Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 5882–5886, July 1992
Biochemistry

Determinant nucleotides of yeast tRNA^{Asp} interact directly with aspartyl-tRNA synthetase

(iodine footprints/phosphorothioate tRNA/tRNA identity/RNA–protein interaction)

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Communicated by Marianne Grunberg-Manago, March 16, 1992

ABSTRACT **The interaction of wild-type and mutant yeast tRNA^{Asp} transcripts with yeast aspartyl-tRNA synthetase (AspRS; EC 6.1.1.12) has been probed by using iodine cleavage of phosphorothioate-substituted transcripts. AspRS protects phosphates in the anticodon (G34, U35), D-stem (U25), and acceptor end (G73) that correspond to determinant nucleotides for aspartylation. This protection, as well as that in anticodon stem (C29, U40, G41) and D-stem (U11 to U13), is consistent with direct interaction of AspRS at these phosphates. Other protection, in the variable loop (G45), D-loop (G18, G19), and T-stem and loop (G53, U54, U55), as well as enhanced reactivity at G37, may result from conformational changes of the transcript upon binding to AspRS. Transcripts mutated at determinant positions showed a loss of phosphate protection in the region of the mutation while maintaining the global protection pattern. The ensemble of results suggests that aspartylation specificity arises from both protein–base and protein–phosphate contacts and that different regions of tRNA^{Asp} interact independently with AspRS. A mutant transcript of yeast tRNA^{Phe} that contains the set of identity nucleotides for specific aspartylation gave a phosphate protection pattern strikingly similar to that of wild-type tRNA^{Asp}. This confirms that a small number of nucleotides within a different tRNA sequence context can direct specific interaction with synthetase.**

Accurate aminoacylation of tRNAs requires specific interaction between tRNA and its cognate aminoacyl-tRNA synthetase (aaRS). The complex of yeast tRNA^{Asp} with aspartyl-tRNA synthetase (AspRS; EC 6.1.1.12) has been studied by footprinting with a number of probes in solution (1, 2), and a high-resolution x-ray structure (3) is presently being refined. As a result, regions of contact between protein and tRNA were defined; the tRNA interacts with AspRS along the variable-loop side of the L-shaped tertiary structure, including the anticodon loop and stem, the base of the D-stem, and the 3′ extremity of the acceptor stem. Nucleotides functionally important for specific aspartylation (Fig. 1) were recently identified by kinetic analysis of *in vitro* transcripts (5); they correspond to nucleotides G34, U35, and C36 in the anticodon, G73 at the acceptor end, and base pair G10·U25 at the base of the D-stem. Mutation of these nucleotides resulted in a dramatic loss of aspartylation activity and suggested that the wild-type identity nucleotides are in direct contact with the protein in the enzyme–substrate complex and that these stabilizing interactions are disrupted upon mutation. Unfortunately, the present state of refinement of the crystal structure is not sufficient to identify the exact contacts between tRNA and protein.

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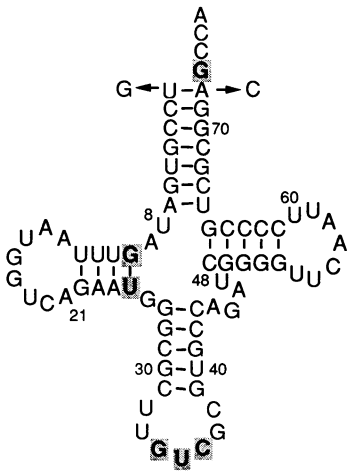

FIG. 1. Cloverleaf structure of wild-type yeast tRNA^{Asp} transcript containing G1·C72 as first base pair; nucleotides are numbered according to ref. 4. Nucleotides corresponding to determinants specific for aspartylation (5) are shaded.

