Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 6132–6136, July 1991
Biochemistry

Interaction of *Escherichia coli* $tRNA^{Ser}$ with its cognate aminoacyl-tRNA synthetase as determined by footprinting with phosphorothioate-containing tRNA transcripts

(phosphorothioate-tRNA/iodine cleavage/sequencing)

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Communicated by John Abelson, April 4, 1991

ABSTRACT **A footprinting technique using phosphorothioate-containing RNA transcripts has been developed and applied to identify contacts between *Escherichia coli* $tRNA^{Ser}$ and its cognate aminoacyl-tRNA synthetase. The cloned gene for the tRNA was transcribed in four reactions in which a different NTP was complemented by 5% of the corresponding nucleoside 5′-*O*-(1-thiotriphosphate). The phosphorothioate groups of such transcripts are cleaved by reaction with iodine to permit sequencing of the transcripts. Footprinting was achieved by performing the same reaction with the phosphorothioate-tRNA–enzyme complex. At 1 mM iodine, selective protection of the tRNA transcripts in the cognate system was observed, with strong protection at positions 52 and 68 and weak protection at positions 46, 53, 67, 69, and 70. It is suggested that these regions of the tRNA interact with the helical arm of the synthetase.**

In recent years significant progress has been made in the identification of nucleotides responsible for the specificity of the charging of tRNAs by their corresponding aminoacyl-tRNA synthetases (for a review see ref. 1). A detailed picture of the interactions between the tRNA and the synthetase in such complexes by x-ray crystallography has so far been obtained only for the *Escherichia coli* glutamine system (2). Various chemical footprinting methods have been employed for the study of tRNAs and their complexes with synthetases in solution, using reagents such as *N*-ethylnitrosourea, dimethyl sulfate, diethylpyrocarbonate, and more recently, photoactivatable aryldiazonium derivatives (3–8). We report here the adaptation of the recently reported method for sequencing of phosphorothioate RNA transcripts (9) to the footprinting analysis of the complex of the phosphorothioate transcripts of *E. coli* $tRNA^{Ser}$ with its cognate aminoacyl-tRNA synthetase, using iodine as cleavage reagent.

MATERIALS AND METHODS

Materials. Synthetic oligonucleotides, consisting of the T7 promoter, the gene for *E. coli* $tRNA^{Ser}$ with UGA as anticodon, and a *Nae* I restriction site, were inserted into the *Eco*RI and *Bam*HI restriction sites of the polylinker of M13mp18 as described (10) to give, after transcription, a tRNA with a 3′-CC terminus. The gene coding for the complete CCA-terminated *E. coli* $tRNA^{Ser}$ with a restriction site for *Bst*NI was obtained by site-directed mutagenesis (11) of the gene described above. *E. coli* seryl-tRNA synthetase (SerRS; EC 6.1.1.1.1) was isolated as described in ref. 12 and stored (1000 units/mg, 15 mg/ml) in 67 mM Tris·HCl, pH 7.6/10 mM $MgCl_2$/1 mM dithiothreitol. *E. coli* aspartyl-tRNA synthetase (AspRS; EC 6.1.1.12) was a generous gift from D. Moras (Strasbourg, France); it was stored (1840 units/mg, 24 mg/ml) in 40 mM 1,3-bis[tris(hydroxymethyl)-methylamino]propane/10 mM Tris·HCl, pH 7.9/1.4 M ammonium sulfate.

T7 RNA polymerase (1190 units/mg, 6 mg/ml) was prepared from an overproducing strain kindly provided by W. Studier (Stonybrook, New York). T4 polynucleotide kinase (30 units/μl) was purchased from United States Biochemical, and ultrapure calf intestinal alkaline phosphatase (3370 units/mg, 1 unit/μl) was from Boehringer Mannheim. Restriction endonucleases *Bst*NI (10,000 units/ml) and *Nae* I (5000 units/ml) were from New England Biolabs. Yeast tRNA nucleotidyltransferase (1500 units/mg, 3 mg/ml) was a gift from H. Sternbach (Göttingen). The S_P diastereomers of NTP[αS]s were prepared as described (13) or obtained from Amersham. Qiagen Pak 100 and Tip 20 were purchased from Diagen (Düsseldorf, F.R.G.) and Centricon 30 microconcentrators were from Amicon. L-[3-^{3}H]Serine (30 Ci/mmol; 1 Ci = 37 GBq), [α-^{32}P]ATP (3000 Ci/mmol), and [γ-^{32}P]ATP (5000 Ci/mmol) were purchased from Amersham.

