Study of the Interaction of *Escherichia coli* Methionyl-tRNA Synthetase with $tRNA^{fMet}$ Using Chemical and Enzymatic Probes†

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Received December 6, 1985; Revised Manuscript Received March 21, 1986

ABSTRACT: The accessibility of nucleotides in *Escherichia coli* $tRNA^{fMet}$ to chemical and enzymatic probes in the presence and absence of methionyl-tRNA synthetase has been investigated. Dimethyl sulfate was used to probe the reactivity of cytosine and guanosine residues. The N-3 position of the wobble anticodon base, C_{34}, was strongly protected from methylation in the tRNA–synthetase complex. A synthetase-induced conformational change in the anticodon loop was suggested by the enhanced reactivity of C_{32} in the presence of enzyme. Cytosine residues in the dihydrouridine loop and in the 3′-terminal CCA sequence showed little or no change in reactivity. Methylation of the N-7 position of guanosine residues G_{42}, G_{52}, and G_{70} was partially inhibited by the synthetase. Nuclease digestion of $tRNA^{fMet}$ with α-sarcin in the presence of 1–2 mM Mg^{2+} resulted in cleavage mainly at C_{71} in the acceptor stem and was strongly inhibited by synthetase. Other nuclease digestion experiments using the single strand specific nucleases RNase A and RNase T_1 revealed weak protection of nucleotides in the D loop and strong protection of nucleotides in the anticodon on complex formation. The present data, together with previous structure–function studies on this system, indicate strong binding of methionyl-tRNA synthetase to the anticodon of $tRNA^{fMet}$, leading to a change in the conformation of the anticodon loop and stem. We propose that this, in turn, produces more distant, and possibly relatively subtle, conformational changes in other parts of the tRNA structure that ultimately lead to proper orientation of the 3′ terminus of the tRNA with respect to the active site of the enzyme.

Previous chemical modification studies from this laboratory indicated a role for the anticodon sequence in recognition of *Escherichia coli* methionine tRNAs by *E. coli* methionyl-tRNA synthetase (MetRS)[1] (Schulman & Goddard, 1973; Schulman & Pelka, 1977a; Stern & Schulman, 1977). The data suggested a positive interaction between the synthetase and the wobble cytidine base and pointed to the N-3 position of the pyrimidine ring as a probable binding site for the enzyme. More recent studies involving the synthesis of an extensive series of methionine tRNA derivatives having single base changes in this region have confirmed the importance of the anticodon sequence for recognition of tRNAs by methionyl-tRNA synthetase and have dramatically demonstrated the absolute requirement for cytidine in the wobble position (Schulman et al., 1983; Schulman & Pelka, 1983, 1984). The present experiments have been undertaken to provide evidence for direct physical interaction of MetRS with the anticodon and possibly other regions of $tRNA^{fMet}$. We have investigated the topology of the $tRNA^{fMet}$–MetRS synthetase complex using dimethyl sulfate, a small chemical probe known to react readily with the N-3 position of single-stranded cytidine residues, and RNase A, a low molecular weight ribonuclease known to cleave $tRNA^{fMet}$ primarily in the anticodon sequence. In addition, we have probed the complex using α-sarcin, a nuclease that cleaves both single- and double-stranded purine residues in RNAs (Endo et al., 1983) and that has been used to study the binding of ribosomal proteins to 5S rRNA (Huber & Wool, 1984).

Experimental Procedures

Materials

RNase A was obtained from Worthington and RNase T_1 (Sankyo) from Calbiochem. PhyM and *Bacillus cereus* RNases and T4 polynucleotide kinase were purchased from P-L Biochemicals. *E. coli* $tRNA^{fMet}$ and calf intestinal alkaline phosphatase were obtained from Boehringer Mannheim. *E. coli* methionyl-tRNA synthetase was purified from *E. coli* K12 strain EM 20031 as described previously (Schulman & Pelka, 1977b). α-Sarcin was a gift of Ira Wool (University of Chicago). The nuclease was dissolved in water at a concentration of 1.79 mg/mL and stored at 4 °C. [γ-^{32}P]ATP and [α-^{32}P]ATP were purchased from Amersham. Dimethyl sulfate was obtained from Aldrich, anhydrous hydrazine was from Eastman Kodak, and sodium borohydride and aniline were purchased from Fisher. The aniline was twice redistilled from zinc and stored at −20 °C under nitrogen. Phenol was once distilled and stored at 4 °C. Other chemicals were treated as described by Peattie (1983).

Methods

5′ and 3′ End Labeling of $tRNA^{fMet}$. $tRNA^{fMet}$ was dephosphorylated by incubation with calf intestinal phosphatase at 65 °C and labeled with ^{32}P at the 5′ terminus by incubation with T_4 polynucleotide kinase and [γ-^{32}P]ATP at 37 °C, as described elsewhere (Schulman et al., 1983).

The 3′-terminal adenosine residue of $tRNA^{fMet}$ was labeled with ^{32}P by using an exchange reaction catalyzed by *E. coli* tRNA nucleotidyl transferase in the presence of [α-^{32}P]ATP and sodium pyrophosphate, as described by Francis et al. (1983).

Methylation Protection Experiments. Methylation experiments were carried out on a sample of $tRNA^{fMet}$ which was highly enriched for the isomer containing adenosine at position 46 in the variable loop (Dube et al., 1968). This isomer was prepared by chromatography of total *E. coli* K12 $tRNA^{fMet}$ on RPC-3 (Weeken et al., 1972). The tRNA was labeled with

† This work was supported by Grant GM-16995 from the National Institutes of Health. Partial salary support for L.H.S. was provided by National Cancer Institute Grant P30 CA1 333 0-10.
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[1] Abbreviations: MetRS, *E. coli* methionyl-tRNA synthetase; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

0006-2960/86/0425-4450$01.50/0

^{32}P in the 3′-terminal adenosine as described earlier.

