c and d). In addition, the serylation activity of the extract, determined by standard aminoacylation assay (25 \( \mu \)M serine) was very low. It seems, therefore, that the C-terminal fragment of yeast SerRS influences the expression of the protein or its accumulation in the cell. This poor level of overexpression of the truncated protein, together with the necessity to separate the enzyme from endogenous wild-type SerRS present in the yeast strain S2088, made the purification procedure much more difficult and inefficient. pCJ11SES1C20 transformants of BR2727 could not be used as a source of truncated protein, because the strain carries a mutation in the galactose permease gene and is not fully galactose inducible (26). Full-length SerRS was purified to apparent homogeneity (Fig. 3A, lanes e and f) by a quick two-step chromatographic procedure that involves fractionation on DEAE-cellulose and phosphocellulose columns (20, 21) as the important steps in isolation of SerRS from non-overproducing yeast strains. The procedure allows the purification of 1 mg of protein from 1 g of yeast cells, with a specific activity of 115 nmol mg⁻¹ min⁻¹, as determined in the standard aminoacylation assay with 25 \( \mu \)M serine. By increasing the concentration of serine to 1 mM (see

|        | I  | II | III | 430 |
|--------|----|----|-----|-----|
| E. coli| 1  | I  | II  | 421 |
| T. thermophilus| 1 |    |     | 425 |
| B. subtilis| 1 |    |     | 423 |
| C. burnetii| 1 |    |     | 423 |
| S. cerevisiae| 1 |    |     | 423 |
| Chinese hamster| 1 |    |     | 423 |
| Human    | 1  |    |     | 514 |

**Fig. 1. Schematic representation of the homology on the amino acid level between SerRS from E. coli, T. thermophilus, B. subtilis, C. burnetii, S. cerevisiae, Chinese hamster, and human.** Numbers above the line indicate the position of the amino acids. The composition of the C-terminal extensions in the yeast enzyme (443–462) and the corresponding sequences of Chinese hamster and human SerRS (469–493) are shown by one-letter code. Only the sequence of 199 C-terminal amino acids of Chinese hamster SerRS is known. Horizontal bars indicate the approximate position of three motifs characteristic for class II synthetases. There is extensive homology between all SerRS proteins in motifs I, II, and III.
below), a specific activity for the wild-type enzyme of 280 nmol mg⁻¹ min⁻¹ was obtained. The purified enzyme has two active sites for seryl-AMP per protein dimer (i.e. full site reactivity), as determined by the formation of the SerRS-bound intermediate seryl-AMP according to the method of Borel et al. (22).

The purification procedure for the truncated SerRS mutant was essentially the same as for SerRS, except the overall yield was 2 orders of magnitude lower (about 8 mg of protein/g of cells), both due to lower expression and to the narrow pools taken from both ion exchangers (see below). The separation of truncated enzyme from the wild-type SerRS by fractionation on DEAE-cellulose column was analyzed by the aminoaacylation assay and by SDS-PAGE followed by Western blot analysis (Fig. 3B). The activity peak was found in fractions j and k. Since the best separation of full-length and truncated SerRS enzymes was found in fraction k, this material was applied on the phosphocellulose column after careful desalting. Truncated protein was eluted with 10 mM KCl, immediately following the majority of unbound proteins, indicating very weak interaction of SerRSC20 with the anionic resin. Fig. 3C shows the separation of the proteins from the P-11 eluted fractions on a silver-stained SDS gel. Apparently homogeneous truncated enzyme eluted in fraction d, was concentrated and used in additional experiments. When assayed in the presence of elevated serine
TABLE II

Relative overexpression levels of wild-type and truncated SerRS

| Plasmid          | Overproduction |
|------------------|----------------|
| pVTU-103         | 1              |
| pVTU-103SES1     | 4              |
| pVTU-103SES1C20  | 1              |
| pCJ111           | 1              |
| pCJ115SES1       | 150            |
| pCJ115SES1C20    | 10             |

Concentration (1 μM), SerRSC20 shows specific activity similar to that of the full-length SerRS (257 nmol mg⁻¹ min⁻¹).