Agarose gel stop mix contained 10 mM Na_2EDTA at pH 7.4, 50% (vol/vol) glycerol, and 0.1% xylenecyanol FF. Urea stop mix contained 8 M urea, 0.09 M Tris borate at pH 8.3, 2.5 mM Na_2EDTA, and 0.1% xylenecyanol FF. Kodak X-Omat XAR-5 films were employed for autoradiography. The exposed films were scanned with a Pharmacia LKB Ultroscan XL laser densitometer equipped with Gelscan XL software. All solutions were made up in diethylpyrocarbonate-treated sterile distilled water with the exception of those containing enzymes. Generally, solutions were sterilized prior to any reaction by autoclaving or filtering through a 0.22-μm pore Millipore filter.

One A_{260} unit was taken as representing 50 μg of DNA or 42 μg of tRNA (ref. 14, p. E5). The molecular weight of $tRNA^{Ser}$, consisting of 88 nucleotides, was calculated to be approximately 28,000.

Transcription. M13 replicative form I DNA was prepared essentially as described (ref. 14, p. 1.34). DNA was linearized by reaction with *Bst*NI or *Nae* I in a 1.5-ml reaction volume and transcribed in a volume of 500 μl with 60,000 units of T7 RNA polymerase (15). For the preparation of the phosphorothioate-containing transcripts, four transcription reactions in which one NTP was supplemented by 5% of the corresponding NTP[αS] were performed in parallel. They were stopped by the addition of Na_2EDTA to a final concentration of 40 mM. The transcripts were separated from nucleotides, enzymes, and DNA by Qiagen chromatography according to

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Abbreviations: NTP[αS], nucleoside 5′-*O*-(1-thiotriphosphate); Ns-transcript, transcript obtained with an NTP[αS]; AspRS, aspartyl-tRNA synthetase; SerRS, seryl-tRNA synthetase.

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the instructions of the supplier. The transcript was then precipitated with ethanol, and the pellet was washed with 70% (vol/vol) aqueous ethanol and dried. A typical transcription reaction yielded 200 μg of product after Qiagen purification. This tRNA was labeled for sequencing or footprinting or was further purified by electrophoresis on denaturing 12% polyacrylamide gels (40 × 20 × 0.04 cm) at 1600 V and 37 W for 4 hr. The nucleic acid band was visualized by UV light and the appropriate gel piece was excised. The gel pieces were frozen in liquid nitrogen, crushed in an Eppendorf tube, and suspended in 500 μl of buffer (50 mM NaOAc/100 mM KCl/10 mM Na_2EDTA, pH 7). The suspension was swirled on a Vortex mixer, frozen in liquid nitrogen, thawed on ice for 15 min, and centrifuged at 14,000 rpm for 10 min in an Eppendorf 5415 centrifuge. The supernatant was collected and the extraction was repeated once. The supernatants were pooled, the RNA was precipitated with ethanol, and the pellet was washed with 70% ethanol, dried, and dissolved in 5 mM Hepes at pH 7.4. The average recovery of a gel-purified transcript was 30–50% as determined by UV absorption at 260 nm.

^{32}P-Labeling. The tRNA transcript was dephosphorylated at the 5′ end (ref. 14, p. 1.60) and isolated by Qiagen chromatography as described above. The transcripts were then labeled at the 5′ position as described (ref. 14, p. 10.60). After reaction for 45 min, the reaction was terminated by the addition of 5 μl of urea stop mix and the crude product was purified by PAGE. The labeled transcripts were visualized by autoradiography, the appropriate gel piece was cut out, and the transcript was eluted as described above. The 3′ labeling of transcripts with CC termini was performed essentially as described (16), using [α-^{32}P]ATP.

