Identification of Active Site Residues of the Tsp Protease*

Kenneth C. Keiler and Robert T. Sauer

From the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139-4307

In a search for active-site residues of the Tsp protease, 20 positions were individually mutated to alanine, the mutant strains were assayed for growth defects in vivo, and the purified proteins were assayed for proteolytic activity in vitro. Alanine substitutions at three positions, Ser-430, Asp-441, and Lys-455, result in inactive proteases that have structures and substrate-binding properties similar to wild type, suggesting that the side chains at these positions participate in catalysis. Replacing Ser-430 with cysteine results in a partially active protease, which is inhibited by cysteine-modifying reagents. Replacing Asp-441 with asparagine does not significantly affect activity. However, other residues, including histidine and arginine, cannot functionally replace Lys-455. These data are consistent with a serine-lyase dyad mechanism, similar to those proposed for the LexA-like proteases, the type I signal peptidases, and the class A β-lactamases.

Tsp is a periplasmic protease of Escherichia coli (1), which has been implicated in the processing of penicillin binding protein 3 (2) and TnB (3) and appears to play some role in fatty acid transport mediated by the FadL protein (3). Deletion of the tsp gene (also known as prc) causes a temperature-dependent growth defect under conditions of osmotic stress (2). Biochemical studies in vitro suggest that Tsp selects substrate proteins based on the identity of side chains and functional groups at their C termini. For example, amidation of the α-carboxyl group of a good peptide substrate prevents cleavage by Tsp (4). Moreover, Tsp cleaves variants of Arc repressor and the N-terminal domain of λ-repressor, which has apolar C-terminal residues but does not cleave variants of comparable stability that have polar C-terminal sequences (1, 4).

Tsp does not share sequence homology with well-characterized proteases or protease active-site sequences and cannot be classified as a serine, cysteine, aspartic, or metallo protease based on the identity of side chains and functional groups at their C termini. For example, amidation of the α-carboxyl group of a good peptide substrate prevents cleavage by Tsp (4). Moreover, Tsp cleaves variants of Arc repressor and the N-terminal domain of λ-repressor, which has apolar C-terminal residues but does not cleave variants of comparable stability that have polar C-terminal sequences (1, 4).

Tsp is a periplasmic protease of Escherichia coli (1), which has been implicated in the processing of penicillin binding protein 3 (2) and TnB (3) and appears to play some role in fatty acid transport mediated by the FadL protein (3). Deletion of the tsp gene (also known as prc) causes a temperature-dependent growth defect under conditions of osmotic stress (2). Biochemical studies in vitro suggest that Tsp selects substrate proteins based on the identity of side chains and functional groups at their C termini. For example, amidation of the α-carboxyl group of a good peptide substrate prevents cleavage by Tsp (4). Moreover, Tsp cleaves variants of Arc repressor and the N-terminal domain of λ-repressor, which has apolar C-terminal residues but does not cleave variants of comparable stability that have polar C-terminal sequences (1, 4).

Tsp does not share sequence homology with well-characterized proteases or protease active-site sequences and cannot be classified as a serine, cysteine, aspartic, or metallo protease based on the identity of side chains and functional groups at their C termini. For example, amidation of the α-carboxyl group of a good peptide substrate prevents cleavage by Tsp (4). Moreover, Tsp cleaves variants of Arc repressor and the N-terminal domain of λ-repressor, which has apolar C-terminal residues but does not cleave variants of comparable stability that have polar C-terminal sequences (1, 4).

Tsp does not share sequence homology with well-characterized proteases or protease active-site sequences and cannot be classified as a serine, cysteine, aspartic, or metallo protease based on the identity of side chains and functional groups at their C termini. For example, amidation of the α-carboxyl group of a good peptide substrate prevents cleavage by Tsp (4). Moreover, Tsp cleaves variants of Arc repressor and the N-terminal domain of λ-repressor, which has apolar C-terminal residues but does not cleave variants of comparable stability that have polar C-terminal sequences (1, 4).