Deletion of the C-terminal Peptide Affects the Kinetics of Seryl-tRNA Synthetase

| Kinetic parameter | Full-length SerRS | Mutant SerRSC20 |
|-------------------|-------------------|-----------------|
| Kₘ (mM)           | 0.05              | 0.02            |
| k₅ (s⁻¹)          | 1500              | 3000            |

SerRSC20 shows a 6-fold higher affinity for tRNA at 30°C. Interestingly, this increased affinity is not detectable at 37°C, which is probably the result of a subtle, temperature-dependent, conformational change of the mutant enzyme (see below).

Another difference in the kinetic parameters between SerRS and SerRSC20 is the increased Km value for serine obtained for the mutant enzyme. There are no significant differences in k₅ values for the substrates, with mutant and wild-type enzyme, when measured at high serine concentration (1 mM). It should be noted that our experiments revealed a higher Km, value for serine for wild-type SerRS (Table III) than reported earlier (10 M) (27), which was the reason for the increase in [¹⁴C]serine concentration in the aminoacylation assay. The observed differences in the kinetic parameters for aminoacylation may be the consequence of the overall change in the enzyme conformation due to deletion of the positively charged C-terminal fragment, which can interact with other parts of the protein via electrostatic interactions. The involvement of the C-terminal peptide in the stabilization of the overall enzyme structure was further demonstrated by the comparison of the thermal stability of the full-length SerRS and the mutant SerRSC20. The proteins were first incubated at 42°C. After various time intervals, aliquots were withdrawn and tested for aminoacylation activity. Much faster heat inactivation of truncated protein compared to the full-length SerRS was observed (Fig. 4), without significant degradation of the protein, as confirmed by Western blotting (not shown). This confirms that removal of the C-terminal fragment of SerRS strongly influences protein stability. The truncated protein may be folded into a less stable conformation and is therefore more accessible to the intracellular proteolytic apparatus, which would then not allow high level accumulation of SerRSC20 in the cell.

C-terminal Truncation of SerRS Does Not Affect Its mRNA Level—This low level of overproduction of truncated SerRSC20 protein, raised the question of whether the transcriptional level of SES1C20 and the stability of its mRNA are altered. Thus, total RNA was isolated from yeast strain BR2727 transformed with either plasmid pVTU-103SES1 or pVTU-103SES1C20, which carry the structural gene SES1 and its truncated form, respectively, distal to the ADH promoter. Northern blot analysis showed that the truncation of 60 bp at the 3'-end of the SES1 coding region, does not influence mRNA production.
extremity of the molecules, usually the N terminus, rather than as insertions within the conserved regions. The exceptions are eukaryotic isoleucyl- (30, 31) and seryl-tRNA synthetases, which carry the extensions at the C terminus. The common feature of N-terminal extensions of eukaryotic synthetases, is a high content of basic amino acids. However, there is no homology or common structural motif between these fragments. As regards the functional significance of N-terminal extensions, it was proposed earlier that this domain confers on the synthetase the ability to bind to polyanionic carriers, promoting the compartmentalization of these enzymes within the cytoplasm, through electrostatic interactions with as yet unidentified cellular components carrying negative charges (28, 32). This hypothesis was experimentally tested on the aspartyl (33–35), glutaminyl (36, 37), lysyl (28, 38), and methionyl (39) systems. It was demonstrated that truncated enzymes lose affinity for anionic carriers (28, 33, 37). Although in some cases the extension, or part of it, was important for structure/activity of the enzyme, extensions were not found to be essential for the viability of the cells (36, 39). These studies reinforce the concept that eukaryotic synthetases have quasi-independent domains not found in their prokaryotic counterparts, which may confer a function distinct from aminoacylation. Recent experiments show that the N-terminal extensions of mammalian aspartyl- and arginyl-tRNA synthetases are responsible for mediating its association with the multisynthetase complex (40, 41), as also shown earlier for rat liver arginyl-tRNA synthetase (42).