Despite a large number of functional studies on interaction of mutant or noncognate tRNAs with synthetases (6–8), little is known about the structural basis of these interactions. In this paper, we compare the interaction of wild-type and mutant tRNA^{Asp} transcripts with AspRS by using footprinting experiments based on cleavage of phosphorothioate-containing transcripts by I_2; this technique has recently been applied to the interaction of *Escherichia coli* tRNA^{Ser} and SerRS (9). The relatively small size and high reactivity of I_2 compared with other phosphate probes such as *N*-ethyl-*N*-nitrosourea (ENU) (10) or aminobenzenediazonium compounds (2) should afford preferential protection at phosphates in direct contact with synthetase. Footprint patterns of wild-type tRNA^{Asp} transcript complexed with AspRS were compared to those of transcripts individually mutated at determinant nucleotides. The results show phosphate protection for the wild-type transcript in the vicinity of determinant nucleotides and local disruption of RNA–protein interactions in the region of mutated determinant nucleotides, due to either a loss of direct contact or a local change of conformation.

MATERIALS AND METHODS

Materials. Yeast AspRS (11) and T7 RNA polymerase (12) were purified according to established procedures. Yeast

Abbreviations: aaRS, aminoacyl-tRNA synthetase; AspRS, etc., aspartyl-tRNA synthetase, etc.; ENU, *N*-ethyl-*N*-nitrosourea; NTP[αS], nucleoside 5′-*O*-(1-thiotriphosphate); phosphorothioate tRNA transcript, transcript obtained in the presence of an NTP[αS].
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PheRS was a gift of P. Remy (Strasbourg, France), and *E. coli* SerRS was a gift of R. Leberman (Grenoble, France). T4 polynucleotide kinase and restriction enzyme *Bst*NI were from New England Biolabs, bovine alkaline phosphatase was from Appligene (Strasbourg, France), and I_2 was from Merck. Nucleoside 5′-*O*-(1-thiotriphosphates (NTP[αS])s, L-[^{3}H]aspartic acid, and [γ-^{32}P]ATP (3200 Ci/mmol; 1 Ci = 37 GBq) were purchased from Amersham.

Preparation of tRNA Transcripts. Plasmids containing genes of wild-type and mutant $tRNA^{Asp}$ were prepared as described (5, 13). tRNA transcripts were obtained by incubation of *Bst*NI-linearized plasmid with T7 RNA polymerase at 3000 units/ml in 40 mM Tris·HCl, pH 8.1/22 mM $MgCl_2$/1 mM spermidine/5 mM dithioerythritol/0.01% Triton X-100/40 units of RNAsin (Promega)/4 mM each NTP/0.2 mM one of the four NTP[αS]s (5% of the corresponding NTP) for 4 hr at 37°C. Thus, for each plasmid, four transcriptions with the S_P diastereomer of ATP[αS], CTP[αS], GTP[αS], or UTP[αS] were performed in parallel, yielding full-length transcripts containing statistically one phosphorothioate per molecule (9); transcripts contain phosphorothioates in the R_P configuration (14, 15). After extraction with phenol and precipitation with ethanol, transcripts were purified by HPLC on a TSK-250 column (Bio-Rad) in 0.2 M sodium acetate, pH 6.5/1% methanol.

The different transcripts were 5′-labeled with polynucleotide kinase and purified by electrophoresis on 12% polyacrylamide/8 M urea gels. Labeled transcripts were eluted from the gel, precipitated, and redissolved in a 10 mM $MgCl_2$/10 mM NaCl/10 mM Hepes–KOH, pH 7.4, buffer.

Aminoacylation Assays. Aspartylation of the four wild-type phosphorothioate transcripts and determination of kinetic parameters were performed as described (5).

Footprinting. For footprinting experiments, aaRSs were dialyzed against 10 mM $MgCl_2$/10 mM NaCl/10 mM Hepes–KOH, pH 7.4, buffer in a Centricon 30 (Amicon). tRNA transcripts with different phosphorothioates were tested individually. All footprinting experiments were performed in 10 μl total volume in 10 mM $MgCl_2$/10 mM NaCl/10 mM Hepes–KOH, pH 7.4, at 20°C. Unlabeled and labeled transcripts were mixed to give a total concentration of 1 μM and 30,000 cpm (Cerenkov). Yeast AspRS was added to give a total concentration of 4 μM or replaced by 10 mM $MgCl_2$/10 mM NaCl/10 mM Hepes–KOH, pH 7.4, buffer for sequencing. The concentration of 4 μM AspRS assures nearly complete binding (>90%) for all transcript sequences tested (J.P., unpublished results). Transcripts and synthetase were incubated for 2 min to allow complex formation and were subsequently cleaved by addition of 1 μl of 10 mM I_2 in 100% ethanol. After 2 min, reactions were stopped by dilution with 40 μl of 0.4 M sodium acetate, pH 6.0, and extraction with phenol. The precipitated transcripts were resuspended in 10 μl of 8 M urea/20% sucrose and dyes, and separated by denaturing PAGE on 12% polyacrylamide gels. Band intensities in the presence and absence of synthetase were compared and quantitated by laser densitometry of the autoradiograms. Protection is considered as weak, moderate, or strong if intensity of bands is decreased 25–50%, 50–75%, or 75–100%, respectively. To have better accuracy by densitometry, autoradiograms were not overexposed.

