Peptides at the tRNA binding site of the crystallizable monomeric form of *E. coli* methionyl-tRNA synthetase

LaDonne H. Schulman, Heike Pelka and Oscar Leon

Department of Developmental Biology and Cancer, Division of Biology, Albert Einstein College of Medicine, Bronx, NY 10461, USA

Received August 21, 1987; Revised and Accepted November 10, 1987

ABSTRACT

A protein affinity labeling derivative of *E. coli* tRNA^{fMet} carrying lysine-reactive cross-linking groups has been covalently coupled to monomeric trypsin-modified *E. coli* methionyl-tRNA synthetase. The cross-linked tRNA-synthetase complex has been isolated by gel filtration, digested with trypsin, and the tRNA-bound peptides separated from the bulk of the free tryptic peptides by anion exchange chromatography. The bound peptides were released from the tRNA by cleavage of the disulfide bond of the cross-linker and purified by reverse-phase high-pressure liquid chromatography, yielding three major peptides. These peptides were found to cochromatograph with three peptides of known sequence previously cross-linked to native methionyl-tRNA synthetase through lysine residues 402, 439 and 465. These results show that identical lysine residues are in close proximity to tRNA^{fMet} bound to native dimeric methionyl-tRNA synthetase and to the crystallizable monomeric form of the enzyme, and indicate that cross-linking to the dimeric protein occurs on the occupied subunit of the 1:1 tRNA-synthetase complex.

INTRODUCTION

Native *E. coli* methionyl-tRNA synthetase (MetRS) is a dimeric enzyme containing identical subunits of molecular weight 76,000 (1). Controlled proteolysis of the native enzyme releases approximately 130 amino acids from the carboxy terminus of each protomer and yields a monomeric fragment (MetRS_F) of molecular weight 64,000 which retains full biological activity (2). This form of the enzyme has been crystallized (3) and its structure at 2.5Å resolution reported (4).

We have previously achieved stoichiometric cross-linking of lysine-reactive affinity labeling derivatives of *E. coli* tRNA^{fMet} to the tRNA binding site of native MetRS and have sequenced peptides cross-linked to five different sites in the tRNA (5-10). In order to assist in developing a three-dimensional model of the interaction between tRNA^{fMet} and the crystallized protein, it is important to determine whether covalent coupling of the tRNA occurs to the same sites in the monomeric and dimeric forms of the synthetase. In this paper, we report the results of such cross-linking experiments with MetRS_F.

MATERIALS AND METHODS

Native MetRS was purified from *E.coli* K12 strain EM 20031 as described before (11). MetRS_F was generated by limited digestion of the native enzyme with trypsin and purified by chromatography on hydroxyapatite (2). *E.coli* tRNA^{fMet} having a specific activity of 1720 pmol/A₂₆₀ unit was purchased from Boehringer Mannheim. TPCK-treated trypsin was obtained from Worthington Biochemical Corp. and further purified as described before (7). Dithiobis(succinimidyl propionate) (DTSP) was purchased from Pierce Chemical Co. and ³⁵S-labeled DTSP was synthesized by the method of Lomant and Fairbanks (12). Procedures for preparation of the lysine-reactive derivative of tRNA^{fMet}, for cross-linking the modified tRNA to MetRS_F, and for isolation of the cross-linked peptides were the same as those described previously for MetRS (5-7). Reaction mixtures for assay of methionine acceptor activity of MetRS_F contained 20 mM imidazole-HCl, pH 7.5, 0.1 mM EDTA, 4 mM MgCl₂, 150 mM NH₄Cl, 2 mM ATP, 17 μM [³⁵S] methionine (1300 cpm/pmole) and 85 A₂₆₀/ml of crude *E.coli* K-12 tRNA. Samples were incubated at 25° C and reactions were initiated by addition of enzyme to a final concentration of 200 pM. Under these conditions, methionine incorporation was proportional to enzyme concentration and linear with time for at least 10 min.