The 105 variant of the N-terminal domain of λ-repressor, a good protein substrate for Tsp (1), was purified by ion-exchange chromatography, affinity chromatography, and gel filtration as described previously (12).
Activity and Structural Assays—To assay proteolytic cleavage activity, 0.5 μg of Tsp and 2 μg of the λ-repressor substrate were incubated at 37 °C for 20 h in 30 μl of cleavage buffer (10 mM Tris-HCl (pH 8.0) plus 20 mM NaCl). The reaction products were then electrophoresed on 15% polyacrylamide SDS-Tricine gels (13) and visualized by staining with Coomassie Blue. To quantify differences in cleavage rates, 10 μM Arc repressor was incubated with 290 mM Tsp or a Tsp variant in cleavage buffer at 37 °C in an Aviv model 60DS circular dichroism spectrometer, and the change in ellipticity at 222 nm was monitored over time (4). The activity of Tsp variants in vivo was assayed by streaking E. coli strain KS1000 transformed with wild-type or mutant versions of pKK101 on low salt agar plates containing 0.5% Tryptone, 0.25% yeast extract, and 150 mM ampicillin. Duplicate plates were incubated for 2 days at the permissive (37 °C) or the restrictive temperature (45 °C).

The CD spectra of Tsp or variants at concentrations of 50 μg/ml were recorded at 37 °C (to prevent autodegradation), in buffer containing 10 mM Tris-HCl (pH 8.0), 10 mM NaCl, using an Aviv model 60DS circular dichroism spectrometer.

Sequence Alignments—Sequences with homology to Tsp in GenBank release 89 were identified using the program BLAST (14), and multiple sequence alignments were performed using the PileUp program in the GCG package (15).

Substrate Binding Assay—The B chain of bovine insulin labeled with FITC at its amino terminus was purified from material purchased from Sigma using reverse-phase chromatography under reducing conditions. Incubation of the FITC-labeled insulin B chain with Tsp in cleavage buffer (2 h, 37 °C, enzyme:substrate ratio, 1:50) at 37 °C resulted in approximately 40% cleavage of the labeled protein as assayed by reverse-phase HPLC.

The addition of 2 μg of FITC-insulin to the λ-repressor-degradation reaction described above resulted in 40–60% inhibition of λ-repressor cleavage. For binding assays, equal amounts (667 nm) of Tsp and the FITC-labeled insulin B chain were incubated in cleavage buffer at 5 °C (at this temperature less than 1% of FITC-insulin was cleaved during the assays as determined by reverse-phase HPLC analysis) for 1 h, and the fluorescence emission spectrum was taken following excitation at 496 nm. In this assay, binding results in a 25–50% decrease in the intensity of the fluorescein fluorescence and an approximate 1.5-nm red shift in the fluorescence spectrum. Incubation of Tsp with fluorescein-iodoacetamide results in only 5–10% changes in fluorescence intensity and no red shift, suggesting that binding to FITC-labeled insulin B chain is not mediated solely by the fluorescein dye.

Chemical Inhibition Assays—The ability of diisopropyl fluorophosphatase (DFP) to inhibit Tsp was studied by incubating 1.5 μM Tsp with 1–50 mM DFP in cleavage buffer for 1 h, adding 2 μg of the λ-repressor substrate, and incubating for an additional 4 h. Degradation was assayed by gel electrophoresis, and the degree of inhibition was determined by comparing reactions incubated with DFP to control reactions containing all components except DFP. As a positive control, DFP inhibition of bovine trypsin was assayed under identical conditions and found to be complete at each concentration of DFP tested. Labeling with tritiated DFP (DuPont/NEN) was performed by incubating 1.5 μM Tsp or bovine trypsin with 12.5 μM tritiated DFP for 1 h, adding a second aliquot of tritiated DFP to a final concentration of 25 μM, and incubating for an additional 1 h. Reaction products were separated on an SDS-polyacrylamide gel, stained with Coomassie Blue, soaked in Autoradiol (National Diagnostics) for 1 h, dried, and exposed to film at −80 °C for 5 days. To quantify the extent of labeling, the stained bands were excised from the gel, suspended in scintillation fluid, and counted in a liquid scintillation counter. Inhibition of the S430C variant of Tsp by alkylating reagents was assayed by incubating 0.5 μg of protein in cleavage buffer with 10 mM N-ethylmaleimide or iodoacetamide at room temperature for 20 min, adding 2 μg of the λ-repressor substrate, incubating the mixture for 20 h at 37 °C, and assaying for substrate cleavage by gel electrophoresis.