Effect of I_2 on AspRS Activity. To check the effect of iodine on AspRS, control experiments were performed with enzyme pretreated with I_2 as described (9).

RESULTS

I_2 Footprint of Wild-Type Transcript Complexed to AspRS. Footprint experiments were performed on phosphorothioate-substituted wild-type $tRNA^{Asp}$ transcript complexed to yeast AspRS. Strong differences between the intensities of bands obtained in the absence and presence of AspRS (Fig. 2*A*) reveal local protection of phosphates in the wild-type transcript by AspRS. There is no protection in the presence of yeast PheRS (Fig. 2*A*) or *E. coli* SerRS (data not shown), indicating that the protection found with AspRS is specific. Phosphates discussed

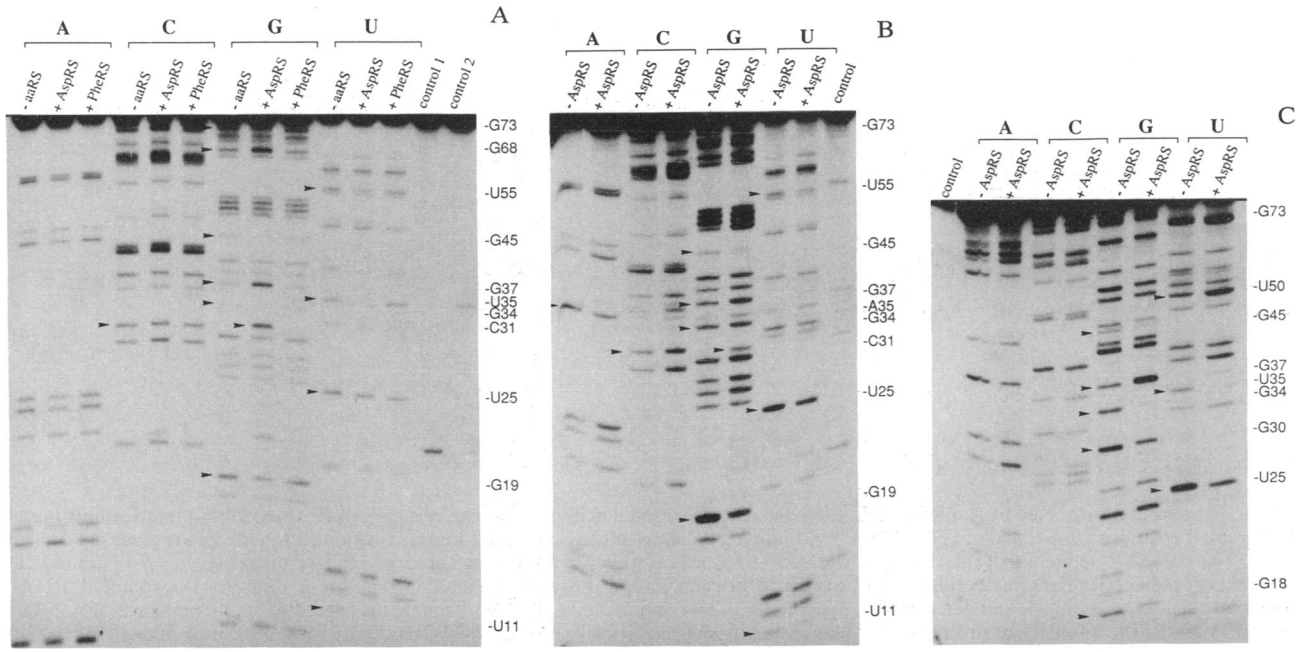

FIG. 2. Autoradiograms of I_2 footprint experiments obtained with 5′-labeled transcript on 12% polyacrylamide denaturing gels. Footprinting was performed on transcripts substituted with ATP[αS] (A lanes), CTP[αS] (C lanes), GTP[αS] (G lanes), or UTP[αS] (U lanes). Some of the positions discussed in the text are indicated by arrowheads on the autoradiograms. (*A*) Wild-type yeast $tRNA^{Asp}$ in the absence of synthetase (−aaRS) or presence of either yeast AspRS (+AspRS) or yeast PheRS (+PheRS). Control lanes 1 and 2, transcript and AspRS or PheRS, respectively, without I_2 treatment. (*B*) $tRNA^{Asp}$ transcript mutated at position 35 (U35 → A35): Footprints in the absence (−AspRS) or presence (+AspRS) of yeast AspRS. Control lane, transcript and AspRS without I_2 treatment. (*C*) $tRNA^{Phe}$ transcript with the determinants specific for aspartylation in the absence (−AspRS) or presence (+AspRS) of yeast AspRS. Control lane as in *B*.

in the text are those located 5′ to the indicated nucleoside. The intensity of protection varies; the strongest protection is found in the anticodon loop (G34, U35). Moderate protection is observed at the end of the amino acid acceptor arm (G73) and in the variable loop (G45). Weak protection is found in the D-loop (G18, G19) and arm (U11, U12, U13, U25), in the anticodon stem (C29, U40, G41), T-stem, and loop (G53, U54, U55), and in the acceptor arm (G71). Interestingly, phosphate at G37 was considerably more accessible in the presence of AspRS. Phosphates A14, G27, G50, U66, G68, and C69 are slightly more reactive in the presence of AspRS. Phosphates protected are shown schematically in Fig. 3*A*. The unexplained presence of a band in the G lane at the position of C31 in addition to the corresponding band at position G30 requires the presence of AspRS as well as a phosphorothioate-substituted guanosine within the transcript.

