Isolation and Characterization of an *Escherichia coli* Seryl-tRNA Synthetase Mutant with a Large Increase in $K_m$ for Serine

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A mutant of *Escherichia coli* resistant to serine hydroxamate which has a large increase in $K_m$ for serine of seryl-tRNA synthetase is described. The mutant ser5 gene was cloned and sequenced and was found to contain a single-base-pair mutation, resulting in the substitution of the residue alanine 262 by valine in motif 2. The methyl side chain of alanine 262 is not exposed at the active site, and molecular modeling indicated that replacement of alanine 262 by valine does not significantly affect the configuration of amino acids at the active site. This finding suggests that the residue at this position may be involved in a conformational change (possibly induced by ATP binding) which is necessary for optimal binding of the cognate amino acid.

The maintenance of fidelity of translation in protein biosynthesis is strongly dependent on the correct recognition of amino acids by their cognate aminoacyl-tRNA synthetases. This recognition process has been finely tuned by evolution so that the amino acid binding sites can accommodate only the cognate substrate, albeit with rather small affinity constants. Directed mutagenesis has been used to study amino acid recognition by *Bacillus stearothermophilus* tyrosyl-tRNA synthetase, a class I aminoacyl tRNA synthetase, and mutants with an increased discrimination between tyrosine and phenylalanine have been isolated (5).

A strategy to identify amino acid binding sites in aminoacyl-tRNA synthetases is the characterization of mutant strains resistant to amino acid analogs. This approach has been used for phenylalanyl-tRNA synthetase (PheRS) (9). Mutants of *Escherichia coli* resistant to p-fluorophenylalanine were analyzed genetically, and the pheC5 mutations were found to cause an Ala-294→Ser replacement within motif 3 of the α subunit. Exchanges at position 294 altered the binding of phenylalanine, and certain mutants showed changes in specificity toward phenylalanine analogs. Another strategy to identify residues involved in amino acid binding is the characterization of mutations in aminoacyl-tRNA synthetase genes leading to an auxotrophic or temperature-sensitive phenotype. A Gly-191→Asp exchange in motif 2 of PheRS (10) and a Pro-231→Leu exchange in the same motif of asparaginyl-tRNA synthetase (AsnRS) (13) decrease strongly the affinity for the cognate amino acid.

Seryl-tRNA synthetase (SerRS), like PheRS and AsnRS, belongs to class II of the aminoacyl-tRNA synthetases (4, 6). The *E. coli* SerRS has been crystallized, and the resolution of its three-dimensional structure (3) defined the roles of the three primary structural motifs for this class of enzymes (6). The striking features of its structure are a protruding $\alpha$-helical arm, which is the N-terminal domain of the enzyme required for tRNA binding through the long variable arms of tRNA$^{Ser}$ (17), and a globular domain of seven antiparallel $\beta$ strands forming the C-terminal catalytic domain.

Recent studies from this laboratory have defined the residues in the active site of the *Thermus thermophilus* SerRS, and by analogy those in the active site of *E. coli* SerRS, which are involved in the binding of two analogs of seryl adenylate (2). As an approach to identifying functional residues involved in amino acid binding, we have isolated mutants of *E. coli* resistant to py-serine hydroxamate (SerHdx), a competitive inhibitor of SerRS (19). Mutant SerHdx resistant to SerHdx have been described previously, and some of these contained SerRS with a small increase in $K_m$ for serine [$K_m$(Ser)] and/or an increased $K_f$ for SerHdx (20). In the present report, we describe the isolation of a serine hydroxamate-resistant SerRS mutant with a 75-fold increase in $K_m$(Ser) in the aminoacylation reaction. The amino acid change in the mutant enzyme has been identified, and the implications of this observation for the catalytic mechanism of SerRS are discussed.

Isolation of SerHdx-resistant mutants and identification of a mutant with altered SerRS.

In a previous study (20), SerHdx-resistant mutants were isolated after selection on agar plates. We were unsuccessful with this approach, because of the high background growth of the wild-type strain, and we therefore devised an enrichment procedure in liquid culture. The *E. coli* wild-type strain W6 (18) was mutagenized with N-methyl-N'-nitrosoguanidine as described previously (22). After overnight growth in M9 minimal medium containing glucose as a carbon source (14), the mutagenized culture was subjected to several cycles of enrichment in the presence of SerHdx, with stepwise increases in concentration from 200 μg/ml to 1 mg/ml. At each stage of enrichment, the SerHdx concentration was increased when a significant difference in growth rate was observed between the mutagenized culture and a nonmutagenized control. Enriched cultures were then streaked onto M9 minimal agar plates, and individual colonies were tested for resistance to 1 mg of SerHdx per ml. To avoid the isolation of siblings, one resistant mutant was taken from each independent mutagenesis experiment.