Methylation reactions were carried out in 50 mM Hepes, pH 7.0, and 10 mM $MgCl_2$ in a total volume of 50 μL. The tRNA alone (1 μM) was preincubated in this buffer for 20 min at 37 °C. Methionyl-tRNA synthetase (0.5–10 μM) was added and the mixture incubated at 25 °C for 10 min. Parallel samples were run in the absence of enzyme. Concentrated dimethyl sulfate was diluted 1:10 with absolute ethanol just before use. One microliter of the diluted reagent was added to the tRNA–synthetase complex or tRNA alone, and incubation was continued at 25 °C. Fifteen-microliter aliquots were removed at various times and added to 135 μL of 0.2 M Tris–acetate, pH 7.5, 0.2 M 2-mercaptoethanol, and 0.3 M sodium acetate containing 2 A_{260}/mL crude carrier tRNA. The mixture was extracted with an equal volume of phenol saturated with the same buffer. Three volumes of ethanol was added to the aqueous phase, the reaction chilled for 10 min at −70 °C, and the precipitate collected by centrifugation. The pellet was redissolved in 0.3 M sodium acetate and precipitated with 3 volumes of ethanol as above. Cleavage of the tRNA at the sites of methylated cytidine and guanosine residues and analysis on polyacrylamide gels were carried out as described by Peattie (1983). In control experiments, tRNA methylated in the absence of methionyl-tRNA synthetase was subsequently incubated with 4 μM synthetase for 10 min at 25 °C. The samples were then quenched, extracted, and analyzed for the extent of cleavage as described before.

Nuclease Protection Experiments. (*1*) *RNase A.* Reactions were carried out in 50 μL of 100 mM Tris-HCl, pH 7.5, and 5 mM $MgCl_2$. 5′- or 3′-^{32}P-labeled Boehringer tRNA^{fMet} (1 μM) was preincubated in this buffer for 20 min at 37 °C. Methionyl-tRNA synthetase (1–10 μM) was added and the solution incubated at 25 °C for 20 min. Parallel reactions were run in the absence of synthetase. RNase A (1–10 ng) was added and the incubation continued at 25 °C. Fifteen-microliter aliquots were withdrawn at various times and added to 30 μL of phenol saturated with reaction buffer. After extraction of protein, aliquots were removed from the aqueous phase, mixed with an equal volume of 10 M urea and 0.02% xylene cyanol dye, and analyzed on polyacrylamide gels by the method of Donis-Keller et al. (1977).

(*2*) *RNase T_1.* Reactions were carried out as described except that incubation mixtures contained 0.015 unit of RNase T_1 in a final volume of 70 μL.

(*3*) *α-Sarcin.* 5′- or 3′-^{32}P-labeled Boehringer tRNA^{fMet} (2.5 μM) was preincubated for 20 min at 37 °C in 100 mM Tris-HCl, pH 7.5, and 5 mM $MgCl_2$. Methionyl-tRNA synthetase was added, and the solution was diluted to a final concentration of 1 μM tRNA and 4 μM enzyme in 40 mM Tris-HCl, pH 7.5, and 2 mM $MgCl_2$ and incubated for an additional 20 min at 37 °C. Parallel reactions were run in the absence of synthetase. α-Sarcin (1–10 μM) was added and the reaction mixture (22 μL) incubated for 15 min at 37 °C. Protein was extracted with 50 μL of phenol saturated with reaction buffer. Fifteen microliters of the aqueous phase was diluted with an equal volume of 10 M urea and 0.02% xylene cyanol dye, followed by $^1/_{10}$ volume of 0.1 M EDTA, and aliquots were analyzed on polyacrylamide gels as above.

For analysis of the sites of cleavage at the 3′ terminus of tRNA^{fMet} by column chromatography, reactions were carried out with 1 μM 3′-^{32}P-labeled tRNA^{fMet}, 4 μM methionyl-tRNA synthetase, and 10 μM α-sarcin in a total reaction volume of 0.18 mL. Protein was extracted with 2 volumes of phenol, and the aqueous phase was diluted with an equal volume of 10 M urea and $^1/_{10}$ volume of 0.1 M EDTA. A_{260} oligonucleotide markers were added, and the mixture was chromatographed on a 0.5 × 100 cm column of RPC-5 by using a linear gradient of 0.075–1.6 M ammonium acetate, pH 7.5, per 400 mL at a flow rate of 36 mL/h. Absorbance patterns were recorded at 260 nm with a Gilford Model 2400 absorbance recorder. Three-milliliter fractions were collected and 1-mL aliquots counted in 15 mL of ACSII (Amersham) in a liquid scintillation counter.

Digestion of tRNAs with α-Sarcin in the Presence and Absence of Mg^{2+}. 5′- or 3′-^{32}P-labeled *E. coli* tRNA^{fMet} (1 μM) was digested with α-sarcin (1–10 μM) as described except that no methionyl-tRNA synthetase was present and the final reaction mixtures contained 50 mM KCl, 50 mM Tris-HCl, pH 7.5, and various amounts of Mg^{2+} or EDTA.

Results

Chemical Probing of Cytidine and Guanosine Residues in tRNA^{fMet} Complexed with Methionyl-tRNA Synthetase. The use of dimethyl sulfate to probe the ordered structure of RNA has been described in detail by Peattie (1983). The reagent methylates single-stranded cytosine residues at the N-3 position of the pyrimidine ring. Double-stranded cytosines are unreactive due to the participation of N-3 in hydrogen bond formation. The sites of methylation of cytosines in 3′-^{32}P-labeled RNAs can be detected by reaction of m^3C residues with aqueous hydrazine, followed by aniline-catalyzed chain cleavage, electrophoresis of the cleavage products on polyacrylamide gels, and visualization by autoradiography (Peattie & Gilbert, 1980). 5′-End-labeled RNAs cannot be used to investigate cytosine methylation since the cleavage reactions do not produce unique 3′ termini and blurred banding patterns are obtained (Peattie, 1983).

Dimethyl sulfate reacts with guanosine residues at the N-7 position of the purine base in both single- and double-stranded nucleic acids; however, not all G residues in native RNAs are methylated, indicating that structural features other than participation in hydrogen bonding influence reaction at this site (Peattie & Gilbert, 1980; Peattie & Herr, 1981; Peattie et al., 1981). The location of methylated guanosines can be determined by reduction of m^7G with sodium borohydride, followed by aniline-induced chain cleavage and gel analysis (Peattie, 1983). Many *E. coli* tRNAs contain m^7G as a naturally occurring minor base at position 46 from the 5′ terminus [numbering based on the convention used in Gauss et al. (1979)]. Such tRNAs are extensively cleaved by treatment with sodium borohydride and aniline in the absence of chemical methylation. *E. coli* tRNA^{fMet} exists in two isomeric forms that differ by a single nucleotide at position 46 (Dube et al., 1968). One isomer contains m^7G at this position, while the other contains an A residue. The methylation experiments were carried out on a 3′-^{32}P-labeled tRNA^{fMet} sample that was highly enriched for the A_{46} isomer in order to reduce the background cleavage of the tRNA at m^7G.