Aminoacylation of Transcripts. Kinetic parameters of the aminoacylation were determined in Hepes buffer as described (15) but without preheating at 60°C.

Sequencing. The ^{32}P-labeled phosphorothioate transcripts were sequenced using a modified procedure of Gish and Eckstein (9). The sequencing reactions were carried out with the four phosphorothioate transcripts. Approximately 50 nM transcript was treated with 500 μM I_2 (dissolved to 5 mM in ethanol) in a total volume of 10 μl. Sequencing under denaturing conditions was achieved by heating the transcript in 20 mM Hepes, pH 7.5, at 70°C for 3 min prior to the addition of iodine. For sequencing under nondenaturing conditions the transcripts were dissolved in footprinting buffer (10 mM NaCl/10 mM Hepes, pH 7.4/10 mM $MgCl_2$) and incubated at 37°C for 3 min prior to the addition of the cleavage reagent. The mixtures were incubated at room temperature for 1 min and the cleavage reactions were stopped by the addition of NaOAc solution (pH 6) to a final concentration of 0.25 M. The fragments were precipitated with ethanol and then washed with 70% ethanol. The dried fragments were dissolved in 3 μl of water, 3 μl of urea stop mix was added, and the solution was loaded directly, without prior heating, onto a denaturing 8% or 12% polyacrylamide gel. After electrophoresis for 90 min the bands were visualized by standard autoradiography techniques.

Footprinting. Prior to footprinting assays the SerRS and AspRS were diluted with the footprinting buffer to the desired concentration. These solutions were stored at 4°C and used for up to 3 days. The reaction solution (total volume of 10 μl of footprinting buffer) contained 3000 cpm of ^{32}P-labeled phosphorothioate transcripts, either 1 μM unlabeled unsubstituted phosphate transcript (referred to as oxy-transcript) or 1 μM unlabeled phosphorothioate transcript (referred to as Ns-transcript), and 4 μM synthetase. The mixture was incubated for 3 min at room temperature and then iodine was added as a 10-fold concentrated solution in ethanol to a final concentration between 100 μM and 1 mM. The mixture was incubated at room temperature for 1 min and the cleavage reaction was terminated by the addition of NaOAc solution (pH 6) to a final concentration of 0.25 M. After extraction with phenol, the sample was precipitated with ethanol and the pellet was subjected to PAGE as described above. Band intensities of the gels obtained after the cleavage reactions in the presence and absence of enzyme were scanned by laser densitometry and compared by superimposing their densitometric traces.

RESULTS

The S_P diastereomers of NTP[αS]s are substrates for T7 RNA polymerase and the phosphorothioate group is incorporated into the transcripts with the R_P configuration (17). *In vitro* transcription of the tRNA^{Ser} gene with the four NTPs in the presence of 5% of one of the NTP[αS]s yields 200–250 μg of transcript and is comparable to the yield of the oxy-transcript reported previously (15). Qiagen column chromatography of the reaction mixture results in separation of the transcript from NTPs and DNA as monitored by PAGE. However, this material was a poor substrate in the charging reaction and had to be further purified by PAGE. In most cases the transcript consisted of a single band of the expected size, which was only occasionally accompanied by a transcript one nucleotide shorter. Extraction of the transcript from the gel resulted in a 30–50% recovery. The gel-purified full-length transcript was fully chargeable (Table 1). The K_m and V_{max} values for the oxy- and the 5% phosphorothioate transcripts are comparable. The K_m values reported here are in reasonable agreement with the value determined for the native tRNA of 2 μM (18). In contrast to charging of the tRNA^{Phe} transcript, the charging of the tRNA^{Ser} transcripts was not altered by a heating–cooling cycle (15). Transcripts in general showed little degradation except for positions 26 and 50 (Fig. 1, lane C). Both these positions represent YpA sequences, which others (19) have also reported to be particularly susceptible to self-cleavage.