RESULTS

Candidates for Functionally Important Positions—The mature Tsp protein contains 660 amino acids (1). Positions for mutagenesis were chosen in several ways. First, each of the five histidine residues in Tsp was mutated to alanine because histidine is a common component of known proteolytic mechanisms (16). Second, Arg-371 and Arg-444 were mutated to alanine because these positions are conserved between Tsp and each of the known IRBP repeats, suggesting that these basic residues may play a role in binding substrate. Third, Ser-372, Gly-375, and Gly-376 were mutated because these residues (as well as Arg-371) fall in a small region that shows weak homology with an active-site region of chymotrypsin (RSGGGL in Tsp; MGDSGGL in chymotrypsin). Finally, the alignment between Tsp and the presumed protease sequences shown in Fig. 1 was used as a guide, since these proteins share 53% identity in the two regions shown but have little significant homology in other regions. Each conserved residue with a hydroxyl, carboxyl, or amino group within the homology region was mutated to alanine since these residues could potentially participate in proteolytic chemistry. In all, 20 positions in Tsp were selected, alanine-substitution mutations were constructed, and the mutant proteins were purified.

Activity of Tsp Variants—13 alanine-substituted Tsp variants showed significant proteolytic activity in vitro, and seven had no detectable activity (Table I, Fig. 2A). The inactive proteins include the G375A, G376A, S430A, E433A, D441A, T452A, and K455A variants. Activity of the mutants in vivo was assayed by rescue of the temperature-sensitive growth defect of a tsp deletion strain on low salt medium. As shown in Table I, growth at the restrictive temperature is rescued by plasmids encoding Tsp variants that are proteolytically active in vitro but is not rescued by plasmids encoding mutants that
are inactive in vitro. This correlation suggests strongly that the conditional growth phenotype of a tsp deletion strain is related to proteolytic activity and not to some other function of Tsp.

Structure and Substrate Binding—In principle, a Tsp mutant could be inactive because it folds improperly, because it cannot bind substrate, or because it cannot catalyze peptide-bond hydrolysis. To address the folding issue, the CD spectra of all inactive variants and several active variants were determined. Three inactive mutants (S430A, D441A, and K455A) had CD spectra that were similar to wild type (Fig. 3A). Although this assay cannot rule out small conformational changes that could cause inactivity, Ser-430, Asp-441, and Lys-455 are the best candidates for residues that are involved directly in substrate binding or catalytic activity. The remaining four inactive mutants (G375A, G376A, E433A, and T452A) had spectra that differed markedly from that of wild-type Tsp (Fig. 3B), suggesting significant structural perturbations.

As an assay for substrate binding, wild-type Tsp and the S430A, D441A, and K455A mutants were incubated with FITC-labeled insulin B chain, and the fluorescence spectra were recorded. As shown in Fig. 4, the spectrum of the FITC-labeled substrate shows decreased intensity and a small red shift in the presence of each of these proteins. No significant change in intensity or red shift is observed when FITC-labeled