Figure 1 shows the results of cytosine-specific methylation protection experiments. *E. coli* tRNA^{fMet} contains six cytosines that react readily with single strand specific reagents: C_1, C_{16}, C_{17}, C_{34}, C_{74}, and C_{75} (Goddard & Schulman, 1972; Chang, 1973; Schulman et al., 1974). We were unable to determine whether C_1 was reactive with dimethyl sulfate using 3′-^{32}P-labeled tRNA^{fMet}; however, the other cytosine residues were methylated as expected in the absence of methionyl-tRNA synthetase. Methylation of 1 μM tRNA^{fMet} in the presence of 1–10 μM synthetase gave little or no protection of C_{74} or C_{75} in the 3′-terminal CCA sequence (Figure 1A) or of C_{16} and C_{17} in the D loop (Figure 1B). In contrast, strong pro-

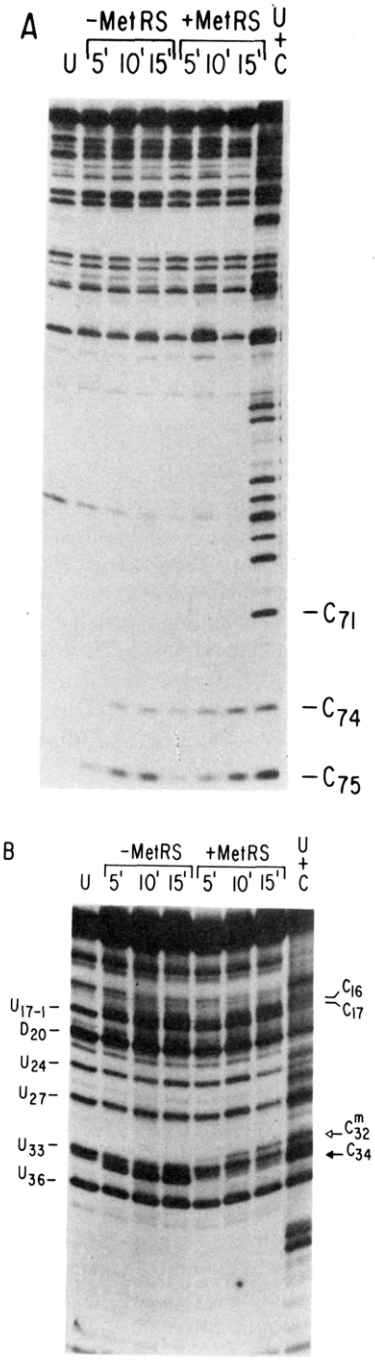

FIGURE 1: Cytosine-specific methylation of $tRNA^{fMet}$. 3'-^{32}P-Labeled $tRNA^{fMet}$ (1.1 μM) was treated with dimethyl sulfate (approximately 23 mM) for 5, 10, and 15 min at 25 °C in the presence (+) and absence (−) of 2.2 μM methionyl-tRNA synthetase (MetRS) in 50 mM Hepes buffer, pH 7.0, and 10 mM $MgCl_2$ as described under Experimental Procedures. Cleavage products were electrophoresed on 20% polyacrylamide and 7 M urea sequencing gels. Increased methylation in the presence of synthetase is indicated by an open arrow and decreased methylation by a closed arrow. The conditions used to cleave m^3C residues also result in cleavage at all U residues in the tRNA. Lanes marked U contain $tRNA^{fMet}$ reacted with hydrazine and aniline under standard conditions for U-specific RNA strand cleavage (Peattie, 1983). Lane U + C contains $tRNA^{fMet}$ treated with dimethyl sulfate under denaturing conditions, followed by aqueous hydrazine and aniline cleavage to generate both U-specific and C-specific bands (Peattie, 1983). Some nonspecific cleavage is also observed in the absence of methylation. Note that a nonspecific cleavage band appears between the bands corresponding to U_{33} and m^3C_{34} in Figure 1B. This band is present in all lanes and does not change in intensity, while the band resulting from methylation of C_{34} is drastically reduced in reaction mixtures containing methionyl-tRNA synthetase.

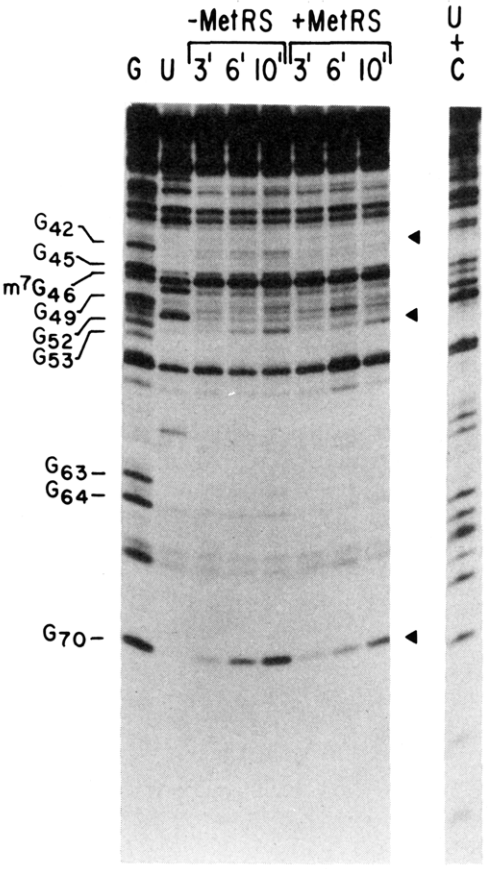

FIGURE 2: Guanosine-specific methylation of $tRNA^{fMet}$. 3'-^{32}P-Labeled $tRNA^{fMet}$ (1.1 μM) was treated with dimethyl sulfate in the presence (+) and absence (−) of 2.2 μM methionyl-tRNA synthetase (MetRS) as described in the legend to Figure 1. Bases showing decreased reactivity in the presence of synthetase are indicated by closed arrows in the figure. Lane G contains $tRNA^{fMet}$ treated with dimethyl sulfate under denaturing conditions, followed by sodium borohydride and aniline treatment to generate G-specific bands (Peattie, 1983). A considerable background of nonspecific cleavage in the absence of methylation was also observed. Lane U + C was generated by the U- and C-specific sequencing protocol (Peattie, 1983).