Sequencing of transcripts was carried out by a modification of the phosphorothioate alkylation reaction (9), using iodine as the cleavage reagent (D. Speckard and F.E., unpublished results). Nucleotide positions 4–65 were identified after electrophoresis and autoradiography of the 3′-labeled transcript, whereas positions 11–72 were obtained from the 5′-labeled transcript. For footprinting, sequencing was carried out with the complex of tRNA and the cognate synthetase with 1 mM iodine. The relative intensities of only a few bands were changed in comparison to the pattern obtained in the absence of the enzyme (Fig. 1). Densitometer traces of these gels are presented in Fig. 2. Protection at A52 and C68 is >50% and considered strong. That at A46, U69, U70, G53, and G67 is 20–50% and considered weak. The positions at which cleavage was independent of phosphorothioate and iodine are marked in the densitometer traces. These are mainly positions 50 and 26 and, less pronounced, at position 61. Thus, a reliable footprinting analysis requires a control without cleavage reagent. Positions 1–4 and 73–76 could not

Table 1. Kinetic parameters of aminoacylation of tRNA^{Ser} transcripts by *E. coli* SerRS

Transcript	K_m, μM	V_{max}, pmol/min per μg	V_{max}/K_m (relative)
Oxy-	1.2	6800	1.0
5% As-	0.7	4600	1.2
5% Cs-	0.7	4500	1.1
5% Gs-	0.6	3000	0.9
5% Us-	0.7	4500	1.1

Values were obtained from Lineweaver–Burk plots at five different tRNA concentrations between 0.3 and 3.0 μM; synthetase concentration was 0.52 nM.

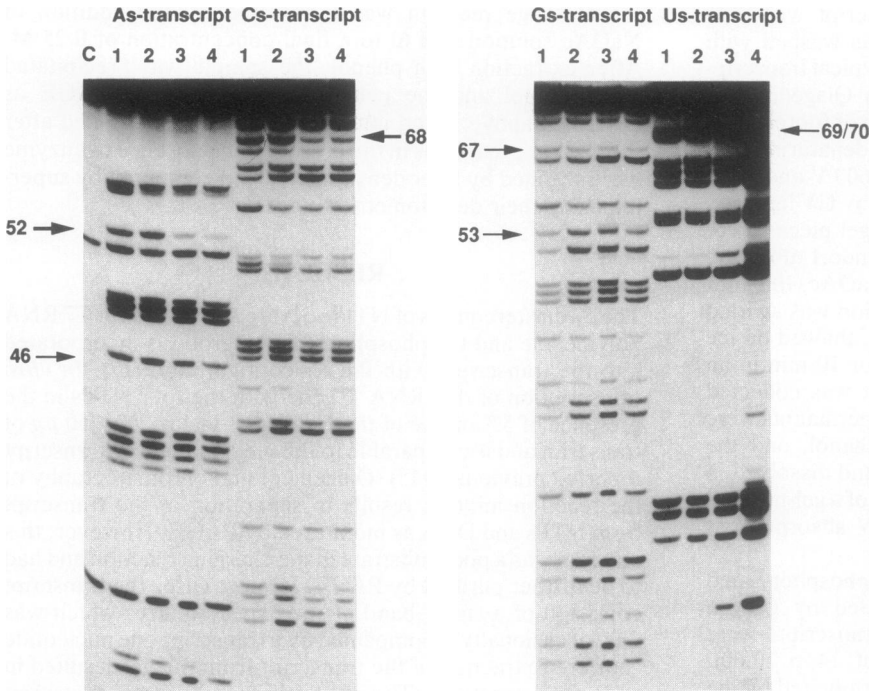

FIG. 1. Sequencing and footprinting of 5′-^{32}P-labeled phosphorothioate tRNA^{Ser} transcripts. Lane C, As-transcript in presence of SerRS, no iodine; lanes 1, sequencing under denaturing conditions without Mg^{2+}; lanes 2, sequencing under nondenaturing conditions with 10 mM Mg^{2+}; lanes 3 and 4, footprinting of synthetase–transcript complexes with 10 mM Mg^{2+}; all sequencing reactions with 1 mM iodine. Bold arrows, strong protection; others, weak protection.

be analyzed as they appeared at the extreme ends of the gels. The sequencing and footprinting results are derived from several repetitions of the experiments. The protection pattern is schematically summarized in Fig. 3.