![Fig. 2. Substrate cleavage by wild-type Tsp and selected variants. A, gel assays for degradation of the λ-repressor 105 substrate by wild-type Tsp and variants after a 20-h incubation at 37 °C. B, effects of incubation of wild-type Tsp or the S430C variant with 10 mM iodoacetamide or 10 mM N-ethylmaleimide for 20 min at room temperature prior to the cleavage assay. Arrows indicate the positions of the intact Tsp and λ-repressor substrate bands. Approximately the same initial concentration of Tsp or variant was used in each assay. The final concentrations of enzyme are lower for wild-type Tsp and the S430C and D441N variants because of autodigestion. Some Tsp variant bands appear to have different mobilities because they were electrophoresed on gels run for different periods of time. C, time courses of degradation of Arc repressor (10 μM) by wild-type Tsp and variants (250 nM) at 37 °C monitored by loss of CD signal at 222 nm. D, effects of incubation of wild-type Tsp or the S430C variant with 10 mM iodoacetamide and 18.5 mM ethanol for 20 min at room temperature prior to the Arc cleavage assay (conditions as in panel C). Control reactions in which the protease is incubated with 18.5 mM ethanol alone are shown. Incubation with ethanol alone results in a reduction in activity of about 5% for both wild-type Tsp and S430C.

| Variant | Proteolysis of λ-repressor substrate | Growth on 1/2 LB no salt at 42 °C | Wild-type CD spectrum |
|---------|-------------------------------------|-----------------------------------|----------------------|
| Wild-type | + | + | yes |
| H19A | + | + | |
| H34A | + | + | |
| H60A | + | + | |
| H203A | + | + | yes |
| D369A | + | + | |
| R371A | + | + | |
| S372A | + | + | |
| G375A | – | – | no |
| G376A | – | – | no |
| S428A | + | + | |
| S430A | – | – | yes |
| S430C | – | – | yes |
| S432A | + | + | yes |
| E433A | – | – | no |
| D441A | – | – | yes |
| D441N | + | + | yes |
| R444A | + | + | |
| E449A | + | + | yes |
| T452A | – | – | |
| K455A | – | – | yes |
| K455H | – | – | yes |
| K455R | – | – | no |
| D508A | + | + | |
| H553A | + | + | |

**TABLE I** Properties of wild-type Tsp and variants

Identification of Active Site Residues of Tsp Protease

---

**Fig. 2. Substrate cleavage by wild-type Tsp and selected variants.** A, gel assays for degradation of the λ-repressor 105 substrate by wild-type Tsp and variants after a 20-h incubation at 37 °C. B, effects of incubation of wild-type Tsp or the S430C variant with 10 mM iodoacetamide or 10 mM N-ethylmaleimide for 20 min at room temperature prior to the cleavage assay. Arrows indicate the positions of the intact Tsp and λ-repressor substrate bands. Approximately the same initial concentration of Tsp or variant was used in each assay. The final concentrations of enzyme are lower for wild-type Tsp and the S430C and D441N variants because of autodigestion. Some Tsp variant bands appear to have different mobilities because they were electrophoresed on gels run for different periods of time. C, time courses of degradation of Arc repressor (10 μM) by wild-type Tsp and variants (250 nM) at 37 °C monitored by loss of CD signal at 222 nm. D, effects of incubation of wild-type Tsp or the S430C variant with 10 mM iodoacetamide and 18.5 mM ethanol for 20 min at room temperature prior to the Arc cleavage assay (conditions as in panel C). Control reactions in which the protease is incubated with 18.5 mM ethanol alone are shown. Incubation with ethanol alone results in a reduction in activity of about 5% for both wild-type Tsp and S430C.
Identification of Active Site Residues of Tsp Protease

insulin B chain is incubated with the structurally perturbed G376A mutant or when the fluorescein dye is incubated with wild-type Tsp. These results suggest that the S430A, D441A, and K455A variants bind substrate in a fashion similar to wild-type Tsp, making it likely that the side chains altered by these mutations are involved directly in the catalytic mechanism.