tection was seen at the anticodon wobble base, C_{34} (Figure 1B). This protection was virtually the same at enzyme:tRNA ratios ranging from 1:1 to 10:1. At concentrations of 0.5 μM synthetase and 1 μM tRNA, the level of methylation was approximately half of that observed in the absence of enzyme (data not shown). In addition to the strong protection of N-3 of the wobble base, a new site of methylation was observed at mC_{32} in the anticodon loop in the presence of methionyl-tRNA synthetase, indicating a change in the accessibility of this base to dimethyl sulfate (Figure 1B). Control experiments indicated that incubation of 1 μM premethylated $tRNA^{fMet}$ with 4 μM methionyl-tRNA synthetase resulted in no change in the methylation pattern of the tRNA (data not shown).

Figure 2 shows the results of guanosine-specific methylation protection experiments. We were able to detect methylation of only seven of the potentially reactive G residues in $tRNA^{fMet}$ in the absence of methionyl-tRNA synthetase. A similar limited reaction of G residues with dimethyl sulfate has been observed under native conditions with yeast $tRNA^{Phe}$ and yeast $tRNA^{Asp}$ (Peattie & Gilbert, 1980; Barciszewski et al., 1982). We also observed a high level of nonspecific RNA chain scission under conditions used to cleave m^7G residues, and considerable variation in band intensities from experiment to experiment. Similar problems have been reported by other laboratories (Barciszewski et al., 1982; Swerdlow & Guthrie, 1984). These factors make quantitative assessment of G-

Table I: Reaction of Cytosine and Guanosine Residues in tRNA^{fMet} with Dimethyl Sulfate[a]

nucleotide	methylation of tRNA^{fMet}	methylation of tRNA^{fMet}–synthetase complex
C_{16}	+	little or no change
C_{17}	+	little or no change
G_{18}	(+)	uncertain
G_{19}	+	little or no change
G_{26}	(+)	uncertain
mC_{32}	–	enhanced
C_{34}	++	greatly reduced
G_{42}	+	reduced
G_{49}	(+)	uncertain
G_{52}	+	reduced
G_{70}	++	reduced
C_{74}	++	little or no change
C_{75}	++	little or no change

[a] Incubation conditions are described under Experimental Procedures. Data represent the average of a number of different experiments. (+) indicates minor site of reaction. Methylation of C_1 and G_2 could not be determined. Other C and G residues not listed are not methylated by dimethyl sulfate under the conditions described.

methylation protection difficult. Nevertheless, it is clear that no sites of N-7 guanosine methylation are as strongly protected by Met-tRNA synthetase as the N-3 position of the anticodon wobble base. A partial reduction in the extent of reaction at G_{42}, G_{52}, and G_{70} can be seen in the presence of the enzyme (Figure 2).

Table I summarizes the sites of reaction of cytosine and guanosine residues in tRNA^{fMet} with dimethyl sulfate and the effect of tRNA–synthetase complex formation on the accessibility of each site to the reagent.

Probing of the tRNA^{fMet}–Methionyl-tRNA Synthetase Complex with Single Strand Specific Ribonucleases. A pyrimidine-specific ribonuclease (RNase A) and a guanosine-specific ribonuclease (RNase T_1) were used to investigate the accessibility of single-stranded nucleotides in tRNA^{fMet} to enzymatic cleavage in the presence and absence of methionyl-tRNA synthetase. Both 3′- and 5′-^{32}P-labeled tRNAs were used in order to distinguish primary from secondary cleavage sites. Radioactive bands corresponding to primary cleavage sites have the same relative intensities on sequencing gels run with either end-labeled tRNA, while the intensities of bands corresponding to secondary cleavage sites depend on the distance of the cleavage site from the labeled terminus of the tRNA.

Figure 3 shows the results of limited digestion of 5′-^{32}P-labeled tRNA^{fMet} with RNase A. The strongest cleavage is observed at the cytidine of the anticodon in the absence of methionyl-tRNA synthetase, with weaker cleavages at anticodon base U_{36} and at pyrimidine residues in the D loop and a still weaker cleavage at C_{56} in the T loop. Experiments carried out with 3′-end-labeled tRNA gave rise to an additional band migrating at a position corresponding to nucleotide 37 on the sequencing gel. This band was due to double cleavage of the tRNA at both C_{34} and C_{75} (data not shown). Nuclease attack at C_{75} releases the unlabeled terminal adenosine and produces a labeled fragment having an increased charge due to the generation of a 3′-terminal phosphate. Synthetase complex formation significantly inhibits RNase A cleavage in the anticodon and reduces the rate of nuclease attack in the D loop and 3′ terminus.

Figure 4 shows the results of limited digestion of 5′-^{32}P-labeled tRNA^{fMet} with RNase T_1. Strong cleavages are observed only in the D loop. Bands corresponding to G residues in the D stem appear to arise from secondary cleavages. Weak primary cleavages are also observed at G_{45} and G_{49}. Limited