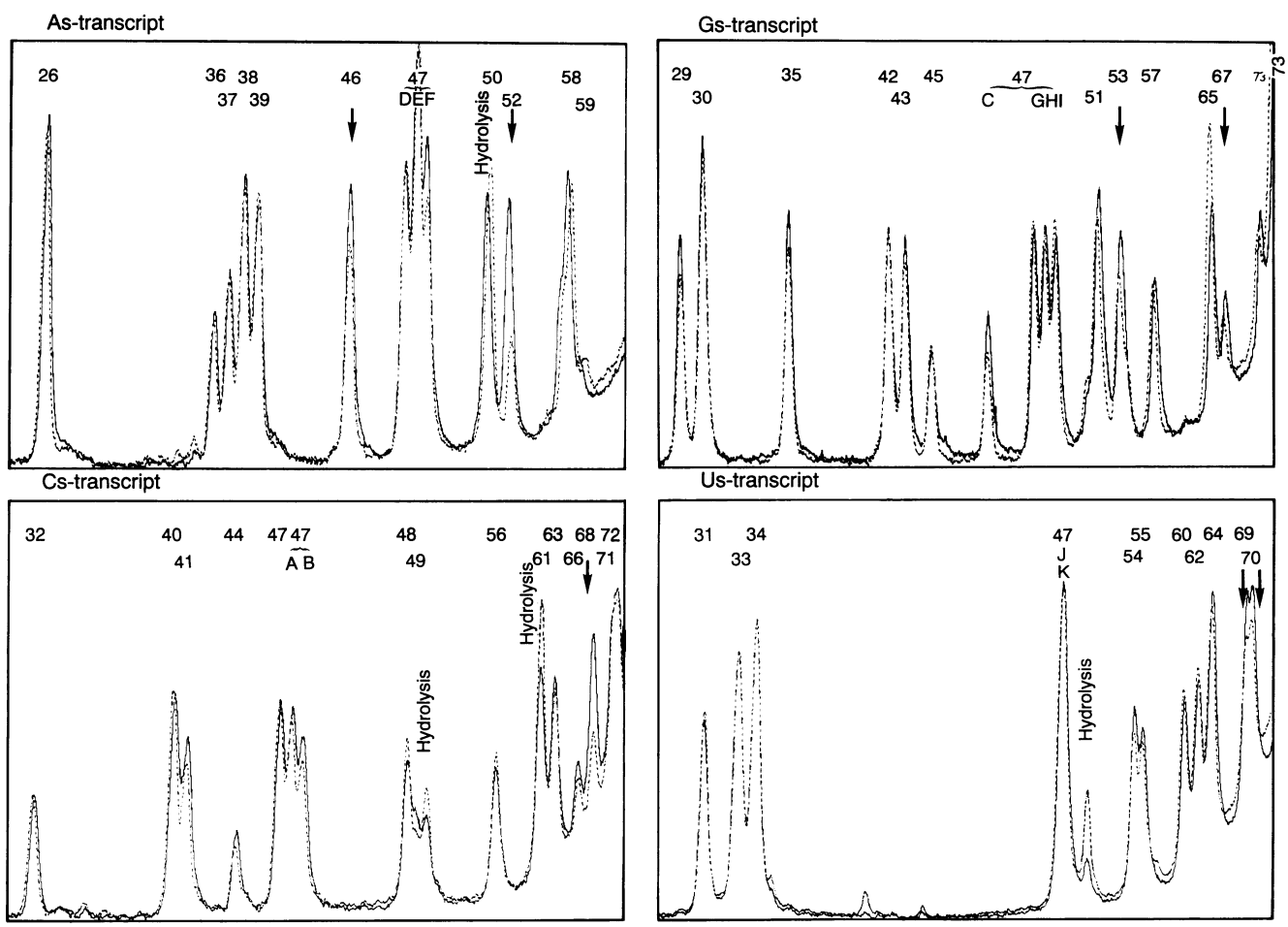

FIG. 2. Densitometry of autoradiograms of reactions of the Ns-transcripts with and without SerRS. The autoradiograms are from the gel electrophoretic analysis of the 5′-^{32}P-labeled transcripts; solid lines, iodine reaction without synthetase; broken lines, with synthetase; arrows, positions of protection; Hydrolysis, spontaneous hydrolysis.