Controls. Various control experiments assessed the validity of the I_2 footprint technique. (*i*) No significant differences were observed in kinetic parameters (K_m and k_{cat}) for aspartylation between phosphorothioate-substituted and nonsubstituted transcripts (data not shown). Since these transcripts are only minimally substituted (ca. 5%) at each position, this control only shows that phosphorothioate nucleotides do not grossly affect the properties of the transcript and not whether the presence of a phosphorothioate at a specific position disturbs an RNA–protein interaction (16). (*ii*) Since I_2 can react with certain amino acids (17), which may be important for the structure or activity of the protein, the protection pattern observed may be due to an inactive synthetase. AspRS was indeed inactive when treated with I_2 and no protection was observed in the presence of inactive AspRS. Partial activity, consistent with the binding stoichiometry (18), is retained when the enzyme is complexed with wild-type transcript before I_2 treatment. Thus, interaction with the transcript blocks AspRS inactivation by I_2. (*iii*) The lack of protection in the presence of two noncognate synthetases demonstrates that the observed footprint is specific to AspRS. However, nonspecific complexes or aggregates may form at the high concentrations of AspRS and transcript used in these experiments (18, 19). To eliminate this possibility, footprinting experiments were performed on wild-type transcript over a range of AspRS concentrations (between 40 nM and 4 μM). All characteristics of the footprint pattern, including intensifications and the band at position 31, are observed at 0.4 μM AspRS. Relative band intensities follow a binding curve similar to that found by filter binding experiments (J.P., unpublished results). Footprint experiments at high AspRS concentration (4 μM) but at increased NaCl concentrations (between 10 mM and 100 mM) gave patterns similar to those in low salt, although the weak protections in the D-loop (G18, G19) are reduced at high NaCl concentrations, which preferentially favor specific complex formation (20).

In summary, these controls demonstrate that the footprint pattern of $tRNA^{Asp}$ transcript complexed to AspRS reflects the functional interaction between two active molecules, as shown previously for $tRNA^{Ser}$ and SerRS (9).