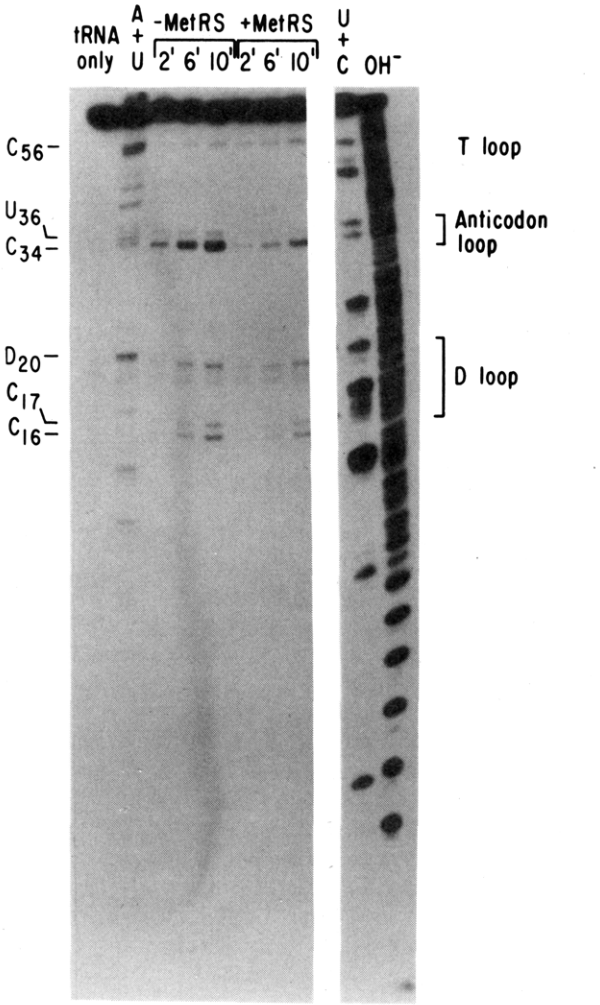

FIGURE 3: Partial digestion of tRNA^{fMet} with RNase A. 5′-^{32}P-Labeled tRNA^{fMet} (1 μM) was incubated with RNase A for 2, 6, and 10 min at 25 °C in the presence (+) and absence (–) of 4 μM methionyl-tRNA synthetase (MetRS) in 100 mM Tris-HCl pH 7.5, and 5 mM $MgCl_2$ as described under Experimental Procedures. Cleavage products were electrophoresed on 20% polyacrylamide and 7 M urea sequencing gels. Lane A + U, partial digestion of tRNA^{fMet} with RNase PhyM under denaturing conditions. Lane U + C, partial digest with *B. cereus* RNase. Lane OH^-, partial hydrolysis with alkali.

digestion of the tRNA in the presence of methionyl-tRNA synthetase shows that the enzyme has a small effect on cleavage by this ribonuclease.

Use of α-Sarcin To Probe the Structure of tRNA^{fMet} and the tRNA^{fMet}–Methionyl-tRNA Synthetase Complex. α-Sarcin has been shown by Wool and co-workers to cleave ribosomal RNAs and RNA homopolymers at purine residues, with the generation of products having 3′-phosphate and 5′-hydroxy termini (Endo et al., 1983). The nuclease is inhibited by concentrations of Mg^{2+} above 2 mM and of monovalent cations above 0.1 M. Single- and double-stranded regions are equally attacked, making α-sarcin an attractive reagent for probing protein binding sites on RNAs (Huber & Wool, 1984). To date, no studies of this kind have been reported for tRNAs or tRNA–protein complexes. We therefore initially examined the nuclease activity of α-sarcin on 3′- and 5′-^{32}P-labeled *E. coli* tRNA^{fMet} under a variety of solvent conditions.

Digestions containing 1 μM tRNA^{fMet} in 5 mM EDTA produced strong cleavages at G_{18}, G_{26}, and G_{49}, which were independent of α-sarcin concentration in the range of 1–10 μM nuclease. α-Sarcin at 10 μM also produced significant levels of cleavage of purines in the acceptor and D stems and the D, T, and variable loops. Other purines were unreactive

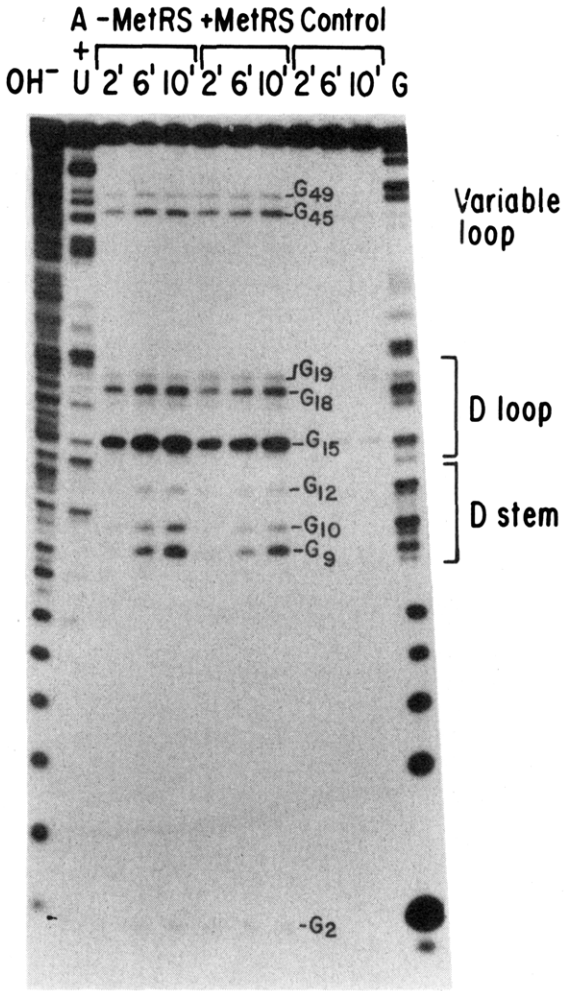

FIGURE 4: Partial digestion of $tRNA^{fMet}$ with RNase T_1. 5′-^{32}P-Labeled $tRNA^{fMet}$ (1 μM) was incubated with 2.2 units/mL RNase T_1 for 2, 6, and 10 min at 25 °C in the presence (+) and absence (−) of 4.8 μM methionyl-tRNA synthetase (MetRS) in 100 mM Tris-HCl, pH 7.5, and 5 mM $MgCl_2$ as described under Experimental Procedures. Cleavage products were electrophoresed on 20% polyacrylamide and 7 M urea sequencing gels. Lanes OH^- and A + U are as indicated in Figure 3. Lane G, partial digest of $tRNA^{fMet}$ with RNase T_1 under denaturing conditions. Control lanes contain 1 μM $tRNA^{fMet}$ and 4.8 μM methionyl-tRNA synthetase but no RNase T_1 incubated as described previously. Part of the band intensity of G_{15} is due to double cuts at G_{18} and G_{15}, as revealed by similar experiments carried out with 3′-end-labeled $tRNA^{fMet}$ (not shown).

or only weakly attacked, the anticodon loop and stem being the most resistant part of the structure (data not shown).