In the serine system, three eukaryotic seryl-tRNA synthetases, from S. cerevisiae (8), Chinese hamster (9), and human (1), share sequence homology with their prokaryotic counterparts from E. coli (4), T. thermophilus (5), C. burnetii (7), and B. subtilis (6), except that they contain a C-terminal fragment of 20 or more mostly basic and hydrophilic amino acids (Fig. 1). Interestingly, the sequence of the first 25 residues (469–493) in the C-terminal extension in human SerRS is almost identical (with the exception of one amino acid) to the sequence of the corresponding peptide of Chinese hamster enzyme. Crystallographic studies have shown that two prokaryotic enzymes, isolated from E. coli and T. thermophilus fold to very similar conformations (5). Since none of the eukaryotic seryl-tRNA synthetases has been crystallized yet, the influence of the C-terminal domain on the overall structure of the protein is unknown. Although the deletion of the C-terminal extension of the yeast enzyme is not essential for the viability of the cell, thermal stability of the truncated enzyme determined in vitro is much below that of the full-length form. The abundance of positively charged (lysines) and hydrophilic (serines) amino acids in the C-terminal peptides of eukaryotic enzymes (Fig. 1), makes it likely that this domain is involved in maintaining the overall structure of the enzyme via electrostatic interactions.

When full-length enzyme showed full activity in the aminoacylation reaction in the presence of elevated serine concentration, as shown by very similar specific activities and $K_m$ values for SerRS and SerRSC20 (Table III), its affinity for the substrates differs from the full-length protein. At 30 °C, the $K_m$ for tRNA is 3.6-fold reduced for truncated enzyme, while the $K_m$ for serine is 4-fold elevated. These relatively small differences in kinetic parameters could be explained by an overall change of protein conformation, rather than by the loss of direct contacts with substrate binding sites, which would be expected to lead to far more dramatic changes.

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| Enzyme | Substrate | $K_m$ ($\mu$M) | $k_{cat}$ ($s^{-1}$) | Relative $k_{cat}/K_m$ |
|--------|-----------|---------------|---------------------|---------------------|
| SerRS  | tRNA<sub>Ser</sub> | 0.19          | 0.55               | 1                   |
| SerRSC20 | tRNA<sub>Ser</sub> | 0.05          | 0.46               | 3.01                |
| SerRS  | Serine    | 62.5          | 0.50               | 1                   |
| SerRSC20 | Serine    | 252           | 0.46               | 0.23                |
| SerRS  | ATP        | 21.0          | 0.52               | 1                   |
| SerRSC20 | ATP       | 14.3          | 0.50               | 1.4                 |

Fig. 4. Heat inactivation curve of full-length (a) and truncated (b) SerRS. An equal amount of protein was incubated at 42 °C in 50 mM Tris-HCl, pH 7.5, 2 mM DTT, 5% glycerol. At various time intervals, aliquots were withdrawn and used in the aminoacylation assay. The remaining seryl transfer activity is shown; 100% activity corresponds to 31 and 29 pmol of charged tRNA for full-length and truncated enzyme, respectively. The concentration of serine in the assay was 1 mM.

Fig. 5. Northern blot analysis of total yeast RNA isolated from strain BR2727 transformed with pVTU-103 (a, 50 µg), pVTU-103SE1 (b, 25 µg), or pVTU-103SE1C20 (c, 25 µg). RNA was fractionated on a 1.5% agarose/formaldehyde gel and transferred to a Nytran membrane (Schleicher & Schuell). Hybridization to a 1.4-kb HI fragment of 32P-labeled yeast DNA, containing the SES1 gene, was at 42 °C, as was the final wash of the membrane.

DISCUSSION

Alignments of primary structures of a number of aminoacyl-tRNA synthetases have established that subunit sizes of eukaryotic enzymes are often larger than those of the corresponding enzymes from prokaryotic sources (28, 29). These polypeptide chain extensions are found to be located at one
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