I_2 Footprints of Mutant $tRNA^{Asp}$ Transcripts Complexed to AspRS. Footprint experiments were performed on transcripts bearing single mutations at determinant positions (5). The

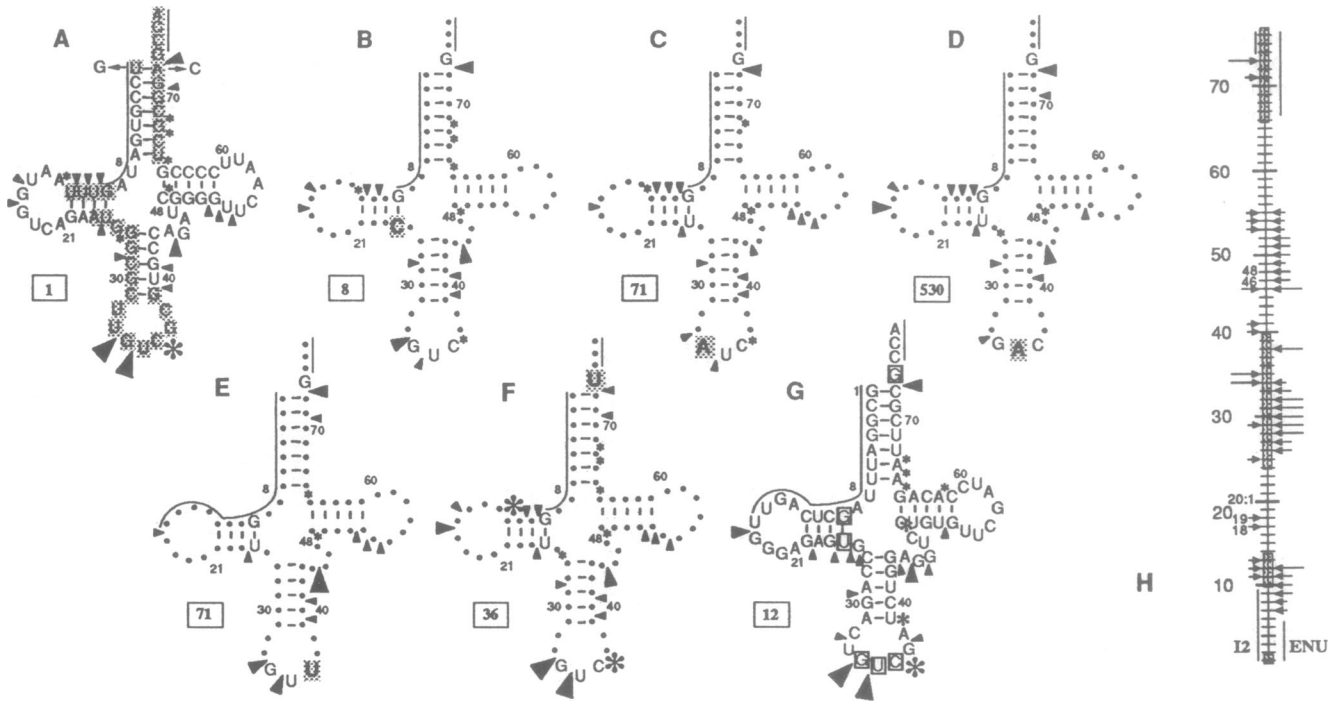

FIG. 3. Phosphates protected by AspRS displayed on the cloverleaf sequences of wild-type yeast $tRNA^{Asp}$ transcript (*A*), of variants mutated at determinant positions (*B–F*), and of yeast $tRNA^{Phe}$ containing the determinants specific for aspartylation (*G*). Untested regions are indicated by lines. The complete sequence of the wild-type $tRNA^{Asp}$ transcript is given. Nucleotides conserved in mutant sequences *B–F* are indicated by dots except the determinant nucleotides shown explicitly for each variant. Positions mutated in *B–F* are shaded. For transplanted $tRNA^{Phe}$, the complete sequence is shown and determinants specific for aspartylation are in squares. Phosphates protected in the presence of AspRS are indicated by triangles. Intensity of protection as determined by densitometry [weak (25–50%), moderate (50–75%), and strong (75–100%)] is proportional to triangle size. Intensity of enhancement as determined by densitometry [weak (20–50%), moderate (50–70%), and strong (>70%)] is proportional to asterisk size (strongest enhancement was 86% in wild-type $tRNA^{Asp}$ transcript). In wild-type $tRNA^{Asp}$ (*A*), shaded nucleotides represent nearest proximities with AspRS as visualized by crystallography (3). The loss of aspartylation specificity for tRNA variants expressed as fold decrease in k_{cat}/K_m (5) relative to wild type is given in the box at the lower left of each cloverleaf sequence. (*H*) Quantitative comparison between footprinting data of $tRNA^{Asp}$ by I_2 (left of line) and ENU (right of line) and the regions of tRNA in nearest proximity with AspRS (shaded). The ENU data are taken from figure 6 in ref. 1 and correspond to the modified tRNA. The extent of protection is proportional to the length of the arrows [weak (25–50%), moderate (50–75%), and strong (75–100%)]. No enhancements were found in ENU footprints (1). (Preliminary ENU footprints on $tRNA^{Asp}$ transcripts gave similar results.)

following mutants were studied: those in which U25 was changed to C25, G34 to A34, U35 to A35, C36 to U36, and G73 to U73. A gel corresponding to footprinting experiments on mutant A35 is shown in Fig. 2*B*. These results and those corresponding to the other mutants studied are summarized in Fig. 3 *B–F*.

The effect of mutations of determinant nucleotides on the footprint pattern is localized in the region of the mutated nucleotide. However, all tRNA mutants did not have the same behavior. Two different cases were encountered: (*i*) For anticodon mutants (G34 mutated to A34, U35 to A35, and C36 to U36), the effect of the single mutation is located not only at the level of the phosphate belonging to the mutated nucleotide but also on the whole anticodon loop. A single mutation in