Overall cleavage of 1 μM $tRNA^{fMet}$ by 10 μM α-sarcin was strongly reduced by addition of 1–2 mM Mg^{2+} to the reaction. In addition, the purine specificity of the nuclease was diminished, and a significant level of cleavage of pyrimidine residues was observed. Cleavage of one site near the 3′ terminus was strongly enhanced. Since the nuclease activity of α-sarcin on other RNA substrates is unaffected by this level of divalent cation (Endo et al., 1983), this result appears to reflect the effect of changes in the ordered structure of the tRNA on cleavage by the enzyme.

Figure 5 shows the results of limited digestion of 5′- and 3′-^{32}P-labeled $tRNA^{fMet}$ with α-sarcin in the presence and absence of methionyl-tRNA synthetase. Reactions were preincubated at 5 mM Mg^{2+} and then diluted to 2 mM Mg^{2+} on addition of the nuclease. This concentration of divalent cation is sufficient to allow complete complex formation between $tRNA^{fMet}$ and native methionyl-tRNA synthetase (Stein & Crothers, 1976; Blanquet et al., 1973). Binding to the

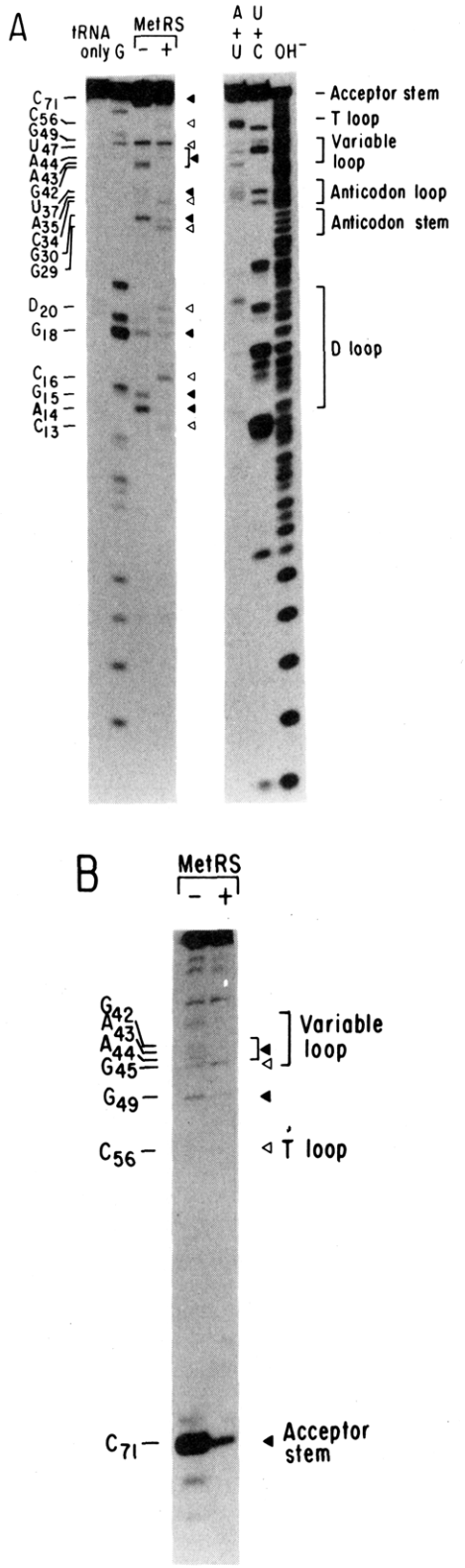

FIGURE 5: Partial digestion of the $tRNA^{fMet}$–methionyl-tRNA synthetase complex with α-sarcin. (A) 5′-^{32}P-Labeled $tRNA^{fMet}$ (1 μM) and α-sarcin (10 μM) were incubated for 15 min at 37 °C in the presence (+) and absence (−) of 4 μM methionyl-tRNA synthetase (MetRS) in 40 mM Tris-HCl, pH 7.5, and 2 mM $MgCl_2$ as described under Experimental Procedures. Cleavage products were electrophoresed on 25% polyacrylamide and 7 M urea sequencing gels. Lanes G, A + U, U + C, and OH^- are as indicated in Figures 3 and 4. (B) 3′-^{32}P-Labeled $tRNA^{fMet}$ (1 μM) incubated as described previously. Open arrows indicate sites of increased nuclease cleavage, and closed arrows indicate sites of decreased nuclease cleavage in the presence of methionyl-tRNA synthetase.

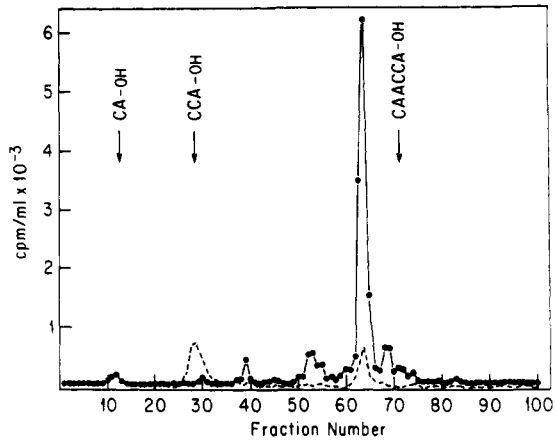

FIGURE 6: Identification of α-sarcin cleavage sites near the 3′ terminus of tRNA^{fMet}. 3′-^{32}P-Labeled tRNA^{fMet} (1 μM) was digested with 10 μM α-sarcin for 15 min at 37 °C in 40 mM Tris-HCl, pH 7.5, and 2 mM $MgCl_2$ in the presence (---) and absence (—) of 4 μM methionyl-tRNA synthetase. Reaction mixtures were chromatographed on RPC-5 with oligonucleotide markers as described under Experimental Procedures.