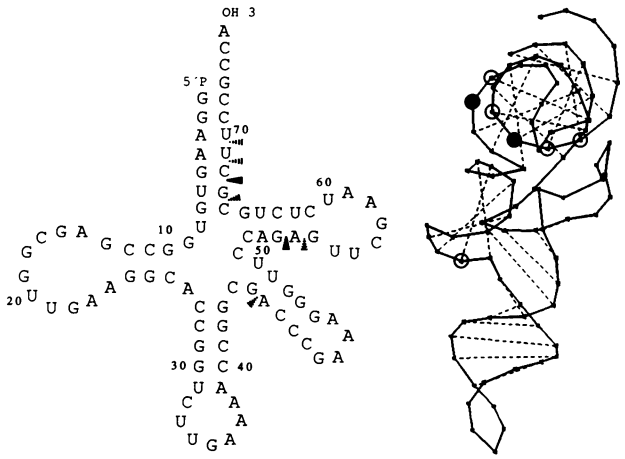

FIG. 3. Summary of protection of *E. coli* $tRNA^{Ser}$-phosphorothioate transcripts by SerRS. (*Left*) Cloverleaf model. Bold arrows, strong protection; broken arrows, weak protection. (*Right*) Projection of protected sites onto model of the yeast $tRNA^{Ser}$ (20). Filled circles, strong protection; open circles, weak protection.

The iodine concentration had no effect on the protection pattern between 1 and 2 mM. A 1 mM concentration was therefore used routinely. However, at iodine concentrations between 100 and 500 μM, additional protected positions appeared, as shown for the 3'-labeled As-transcript in Fig. 4. These were positions 14, 21, 22, 36, 39, 46, 47D, 47E, 52, 58, and 59 (for the cognate complex, lanes 3 and 4). The intensities of all of these bands, with the exception of those at positions 46 and 52, became comparable to those of the control in the absence of enzyme (lane 2) at 1 mM iodine (lane 5). Interestingly, protection at these positions was also seen in the presence of 100 μM iodine in the noncognate complex with AspRS, but all of these disappeared when the iodine concentration was increased to 500 μM or 1 mM (lanes 6, 7, and 8). The results obtained with the Gs-, Cs-, and Us-transcripts were similar in that protection was seen at 100 μM iodine but not at the higher concentration (data not shown). Very similar results were obtained with the noncognate yeast PheRS, with which no protection of any of the phosphorothioate transcripts was seen at the higher iodine concentrations.

A number of experiments were performed to determine whether the enzyme was active under footprinting conditions. Transcripts were preincubated under footprinting conditions for approximately 30 s, then 1 mM iodine and 25 μM radioactive amino acid were added. The amount of charged transcript was then determined after a reaction time of between 30 s and 15 min. A plateau value of 60–70% of charged transcript was obtained after 30 s of incubation. When the enzyme alone was allowed to react with 1 mM iodine for 30 s followed by addition of transcript and labeled amino acid, no charging of transcript could be detected. To determine whether inactivated enzyme would give rise to a footprinting pattern, the enzyme was incubated with 1 mM iodine for 30 s, diluted 10-fold with footprinting buffer, and dialized against this buffer with an Amicon Centricon 30 microconcentrator. This inactive enzyme showed no footprinting pattern when mixed with the transcript and treated with iodine at either 100 μM or 1 mM, whereas the control enzyme yielded a typical footprint. Thus, even though iodine inactivates the enzyme, this inactivation appears to be a slow process in the presence of tRNA in comparison with the cleavage reaction.