DPF Does Not Inhibit Tsp—The properties of the S430A mutant suggest that Ser-430 may be an active-site residue. For this reason, we tested whether DFP, an inhibitor of many serine proteases, could inactivate Tsp. After Tsp was incubated with 50 mM DFP for 1 h, it was still fully active in cleavage of the lac-repressor 105 substrate (data not shown). Moreover, incubation of Tsp with tritiated DFP resulted in less than 1% labeling compared with a bovine trypsin control.

Conservative Mutations at Positions 430, 441, and 455—In some proteases, cysteine can replace the active-site serine with retention of proteolytic activity (7, 8, 17, 18). To test this in Tsp, the S430C mutant was constructed and purified. The S430C variant is active in vitro (Fig. 2A), displaying about 5–10% wild-type activity (Fig. 2C) and can rescue the conditional growth defect of a tsp-deletion strain (Table I). The activity of the S430C mutant is inhibited by the cysteine-modifying reagents N-ethylmaleimide and iodoacetamide (Fig. 2, B and D). By contrast, these alkylating agents have no effect on the activity of wild-type Tsp, which contains no cysteine residues (1). When the S430C mutant was stored for several days at 0 °C in the absence of reducing agents, activity was lost but could be restored by incubating with 50 mM dithiothreitol for 1 h at room temperature. This suggests that the activity of the S430A variant is sensitive to some type of reversible oxidation reaction.

To determine if the carboxyl group of Asp-441 is required for activity, the isosteric D441N variant was constructed and purified. This mutant retains approximately 10% of the wild-type activity in vitro and is active in vivo (Fig. 2, Table I). This result indicates that the carboxylate of Asp-441 is not essential for the proteolytic activity of Tsp. Since the D441A mutant is inactive, we presume that Asn-441 can partially substitute for Asp-441 by participating in similar hydrogen bonding or steric interactions.

To probe the tolerance of position 455 to substitutions, the codon was randomized, and 47 colonies were isolated, tested for activity in vivo, and sequenced. Although all residues except Met, Cys, and Trp were recovered, only genes encoding Lys at position 455 were active. The K455R and K455H variants were purified and found to be inactive when assayed for proteolytic activity in vitro (Fig. 2, Table I). These data indicate that other basic side chains cannot functionally substitute for Lys-455. The K455H variant has a wild-type CD spectrum, while the K455R variant has an altered CD spectrum. The structural disruption caused by the seemingly conservative K455R substitution may indicate that the Lys-455 side chain is partially buried in a way that does not allow larger side chains to be accommodated.

**DISCUSSION**

In this study, we have used site-directed mutagenesis to identify residues important for Tsp activity in vitro and in vivo. Ser-430, Asp-441, and Lys-455 have properties expected of active site residues. Alanine substitution mutations at these positions abolish activity, but do not appear to affect substrate binding or to perturb the Tsp structure. Another group of residues (Gly-375, Gly-376, Glu-433, and Thr-452) seem to be important for maintaining the structure of Tsp. 13 additional residues, including each of the histidine residues in Tsp and two highly-conserved arginines, do not appear to be functionally or structurally important.

The three active site residues in Tsp appear similar in some respects to the residues of the catalytic triad of classical serine proteases. The traditional serine-protease mechanism involves a serine nucleophile, a histidine that acts as a general base to activate the hydroxyl group of the serine and an aspartate, which stabilizes the partial charge assumed by the histidine (19). Substitution of any of the catalytic triad residues with alanine in well-studied serine proteases such as subtilisin re-
duces activity by more than 1000-fold (20). The inactivity and partial activity, respectively, of the S430A and S430C mutants of Tsp, are consistent with the possibility that Ser-430 acts as a nucleophile. Moreover, although Tsp is not sensitive to inhibitors of classical serine proteases, such as DFP and phenylmethylsulfonyl fluoride, the S430C variant is inhibited by modification of the third group. Lys-455 of Tsp could function as a general base, in place of histidine, to activate the serine. However, Asp-441 of Tsp must play a role somewhat different than the aspartate of the classical catalytic triad, since the D441N substitution in Tsp does not affect activity, whereas the analogous substitution in trypsin reduces activity almost completely (21).