the anticodon induced a loss of protection on several phosphates. In addition, no strong intensifications were observed at position 37; the band at position 31 still appeared. For mutant C25, some protections are lost in the D-stem and all in the T-stem, and no strong intensification was observed at G37. (*ii*) For mutant U73, mutation induced a perturbation only at the level of the phosphate belonging to the mutated position; all other protections and enhancements are still observed, with a slightly increased reactivity of phosphate 14.

I_2 Footprints of a Yeast $tRNA^{Phe}$ Transcript Containing Aspartic Acid Determinants Complexed to AspRS. A yeast $tRNA^{Phe}$ transcript containing aspartic acid determinants (U25, U35, C36, and G73; G10 and G34 are already in the yeast $tRNA^{Phe}$ sequence) is an excellent substrate for AspRS, whereas the wild-type $tRNA^{Phe}$ transcript is not aspartylated at a detectable level (5). Footprint experiments were performed on both transcripts to delineate the phosphates in contact with AspRS and to compare these results with those obtained with the wild-type $tRNA^{Asp}$ transcript.

The wild-type $tRNA^{Phe}$ transcript was only weakly protected by AspRS. Minor protection was observed at phosphates 34 and 45. The transcript may interact weakly with AspRS to form a small fraction of complex at high AspRS concentrations. No intensification at position 37 was observed (results not shown). The phosphate protection and intensification pattern for the $tRNA^{Phe}$ transcript with the aspartic acid determinants is strikingly similar to that of wild-type $tRNA^{Asp}$ for the strongest protections and enhancements (Figs. 2*C* and 3*G*); the extra band at position 31 was not observed.

DISCUSSION

The I_2–phosphorothioate technique has been previously used to characterize the interaction of $tRNA^{Ser}$ with SerRS (9). As with any footprinting technique (21), interpretation of protection must proceed with caution. Control experiments demonstrated that both tRNA and synthetase are active under footprinting conditions and that all tRNAs are specifically complexed to AspRS. Certain phosphate positions in transcripts of $tRNA^{Asp}$ are found to be protected in the presence of AspRS. These include phosphates belonging to nucleotides (U25, G34, U35, G73) important for $tRNA^{Asp}$ identity. The crystal structure of the complex between $tRNA^{Asp}$ and AspRS shows that the synthetase interacts in the region of determinant nucleotides (3) (Fig. 3*A*). The weakly protected phosphates at positions 11, 12, and 13 in the D-stem as well as positions 29, 40, and 41 in the anticodon stem correlate with synthetase interaction with these regions. Because of the relatively small size of I_2 as a chemical probe, strongly protected phosphates are probably in regions of interaction with synthetase from which solvent is excluded; these may include phosphates interacting directly with amino acids in the protein.