Table II: α-Sarcin Cleavage of tRNA^{fMet} in the Presence and Absence of Methionyl-tRNA Synthetase[a]

nucleotide	−MetRS	+MetRS	nucleotide	−MetRS	+MetRS
A_{14}	+++	(+)	G_{30}	++	(+)
G_{15}	++	(+)	A_{44}	++	(+)
G_{18}	++	+	G_{49}	+++	++
G_{29}	–	++	C_{71}	++++++	+

[a] tRNA^{fMet} (1 μM) was incubated with 10 μM α-sarcin in 40 mM Tris-HCl, pH 7.5, and 2 mM $MgCl_2$ in the presence and absence of 4 μM MetRS as described under Experimental Procedures. Data represent the average of a number of different experiments. Minor cleavage sites have been omitted from the table. (+) indicates weak cleavage in the presence of synthetase.

synthetase drastically reduced the level of cleavage of tRNA^{fMet} near the 3′ terminus (Figure 5B). Sequencing gels were inadequate to determine the exact cleavage site near the 3′ end since appropriate radioactively labeled oligonucleotide markers could not be generated with PhyM or *B. cereus* RNases. 3′-^{32}P-Labeled tRNA^{fMet} was therefore digested with α-sarcin, and the small oligonucleotides released were identified by column chromatography in the presence of A_{260} oligonucleotide markers (Figure 6). The major site of nuclease attack in the absence of synthetase was at C_{71} in the acceptor stem. Approximately 10% of the input tRNA was cleaved at this site. Addition of methionyl-tRNA synthetase virtually eliminated attack at C_{71}. Much smaller changes were also observed in the cleavage patterns in the variable loop, anticodon loop, D loop, and anticodon stem (Figure 5).

Table II summarizes the significant sites of α-sarcin cleavage in tRNA^{fMet} in the presence and absence of MetRS, and Figure 7 provides a composite illustration of all the data obtained with both chemical and enzymatic probes.

Discussion

We undertook this work in order to obtain information on the accessibility of specific nucleotides in tRNA^{fMet} to chemical and enzymatic probes when complexed to methionyl-tRNA synthetase. The results reported here clearly demonstrate that the N-3 position of C_{34} in the wobble position of the anticodon of tRNA^{fMet} is unavailable for reaction with dimethyl sulfate when the tRNA is bound to the enzyme. Partial digestion of the tRNA–enzyme complex with RNase A, which initially attacks the tRNA primarily at C_{34} and in the 3′-CCA sequence (Wrede & Rich, 1979; Schulman et al., 1983), reveals that the anticodon is also shielded from cleavage by this relatively

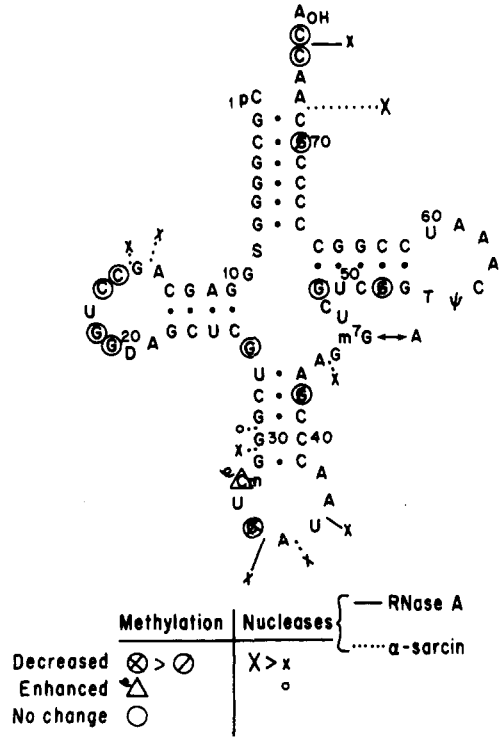

FIGURE 7: Effect of complex formation with methionyl-tRNA synthetase on methylation and nuclease cleavage of tRNA^{fMet}. tRNA^{fMet} was treated with dimethyl sulfate and ribonucleases as described under Experimental Procedures. Bases methylated in the absence of synthetase are circled. C_1 is probably also methylated, although not studied in these experiments (see text). Bases showing reduced reaction in the tRNA–synthetase complex are indicated by (⊗) (large decrease), (⊖) (moderate decrease), or (○) (no change). The base showing enhanced reaction is enclosed in an open triangle. Nuclease cleavage sites are indicated by lines between the bases: RNase A (—); α-sarcin (···). The length of the line is a reflection of the extent of cleavage in the absence of synthetase. Decreased cleavage in the presence of synthetase is indicated by (×) and enhanced cleavage by (○). Only those sites that undergo a significant (greater than 2-fold) change in the extent of cleavage in the presence of synthetase are indicated in the figure.

small nuclease (M_r 13 700). The protection against RNase A cleavage is extensive, but less dramatic than the methylation protection, suggesting that the ribophosphate backbone may be more exposed than the cytidine base itself and/or that interaction of the nuclease with the complex may partially disrupt synthetase binding to the anticodon region. The anticodon nucleotides of free tRNA^{fMet} are also accessible to the single strand specific nucleases S_1 and T_2 (Wrede & Rich, 1979; Wrede et al., 1979; Lockard & Kumar, 1981; Yamashiro-Matsumura & Kawata, 1981; Petersen et al., 1984). Yamashiro-Matsumura and Kawata (1981) reported that C_{34} is not protected from cleavage by either of these nucleases in the presence of methionyl-tRNA synthetase, while Petersen et al. (1984) reported that RNase T_2 cleavages in the anticodon loop of tRNA^{fMet} are significantly reduced in the presence of the synthetase.[2] The reason for this difference is not clear.

[2] Petersen et al. (1984) reported that RNase T_2 cleaves tRNA^{fMet} at nucleotides A_{37}, A_{38}, and C_{39}, while other investigators found no cuts at these sites but a strong cleavage at C_{34} (Wrede & Rich, 1979; Wrede et al., 1979; Lockard & Kumar, 1981; Yamashiro-Matsumura & Kawata, 1981). The reason for this discrepancy is not clear; however, the experiments of Petersen et al. were carried out on tRNA^{fMet} labeled in the phosphate of the 3′-terminal A residue (CpCp*A-OH), while other laboratories used 5′-^{32}P-labeled tRNA. Rapid cleavage of the 3′ end at the penultimate C residue (C_{75}) followed by cleavages in the anticodon nucleotides could produce bands with mobilities apparently corresponding to primary attack at the sites reported by Petersen et al. (1984).