DISCUSSION

X-ray structural analysis provides a very detailed picture of the structure of tRNA–synthetase complexes (2). Enzymatic

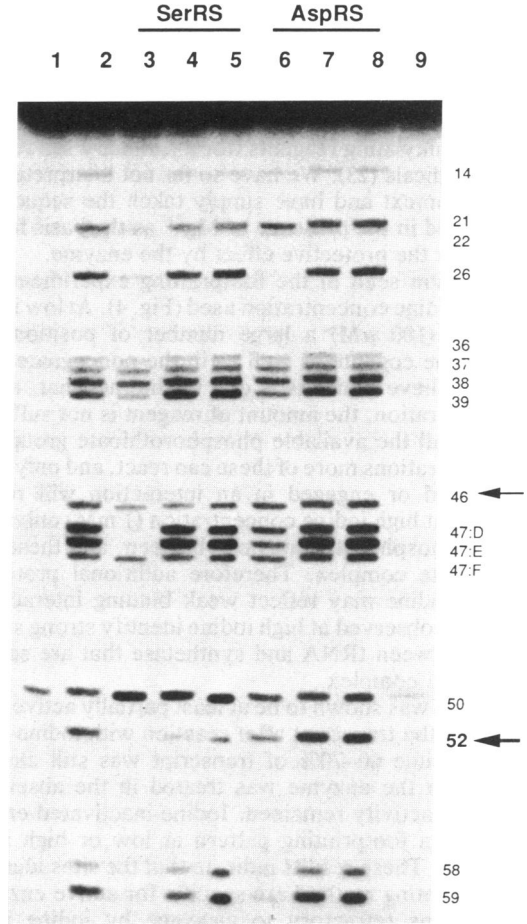

FIG. 4. Footprinting of 3'-^{32}P-labeled As-transcript of $tRNA^{Ser}$ complexed with SerRS and AspRS. Lane 1, transcript and SerRS complex, no iodine; lane 2, sequencing of transcript under nondenaturing conditions with 10 mM Mg^{2+}; lanes 3 and 6, footprinting of complexes with 100 μM iodine; lanes 4 and 7, with 500 μM iodine; lanes 5 and 8, with 1 mM iodine; lane 9, transcript and AspRS complex, no iodine. Lanes 1, 3, 4, and 5 in presence of SerRS, lanes 6, 7, 8, and 9 in presence of AspRS. Arrows as in Fig. 1.

(e.g., see ref. 21) or chemical footprinting (e.g., see refs. 3, 4, 6, and 7) techniques can give information on contacts between the tRNA and a synthetase in solution. The footprinting method employed here is based on the reactivity of internucleotidic phosphorothioate linkages with iodine, resulting in chain breakages that allow the determination of the sequence of phosphorothioate transcripts. Iodine is more reactive than epoxipropanol (9) and produces sufficient cleavage even when the transcripts contain only 5% phosphorothioate linkages. The cleavage depends on the accessibility of the sulfur to the reagent as well as the spatial arrangement of the 2'-OH group such that it can participate in the expulsion of the 5' oxygen. Iodine is ideally suited for footprinting experiments. It is uncharged and reasonably small, it reacts rapidly with phosphorothioates, and its presence does not interfere with migration of the fragments in a gel. The reagent can also be applied in a considerable range of concentration, which permits a titration of weak and strong complexes. A comparison of the K_m and V_{max} values for aminoacylation of the oxy- and the phosphorothioate-tRNA transcripts shows that this low percentage of phosphorothioate groups does not affect this charging reaction.

Sequencing of the tRNA-phosphorothioate transcripts under denaturing conditions and in the native state in the presence of Mg^{2+} differs in that certain bands are weaker in the latter case. This is to be expected, as the presence of Mg^{2+}

leads to the formation of a three-dimensional structure in which the sulfur of certain phosphorothioates is less accessible to the iodine than it is in the denatured state. Such differences in reactivity have also been observed with other reagents and have been the basis for probing the structure of various tRNAs in solution by alkylating reagents (for a summary see ref. 22) or hydroxyl radicals (23). We have so far not interpreted our data in that context and have simply taken the sequencing pattern obtained in the presence of Mg^{2+} as the basis for the comparison for the protective effect by the enzyme.