Tsp may be similar, in some respects, to the class A β-lactamases, which use a serine, lysine, and asparagine in hydrolysis of the lactam bond of penicillins and cephalosporins (9). Biochemical and crystallographic data suggest that the serine of β-lactamase is activated for nucleophilic attack by donating a hydrogen bond to the lysine, which in turn is stabilized by hydrogen bonds with an asparagine and another serine (9).

The conservation of the critical serine, aspartate, and lysine residues of Tsp in CtpA, and the other sequences shown in Fig. 1 suggests that the corresponding residues in these proteins will also be catalytically important. In the case of the IRBP repeats, two of the eight known sequences have a serine and aspartate at positions corresponding to Ser-430 and Asp-441 of Tsp. However, none of the IRBP repeats contains a lysine or other basic residue at positions corresponding to Lys-455 of Tsp. This lack of conservation of key residues is consistent with the fact that proteolytic activity has not been observed for IRBP. Instead, it appears that IRBP retains the fold of the Tsp active-site region, without the catalytic residues.

Acknowledgments—We thank Karen Silber and Bronwen Brown for gifts of protein, Sarah Ades for technical assistance, and Ray Larsen and Karen Postle for communicating unpublished data.

REFERENCES
1. Silber, K. R., Keller, K. C., and Sauer, R. T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 295–299
2. Hara, H., Yamanoto, Y., Higashitani, A., Suzuki, H., and Nishimura, Y. (1991) J. Bacteriol. 173, 4799–4813
3. Azizan, A., and Black, P. (1994) J. Bacteriol. 176, 6653–6662
4. Keller, K. C., Silber, K. R., Downard, K. M., Papayannopoulos, I. A., Biemann, K., and Sauer, R. T. (1995) Protein Sci. 4, 1307–1315
5. Shestakov, S. V., Anbudurai, P. R., Stankevina, G. E., Gadzhiev, A., Lind, L. K., and Pakrasi, H. B. (1994) J. Biol. Chem. 269, 19354–19359
6. Little, J. W. (1993) J. Bacteriol. 175, 4943–4950
7. Tschantz, W. R., Sung, M., Delgado-Partin, V. M., and Dalbey, R. E. (1993) J. Biol. Chem. 268, 27349–27354
8. van Dijl, J. M., de Jong, A., Venema, G., and Bron, S. (1995) J. Biol. Chem. 270, 3611–3618
9. Strynadka, N. C. J., Adachi, H., J. J. Jensen, S. E., Betzel, C., Sutoh, K., and James, M. N. G. (1992) Nature 359, 700–705
10. Silber, K. R., and Sauer, R. T. (1994) Mol. & Gen. Genet. 242, 237–240
11. Higuchi, R. (1990) in PCR Protocols: A Guide to Methods and Applications (Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., eds) pp. 177–183, Academic Press, Inc., San Diego, CA
12. Lim, W. A., and Sauer, R. T. (1991) J. Bacteriol. 173, 359–376
13. Schagger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368–379
14. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403–410
15. Genetics Computer Group (1991) Program Manual for the GCG Package, Version 7, Madison, WI
16. Dunn, B. M. (1989) in Proteolytic Enzymes: A Practical Approach (Beynon, J. R., and Bond, J. S., eds) pp. 57–81, IRL Press, Oxford, UK
17. Nakatsuka, T., Sasaki, T., and Kaiser, E. T. (1987) J. Am. Chem. Soc. 109, 3908–3910
18. Higaki, J. N., Evin, L. B., and Craik, C. S. (1989) Biochemistry 28, 9256–9263
19. Antonov, V. K. (1993) Chemistry of Proteolysis, Springer-Verlag, Berlin
20. Carter, P., and Wells, J. (1988) Science 237, 909–913