In the present study, we also investigated the use of α-sarcin as a probe of tRNA–protein interactions. α-Sarcin is a relatively small (M_r 17 400) nuclease that had previously been shown to cleave purine residues in both single-stranded and double-stranded regions of homopolymers and rRNA substrates (Endo et al., 1983; Huber & Wool, 1984). The enzyme is inhibited by Mg^{2+}, requiring higher concentrations of nuclease for equivalent amounts of cleavage in the presence of the divalent cation. We observed significant changes in the sites of cleavage of free $tRNA^{fMet}$ and a relaxation in the purine specificity of the enzyme in the presence and absence of Mg^{2+}. These results indicated a substantial influence of ordered structure on nuclease activity with the free tRNA. Complex formation with Met-tRNA synthetase in the presence of 2 mM Mg^{2+} also produced significant changes in cleavage sites. In particular, a large decrease in cleavage at C_{71} in the acceptor stem was observed on binding of synthetase to the tRNA. Since removal of Mg^{2+} also leads to a large decrease in cleavage at C_{71} in the absence of synthetase, it is not clear whether enzyme binding and/or tRNA structure governs α-sarcin cleavage at this site. In any case, it is noteworthy that this is the only dramatic change in nucleotide accessibility that occurs outside of the anticodon region on binding of MetRS to the tRNA. Weakly reduced cleavage of $tRNA^{fMet}$ in the D loop is observed in the presence of synthetase with RNase A and RNase T_1, and both reduced and enhanced cleavage sites with α-sarcin. Protection of this region has also been found by others to vary depending on the nuclease probe used (Yamashiro-Matsumura & Kawata, 1981). Methylation experiments showed little or no change in the accessibility of C and G residues in the D loop to the small chemical probe dimethyl sulfate. Other regions such as the anticodon stem and variable loop also show small changes in nuclease digestion patterns on formation of the tRNA–synthetase complex (Figures 4 and 5; Petersen et al., 1984). Such results are consistent with changes in the relative rates of cleavage of specific phosphodiester bonds due to local conformational changes.

The presently available data on the role of anticodon nucleotides in recognition of tRNAs by aminoacyl-tRNA synthetases has recently been reviewed by Kisselev (1985). The available structure–function data on *E. coli* methionyl-tRNA synthetase indicates that a major portion, if not all, of the binding energy for the tRNA–synthetase interaction comes from contacts with the anticodon nucleotides (Schulman et al., 1983; Schulman & Pelka, 1983, 1984). The present results support a model in which strong binding of MetRS to the anticodon of $tRNA^{fMet}$ leads to a conformational change in the anticodon loop and adjoining stem. These changes are accompanied by more distant, and possibly rather subtle, conformational changes in other parts of the structure, ultimately leading to proper orientation of the 3′ terminus of the tRNA with respect to the active site of the enzyme.

ACKNOWLEDGMENTS

We thank Ira Wool for a generous gift of α-sarcin and for helpful discussions on the use of the nuclease. We are grateful to Frank Borriello for carrying out the RNase T_1 digestions. We thank Rita Romita for expert typing.

Registry No. MetRS, 9033-22-1; RNase A, 9001-99-4; RNase T_1, 9026-12-4; $(MeO)_2SO_2$, 77-78-1; α-sarcin, 1407-48-3.

REFERENCES

Barciszewski, J., Romby, P., Ebel, J. P., & Giege, R. (1982) *FEBS Lett. 150*, 459–464.

Blanquet, S., Iwatsubo, M., & Waller, J. P. (1973) *Eur. J. Biochem. 36*, 213–226.

Chang, S. E. (1973) *J. Mol. Biol. 75*, 533–547.

Donis-Keller, H., Maxam, A. M., & Gilbert, W. (1977) *Nucleic Acids Res. 4*, 2527–2538.

Dube, S. K., Marcker, K. A., Clark, B. F. C., & Cory, S. (1968) *Nature (London) 218*, 232–233.

Endo, Y., Huber, P. W., & Wool, I. G. (1983) *J. Biol. Chem. 258*, 2662–2667.

Francis, T. A., Ehrenfeld, G. M., Gregory, M. R., & Hecht, S. M. (1983) *J. Biol. Chem. 258*, 4279–4284.

Gauss, D. H., Gruter, F., & Sprinzl, M. (1979) in *Transfer RNA: Structure, Properties, and Recognition* (Schimmel, P. R., Söll, D., & Abelson, J. N., Eds.) pp 518–519, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Goddard, J. P., & Schulman, L. H. (1972) *J. Biol. Chem. 247*, 3864–3867.

Huber, P. W., & Wool, I. G. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 322–326.

Kisselev, L. L. (1985) *Prog. Nucleic Acid Res. Mol. Biol. 32*, 237–266.

Lockard, R. E., & Kumar, A. (1981) *Nucleic Acids Res. 9*, 5125–5140.

Peattie, D. A. (1983) in *Methods of DNA and RNA Sequencing* (Weissman, S. M., Ed.) pp 261–304, Praeger, New York.

Peattie, D. A., & Gilbert, W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4679–4682.

Peattie, D. A., & Herr, W. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2273–2277.

Peattie, D. A., Douthwaite, S., Garrett, R. A., & Noller, H. F. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7331–7335.

Petersen, H. U., Siboska, G. E., Clark, B. F. C., Buckingham, R. H., Hountondji, C., & Blanquet, S. (1984) *Biochimie 66*, 625–630.

Schulman, L. H., & Goddard, J. P. (1973) *J. Biol. Chem. 248*, 1341–1345.

Schulman, L. H., & Pelka, H. (1977a) *Biochemistry 16*, 4256–4265.

Schulman, L. H., & Pelka, H. (1977b) *J. Biol. Chem. 252*, 814–819.

Schulman, L. H., & Pelka, H. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6755–6759.

Schulman, L. H., & Pelka, H. (1984) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 43*, 2977–2980.

Schulman, L. H., Shapiro, R., Law, D. C. F., & Louis, J. B. (1974) *Nucleic Acids Res. 1*, 1305–1316.

Schulman, L. H., Pelka, H., & Susani, M. (1983) *Nucleic Acids Res. 11*, 1439–1455.

Stein, A., & Crothers, D. M. (1976) *Biochemistry 15*, 157–160.

Stern, L., & Schulman, L. H. (1977) *J. Biol. Chem. 252*, 6403–6408.

Swerdlow, H., & Guthrie, C. (1984) *J. Biol. Chem. 259*, 5197–5207.

Weeren, H. O., Ryon, A. D., & Kelmers, A. D. (1972) *Biotechnol. Bioeng. 14*, 617–627.

Wrede, P., & Rich, A. (1979) *Nucleic Acids Res. 7*, 1457–1467.

Wrede, P., Woo, N. H., & Rich, A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3289–3293.

Yamashiro-Matsumura, S., & Kawata, M. (1981) *J. Biol. Chem. 256*, 9308–9312.