RESULTS

Cross-linking of a lysine-reactive derivative of tRNA^{fMet} to MetRS_F

Lysine-reactive cross-linking groups were attached to *E. coli* tRNA^{fMet} using a previously described two-step procedure (5,13). In the first step, three-carbon side chains terminating in a reactive primary amino group were joined to the N⁴-position of single-stranded cytidine residues by reaction with propane-1,3-diamine (PDA) in the presence of sodium bisulfite. This procedure leads to modification of cytidines in four structural regions of the tRNA: the 5' terminus, the D loop, the anticodon, and the 3' CCA sequence (6). By controlling the extent of modification with PDA, tRNA having an average of one side chain per molecule distributed over these four sites was prepared. In the second step, dithiobis(succinimidyl propionate) (DTSP) radioactively labeled with ³⁵S in the disulfide bond of the cross-linker was coupled to each reactive PDA side chain. The N-hydroxysuccinimide ester group of the resulting [³⁵S] DTSP/PDA-tRNA^{fMet} is capable of reaction with appropriately oriented lysine residues in proteins (Figure 1). Subsequent cleavage of the disulfide bond of the cross-linker with DTT releases the tRNA from the covalent tRNA-protein complex and leaves an ³⁵S-labeled modified lysine residue at the site of each cross-link in the protein (Figure 1).

Covalent coupling of [³⁵S] DTSP/PDA-tRNA^{fMet} to the monomeric tryptic fragment of MetRS (MetRS_F) was detected by retention of ³⁵S on nitrocellulose filters following the cross-linking reaction, as previously described for the native enzyme (5). In

Figure 1. Reaction of [^{35}S] DTSP/PDA-tRNA^{fMet} with lysine residues in MetRS_F.

addition, coupling of MetRS_F to [^{35}S] DTSP/PDA-tRNA followed by cleavage of the cross-linked complex with DTT and SDS polyacrylamide gel electrophoresis yielded ^{35}S -labeled protein which comigrated with the 64,000 M_r monomeric synthetase (data not shown). Cross-linking was accompanied by loss of methionine acceptor activity of MetRS_F. Addition of an eight-fold excess of the modified tRNA resulted in complete inactivation of the enzyme in a 30 min incubation at 25°.

The cross-linked complex was isolated by gel filtration on Sephacryl S-200 following reaction of 3.45 μM MetRS_F with 11 μM [^{35}S] DTSP/PDA-tRNA^{fMet} in a total volume of 2 ml 20 mM Hepes, pH 8, 10 mM MgCl₂ at 25° for 30 min (Figure 2). The MetRS_F-tRNA^{fMet} complex (Peak I) eluted at a position intermediate between that of the corresponding covalent complex formed with native MetRS (indicated by an arrow on the figure) and the elution position of free tRNA (peak II).

Tryptic digestion of the covalent MetRS_F-tRNA^{fMet} complex and HPLC analysis of the cross-linked peptides

Peak I (Figure 2) was concentrated to 175 μl in a collodion bag and dialyzed vs. 0.1 M ammonium acetate, pH 6.5, and 0.1 mM CaCl₂. RNase-free trypsin was added to a concentration of 4% (w/w) trypsin/protein, and the sample was incubated at 25°C for 7 hours. The tRNA-bound ^{35}S -labeled peptides and excess free unreacted tRNA, were precipitated with ethanol. The pellet was dissolved in 1.2 ml of 7M urea, 50 mM sodium acetate, pH 6.5, and 0.1 M NaCl and chromatographed on a 7.5 x 75 mm HPLC ion exchange column of Bio-Gel TSK-DEAE 5PW as described before (7). The radioactive tRNA fraction was pooled, dialyzed, concentrated by evaporation in a siliconized tube, and precipitated with ethanol. The covalently bound peptides were released from the

Figure 2. Isolation of the cross-linked complex of MetRS_E and [³⁵S] DTSP/PDA-tRNA^{fMet} by gel filtration. MetRS_E (3.45 μM) and [³⁵S] DTSP/PDA-tRNA^{fMet} (11 μM) were incubated in 20 mM Hepes, pH 8, 10 mM MgCl₂ at 25° for 30 min. Glycine was added to a final concentration of 50 mM and the incubation at 25° continued for 30 min. The reaction mixture (2 ml) was applied to a 0.9 x 215 cm column of Sephacryl S-200 presaturated with bovine serum albumin and equilibrated with 0.2 M potassium phosphate, pH 6.5, 1 M NaCl at room temperature. The sample was eluted with the same buffer at a flow rate of 12 ml/hr. Fractions (1.5 ml) were collected and 10 μl aliquots were removed for measurement of radioactivity. The arrow on the figure marks the elution position of the corresponding covalent complex formed with native MetRS.