A second category of protected phosphates is observed (G18, G19, G45, G53, U54, U55) that are in regions of the tRNA clearly not in contact with the synthetase as observed in the crystal structure (Fig. 3*A*). Since $tRNA^{Asp}$ undergoes a change in tertiary structure and anticodon conformation upon binding to the synthetase (3), protection at these positions may arise from this conformational change. Another reason for protection can be the formation of nonspecific aggregates that occurs when the concentration of AspRS is in great excess to that of tRNA (18). If so, unspecific protection would affect positions in the complex that are the most exposed to the solvent as are residues in the D-loop and T-arm. The amount of such aggregates is very sensitive to ionic strength (18), and indeed footprinting control experiments (see above) confirm this view. Thus all protection in an I_2 footprint cannot be assigned *a priori* to a direct and specific interaction with the protein.

Enhancement of I_2 reactivity is observed at several phosphates and is especially strong at position G37; this probably arises from conformational changes that result in a preferential geometry for I_2 reactivity at the phosphorothioate or a favorable orientation of the 2′-OH for in-line attack of the phosphothioester bond (9).

The extra band observed at position 31 in the G-substituted transcript correlates with the presence of AspRS. This cleavage of a normal phosphate (corresponding to C31) requires the presence of a phosphorothioate-substituted G. Footprinting experiments as well as the crystal structure of the complex suggest major-groove contacts in this region. The unknown cleavage mechanism may reflect a protein contact in this region.

The complex between yeast $tRNA^{Asp}$ and AspRS has been previously characterized by footprinting experiments using ENU, which also probes phosphate accessibility (1). The two techniques (I_2/phosphorothioate and ENU) give similar regions of protection while emphasizing different specific phosphates (Fig. 3*H*). Phosphates in the anticodon stem of native $tRNA^{Asp}$ (G28–U32, C38) as well as phosphate corresponding to U12 at the base of the D-stem and G45 in the variable loop are the most strongly protected from ENU modification in the presence of AspRS. In contrast, only weak protection at C29, U40, and G41 in the anticodon stem is observed by the I_2 technique. Regions of protection by the two techniques overlap at the base of the D-stem and at G45. Strong protection is observed in the anticodon by the I_2 technique, but it could not be tested by ENU footprinting for technical reasons (1). Only C38 is protected in the anticodon loop by ENU.

The different phosphate protection patterns reflect the relative strengths and weaknesses of each technique. Phosphates protected from ENU in the presence of AspRS are spread over several nucleotides within a certain region of $tRNA^{Asp}$. ENU is much less reactive than I_2. The time of cleavage by the I_2 technique is at most 1–2 min, whereas ENU modifications are performed over a 2-hr time period, with a very low level of modification. A protein–RNA complex is dynamic, and kinetic opening of the complex may contribute to the cleavage pattern if the lifetime of the open I_2-accessible state is significant compared to the I_2 reaction rate; this would increase the number of apparently reactive phosphates in the complex. In addition, ENU can react with both oxygen atoms of a phosphate group, whereas I_2 reacts with only a single position, the sulfur atom, of each phosphorothioate group. Within double-stranded regions of RNA, the substituted oxygen (pro-R_P) points inward in the narrow major groove and may not be as accessible for protein contacts as the pro-S_P oxygen; this may further explain the weak protection observed in the anticodon stem with the I_2 technique. On the other hand, the presence of a sulfur atom may disturb the structure of the transcript (22) or interfere with protein–RNA interactions (16). In the latter case, no protection in the presence of protein would be observed, even though this phosphate is in direct contact with the protein in the unsubstituted transcript. These points emphasize that the protection observed with the I_2 technique represents a minimum set of phosphates in possible contact with the synthetase.