The mutants were initially screened for the resistance of SerRS activity to inhibition by SerHdx. Preliminary experiments showed that in crude extracts of the wild-type, SerHdx either had no effect on or even stimulated SerRS activity (in one experiment, a threefold stimulation of activity was observed in the presence of 1 mM SerHdx). Subsequent experiments were therefore carried out with partially purified preparations of the enzyme.

Crude extracts from 50-ml culture, grown overnight in LB medium (14), were prepared as described previously (7). The
crude extract (5 ml) was then loaded onto a 10-ml DEAE-Sepharose CL-6B column (Pharmacia-LKB) previously equilibrated with buffer A (64.4 mM Tris-HCl [pH 7.6], 10 mM MgCl2, 1 mM sodium azide, 100 μM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol). The column was washed with 30 ml of buffer A and then with 20 ml of buffer A containing 0.1 M NaCl, and the enzyme was eluted with 15 ml of buffer A containing 0.2 M NaCl. Fractions of 1.0 to 1.5 ml were collected and assayed for SerRS aminoacylation activity, and the two to three fractions containing the highest activity were pooled.

Charging of E. coli tRNA with serine was assayed as follows. Partially purified enzyme preparations (1.0 to 2.0 mg of protein per ml) were diluted where appropriate in buffer A. Enzyme (10 μl) was then added to 40 μl of buffer A containing 0.5 mM spermidine, 5 mM disodium ATP, 100 μg of unfractionated E. coli tRNA (Boehringer), either 1 or 4 μCi of L-[3-3H]serine (23 to 29 Ci/mmol), and either 50 or 200 μM L-serine at 37°C. After incubation for 5 to 10 min at 37°C, the reaction was quenched with 50 μl of 5% (wt/vol) trichloroacetic acid, and the mixture was spotted onto a Whatman GF-A filter. The filter was then washed three times with 5% (wt/vol) trichloroacetic acid TCA and subsequently with ethanol, ethanol-ether (1:1), and ether, dried, and counted for radioactivity.

As shown in Fig. 1, the concentration for 50% inhibition of the wild-type enzyme by SerHdx was 50 μM in the presence of 4.7 μM L-serine (it should be noted that only the L isomer is inhibitory, and so the effective inhibitor concentration is half of the total SerHdx concentration [19]). In the SerHdx-resistant mutants, the 50% inhibitory concentration was similar to that of the wild type, except for strain SHDX3; in this mutant, SerRS activity was slightly stimulated by 50 μM SerHdx and was inhibited only 10% by 1 mM SerHdx (Fig. 1). In the presence of 40 μM L-serine, SerRS concentrations as high as 10 mM failed to inhibit enzyme activity (data not shown). It was found in a previous study that not all SerHdx-resistant mutants contain altered SerRS, and alternative mechanisms of resistance have been identified (20).

The Km values for serine, ATP, and tRNA in the aminoacylation reaction were determined for partially purified SerRS from the wild-type strain and the mutant SHDX3 (Table 1). Assays were carried out as described above except that the concentration of one substrate was varied over at least a 10-fold range. To ensure that initial rates were measured, activities were measured over a 5-min time course and enzyme preparations were diluted to give a rate of seryl-tRNA formation, under standard conditions, of 1.5 pmol/min. The amount of tRNA\(^{35S}\) in the crude tRNA was calculated from the amount of seryl-tRNA formed at saturation in the presence of excess serine. The Km(Ser) of SerRS from the mutant was found to be 60- to 90-fold higher than that of wild-type SerRS (Table 1). The SerHdx-resistant mutants isolated in a previous study showed a relatively small increase in Km(Ser) (±60%) but a three- to sixfold increase in Km for L-serine hydroxamate (20).

In the mutant strain SHDX3, the Km(Ser) for ATP was about fourfold higher than in the wild-type, whereas the Km for tRNA was slightly lower (Table 1).

**Growth characteristics of the ser mutant SHDX3.** In M9 medium containing 1 mg of SerHdx per ml, growth of the SerHdx-resistant mutants (measured by the increase in optical density [OD] at 550 nm) was linear with time. Linear growth rates (ΔOD per hour) varied from 0.031/h to 0.054/h for the different mutants and was 0.042/h for SHDX3. In contrast, the wild type showed an initial growth rate of only 0.020/h, and the rate decreased with time.

The growth rates of strain SHDX3 and the wild type (W6) were compared in more detail. In M9 medium, in the absence of SerHdx, the growth of both strains was logarithmic, with doubling times of 69 min (data not shown). On the other hand, in LB medium, growth was nonexponential: strain W6 grew faster than SHDX3 during the initial phases of growth, but the difference in growth rate decreased as the OD of the culture increased (Fig. 2).