The protection seen in the footprinting experiments depends on the iodine concentration used (Fig. 4). At low iodine concentration (100 μM) a large number of positions are protected in the cognate as well as in the noncognate complexes. We believe that this is due to the fact that, at low iodine concentration, the amount of reagent is not sufficient to react with all the available phosphorothioate groups. At higher concentrations more of these can react, and only those that are buried or engaged in an interaction will remain nonreactive. At high iodine concentration (1 mM) only a few nonreactive phosphorothioates can be seen, and these only for the cognate complex. Therefore additional protection seen at low iodine may reflect weak binding interactions, whereas those observed at high iodine identify strong sites of interaction between tRNA and synthetase that are specific for the cognate complex.

The enzyme was shown to be at least partially active in the complex with the transcript after reaction with iodine for 30 s. After this time 60–70% of transcript was still charged, although when the enzyme was treated in the absence of transcript, no activity remained. Iodine-inactivated enzyme did not yield a footprinting pattern at low or high iodine concentration. These results indicate that the sites identified by the footprinting method are specific for active enzyme.

The positions refractory to cleavage by iodine in the cognate complex are located on one side of the acceptor stem (C67–U70), on one side of the TΨC stem (A52, G53), and in the stem of the extra loop (A46) (see Fig. 3). Of these, positions A52 and C68 are clearly less accessible to the cleavage reagent than the others.

The phosphate backbone contacts established here do not coincide with the minimal set of identity elements established for the charging specificity of $tRNA^{Ser}$, which are G73 (which is outside the range that could be probed by the footprinting method employed here), the three base pairs at the end of the acceptor stem, and the base pair C11-G24 in the D-stem (1, 24). The most likely explanation is that the search for identity elements is based on the invariability of nucleotides and thus might reflect base interactions with the protein. Thus the two results do not necessarily contradict each other but may be complementary.

To date no three-dimensional structure for *E. coli* $tRNA^{Ser}$ has been published. However, a model for the yeast $tRNA^{Ser}$ has been constructed, mainly on the basis of chemical modification studies (22). Although the two tRNAs differ slightly in that the *E. coli* tRNA has two additional nucleotides in the extra loop and one in the D-loop, we have projected the positions found protected in the *E. coli* tRNA–synthetase complex described here onto the model structure suggested for the yeast tRNA in solution. It was found that the positions shielded in the acceptor as well as in the TΨC stem are very close to each other and are all on the same side of the tRNA (Fig. 3). Position A46 is separated from this area but also on the same side. Thus, a picture emerges in which the protein interacts with only one side of the tRNA and only in the upper region. This is comparable to the complex of yeast $tRNA^{Asp}$ with its cognate synthetase, although in this system the anticodon is involved in recognition (D. Moras, personal communication).

Recently the three-dimensional structure of the *E. coli* SerRS has been elucidated (20). A distinct feature of this enzyme is two very long solvent-exposed antiparallel α-helices. It has been hypothesized that this arm might support the anticodon stem, although the anticodon is unimportant for specific recognition (24). Preliminary docking experiments have been carried out with the crystal structure of the *E. coli* synthetase and the suggested structure of the *E. coli* $tRNA^{Ser}$. The latter was obtained by model building using the structure suggested for the yeast $tRNA^{Ser}$ (22) as a basis. Taking the protected positions A52 and C68 determined in this report as points of interaction between tRNA and enzyme, docking studies lead to the interaction of the variable loop with the extended helical arm of the enzyme but require a distortion of the tRNA to place the CCA end in the putative active site. This, however, is not without precedent, as the 3′ terminus of *E. coli* $tRNA^{Gln}$ in the complex with its synthetase also undergoes some structural change by forming a hairpin turn toward the inside of the "L" with the disruption of the final base pair in the acceptor stem (2). tRNAs charged by *E. coli* SerRS possess a characteristic large variable loop (25). That it interacts with this remarkable structural domain of the cognate enzyme is an attractive hypothesis.

The authors thank G. Gish, D. Speckard, P. Padmanabhan, O. Uhlenbeck, C. Florentz, and R. Giegé for helpful discussions; A. C. Dock-Bregeon and D. Moras for advice and the preparation of the tRNA representations; and S. Cusack and V. Biou for the docking studies.

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