Specific interaction of AspRS with $tRNA^{Asp}$ likely involves an array of protein contacts with the bases as well as the phosphate backbone. In wild-type transcript, phosphates corresponding to all determinant nucleotides except C36 were protected by AspRS. For determinant mutants, loss of phosphate protection is observed in the region of the mutated nucleotide; this suggests a local alteration in the interaction between tRNA and synthetase. Thus, upon mutation of G73 to U73, protection of phosphate 73 is reduced, but anticodon loop protection is the same as for the wild-type transcript. Enhancement at position 37 is observed for this mutant, suggesting a conformational change similar to that in the wild-type molecule. In contrast, mutation of anticodon nucleotides causes a reduction of protection at several phosphates in the anticodon loop. These results suggest interdependence of protein–base and protein–phosphate contacts, as observed in specific DNA–protein complexes (23, 24).

The conservation of the overall footprint pattern upon mutation can be understood if the different regions of the tRNA in contact with AspRS interact independently with the protein; mutation of a nucleotide in one region (e.g., G73) does not affect the RNA–protein interaction in another (e.g., anticodon). This may be a consequence of the large spatial separation between regions of specific interaction; anticodon nucleotides and the 3′-CCA terminus interact with separate domains of AspRS (3). In contrast, the loss of several phosphate protections within the anticodon upon mutation of a single anticodon nucleotide suggests a greater interdependence among AspRS interactions with anticodon nucleotides. Interestingly, the number of protections lost in different anticodon mutants correlates with the decrease in the kinetic specificity constant (k_{cat}/K_m) for aspartylation (5) (Fig. 3); mutation of U35 to A35 had the greatest effect on the footprinting pattern and aspartylation (530-fold decrease in k_{cat}/K_m). The available binding energy of these interactions is mostly used for transition-state stabilization.

Conformational changes of $tRNA^{Asp}$ upon binding AspRS may contribute to specificity (3). The strongly increased reactivity of position 37 in all sequences tested correlates with the presence of the full complement of protections in the anticodon, since this increased reactivity is reduced or even not observed in anticodon mutants. The conformational change of the anticodon in $tRNA^{Asp}$ appears related to the presence of specific contacts with AspRS in the region of the anticodon. Such contacts would provide the driving force for conformational change, which would contribute to substrate discrimination and aspartylation specificity. Interestingly, the increased reactivities in other parts of the wild-type molecule that we interpret as conformational changes are also significantly perturbed in the $tRNA^{Asp}$ variants (Fig. 3).

Conversion of yeast $tRNA^{Phe}$ into an efficient aspartic acid acceptor is achieved by the mutation of four nucleotides (5). Strikingly, the phosphate protection pattern of this transcript in the presence of AspRS closely resembles that of wild-type $tRNA^{Asp}$ transcript. Thus, the interactions of these two different tRNA sequences appear similar. The same intensifications of phosphate cleavage as in the wild-type $tRNA^{Asp}$ transcript are also observed, which suggests that similar conformational changes are occurring in the $tRNA^{Phe}$ transcript with aspartic acid identity elements as in wild-type $tRNA^{Asp}$. These experiments graphically illustrate that the interactions between tRNA and cognate synthetase required for correct aminoacylation are directed by a small number of nucleotides (7, 8).

The specific recognition of nucleotides spatially dispersed in different regions of tRNA distinguishes tRNA recognition by certain synthetases from sequence-specific DNA recognition. Proper aminoacylation *in vivo* requires discrimination of cognate tRNA from all other tRNAs; the mix of independent recognition of various tRNA domains by synthetase and more interdependent contacts with a certain domain may influence the number of specific interactions required for this discrimination. Recognition of tRNA regions, in particular the 3′-CCA extremity and anticodon, by different protein domains (25) may be an important factor in aaRS evolution (26).

We thank A. Dietrich-Théobald for help in AspRS purification and D. Moras and B. Rees for access on crystallographic data, computer graphics assistance, and discussions. This work was supported by the Centre National de la Recherche Scientifique, Ministère de la Recherche et de l'Enseignement Supérieur (MRES), Université Louis Pasteur (Strasbourg), and the Human Frontier Science Program. J.R. and J.D.P. were supported by fellowships from MRES and the European Molecular Biology Organization, respectively. J.P. was partially supported by a Daimler-Benz grant.

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