To test the possibility that the observed growth effects were due to additional mutations acquired during the selection pro-
procedure, both the mutant and wild-type serS genes were transduced into the temperature-sensitive serS mutant, KL229 (F' serS15 thyA6 rpsL120) (12), obtained from B. Bachmann, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn., using phage P1cml (16). The isogenic, transduced strains showed the same growth pattern as the original wild-type and mutant strains (data not shown), showing that the growth properties of strain SHDX3 can be attributed entirely to the mutation in the serS gene.

The question arises as to how the mutant strain SHDX3 can grow so well, despite the low catalytic efficiency of the mutant enzyme. The intracellular concentration of l-serine was estimated to be around 90 μM (20), which is of the same order as the $K_m$(Ser) for the wild type but far below that of the mutant. It has been suggested that in *E. coli*, growth rates in batch culture may be limited by respiration (1). The large decrease in SerRS activity in strain SHDX3 appears to affect protein synthesis sufficiently to limit growth only at the high growth rates pertaining in rich medium. Under these conditions, the difference in growth rate between the mutant and the wild type decreased as the culture density increased (Fig. 2), presumably as a result of a decrease in oxygen availability and hence in respiration rate.

**Cloning and sequencing of the mutant serS gene.** Genomic DNA from *E. coli* SHDX3 was isolated by the method of Marmur (15) and purified by extraction with phenol-chloroform-isoamyl alcohol (12:12:1) and treatment with RNase A (14). PCR amplification of the serS gene was carried out as described previously (13), using the following oligonucleotide primers: 5'-TCCCATTGAATTCGATAAGC-3' and 5'-TA TAAGCTTGTATACATCG-3'. The PCR product (1.5 kb) was purified with a PCR purification kit (Quiagen; Diagen Inc.), digested with EcoRI and HindIII, and cloned in pUC18. The sequence of the cloned fragment was checked on both strands, using Sequenase version 2.0 enzyme (U.S. Biochemical).

The mutant serS gene (the structural gene for SerRS) in strain SHDX3 was amplified by PCR, and the mutation was identified by sequencing. Clones from two independent PCRs were sequenced, and in each case a single mutation was found: a C→T transition in the coding strand, resulting in the replacement of alanine at position 262 by valine. Examination of the primary (3) and tertiary (2) structures of SerRS showed that although the residue alanine 262 is a semiconserved residue situated at the beginning of motif 2, which forms part of the active site, the methyl side chain of this residue is not exposed in the active site but is buried in a large hydrophobic pocket. Molecular modeling studies suggest that this pocket should be sufficiently large to accommodate the bulkier side chain of valine without perturbing the configuration of amino acids at the active site.

Amino acid changes in motif 2 of PheRS (10) and AsnRS (13) have also been shown to result in a large increase in $K_m$ for the cognate amino acid. These mutations give rise to a temperature-sensitive phenotype and presumably cause long-range structural perturbations; in the case of AsnRS, the Pro-231→Leu exchange also causes an increase in $K_m$ for ATP, which is larger than the increase in $K_m$ for asparagine (13). The mutation in strain SHDX3 does not result in a temperature-sensitive growth phenotype, since the growth rates of both the mutant and wild-type strains were similar at 37°C and at 42°C (data not shown). Nevertheless, some temperature lability of the mutant enzyme was indicated, since the half-life of SerRS activity at 57°C, measured as described by Hill and Konigsberg (8), was 8.5 min, compared with 19 min for the wild type.

Precise determination of the structural effects of the Ala-262→Val exchange will require crystallization of the mutant enzyme. However, a possible explanation for the large increase in $K_m$(Ser) resulting from the mutation is that this residue is involved in a conformational change necessary for substrate binding and catalytic efficiency. Alanine at this position is not strictly conserved, since it is glycine in SerRS from *Saccharomyces cerevisiae* and *T. thermophilus*, which might indicate that a small amino acid is required at this site. Furthermore, recent
kinetic studies have shown that the apparent $K_m$ (Ser) in the formation of seryl-AMP is lower when serine is added after ATP than when the order of addition is reversed (11). ATP binding may therefore induce the required conformational change or stabilize the conformational state required for the optimal binding of serine. This contrast with the mechanism proposed for tyrosyl-tRNA synthetase, a class I aminoacyl-tRNA synthetase, in which the amino acid must bind before ATP, since the prior binding of ATP interferes sterically with the binding of tyrosine at the active site (21).

The mechanism by which the Ala-262→Val exchange confers resistance to SerHdx also remains to be determined. If SerHdx is considered to be a competitive inhibitor of SerRS, then resistance may arise by an improved discrimination of the enzyme between substrate and analog. This possibility is consistent with the data presented above and was also indicated in a previous study of SerHdx-resistant mutants (20). Nevertheless, the recent finding that SerHdx can act as a substrate for the enzyme, with formation of a stable adenylate intermediate, suggests that the mechanism of inhibition may be more complex than previously thought (2). The fact that SerHdx appears to stimulate SerRS activity under some conditions (see above) may also be related to this phenomenon.

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REFERENCES