tRNA by incubation with 50 mM DTT at 37°C for 90 min, and the tRNA precipitated twice from 0.1 M sodium acetate, pH 6.5 with ethanol. The two supernatants containing the released peptides were combined, evaporated several times to a small volume to completely remove the ethanol, and adjusted to 6M guanidinium chloride, 50 mM sodium acetate pH 6.5, and 10 mM DTT. The peptides were applied to a 4.6 x 250 mm Altex-PTH-amino acid ODS reverse-phase HPLC column equipped with a 4.6 x 45 mm precolumn (Rainin) and equilibrated with 0.1% trifluoroacetic acid in water (v/v). Elution was carried out with a gradient of acetonitrile as described before (9), fractions were collected, and aliquots were taken for measurement of radioactivity. Three major ³⁵S-labeled cross-linked peptides were obtained (Figure 3). A portion of each peptide peak was mixed with the previously sequenced peptides cross-linked to native MetRS (7) and rechromatographed as described above. Peak 1 (Figure 3) was found to cochromatograph with a known heptapeptide containing Lys 439, peak 2 coeluted with a known octapeptide containing Lys 402, and peak 3 cochromatographed with a known hexadecapeptide containing Lys 465 (data not shown).

Figure 3. Reverse-phase chromatography of MetRS_F peptides cross-linked to [³⁵S] DTSP/PDA-tRNA^{Met}. The sample in 6M guanidinium chloride was injected onto a reverse-phase HPLC column equilibrated with 0.1% trifluoroacetic acid in water (v/v) and the peptides were eluted with a gradient of acetonitrile (9). One minute fractions were collected at a flow rate of 0.65 ml/min and aliquots (200 μ l) taken for measurement of ³⁵S cpm. Radioactivity eluting prior to fraction 60 is nonpeptide material derived by degradation of the cross-linker side chain released from the uncoupled tRNA (7).

Cross-linking of MetRS_F to free [³⁵S] DTSP

In order to compare the reactivity of lysine residues in MetRS_F with free cross-linker and tRNA-bound cross-linker, the monomeric enzyme was incubated with a 4-fold excess of free [³⁵S] DTSP under the same conditions used for the tRNA cross-linking

Figure 4. Reverse-phase chromatography of MetRS_F peptides cross-linked to free [³⁵S] DTSP. ³⁵S-Labeled tryptic peptides from reaction of free [³⁵S] DTSP with MetRS_F were chromatographed on a reverse-phase HPLC column as described in the legend to Figure 3.

experiments. Following the coupling reaction, the modified enzyme was incubated with DTT and then dialyzed three times vs. 0.1 M sodium acetate, pH 6.5, 0.1 mM CaCl_2 to remove unreacted cross-linker and free ^{35}S -labeled side chains released from the protein by the sulfhydryl reagent. The modified enzyme was digested with trypsin and the ^{35}S -labeled peptides chromatographed as described above. As seen in Figure 4, a complex mixture of products was obtained, with most of the peptides cross-linked to free [^{35}S] DTSP eluting only after a 70% acetonitrile wash of the column. Additional labeled peptides were also released by a subsequent wash with 100% acetonitrile. These data indicate that the bulk of the reaction with the free cross-linking reagent occurs at sites which are distinct from those modified by [^{35}S] DTSP/PDS-tRNA^{fMet}.

DISCUSSION

Cross-linking of DTSP/PDA-tRNA^{fMet} to native MetRS yields a 1:1 covalent complex (5), as expected from the known anticooperative tRNA binding properties of the enzyme (14). Sequencing of the cross-linked peptides has shown that covalent coupling occurs by reaction with five lysine residues in the carboxy-terminal half of the protein sequence (7). Three peptides, containing lysine residues 402, 439 and 596, are coupled to the D-loop of tRNA^{fMet} (9). A single peptide containing Lys 465 is cross-linked to the anticodon wobble base, and a single peptide containing Lys 640 is coupled to the 5' terminus (9).

Cross-linking of DTSP/PDA tRNA^{fMet} to dimeric MetRS could occur by reaction with lysine residues on the unoccupied subunit of the 1:1 tRNA-enzyme complex. The single peptide chain of MetRS_F binds one molecule of tRNA (15), requiring cross-linking to occur at the occupied binding site. Three of the lysine residues previously coupled to native MetRS are present in the primary sequence of the truncated monomeric enzyme. The data presented here show that DTSP/PDA-tRNA^{fMet} couples to these same lysine residues in MetRS_F. In addition, we have shown that free DTSP does not preferentially react with Lys 402, 439 and 465, but rather couples to many accessible amino groups on the surface of the protein, including many which yield large peptides on digestion with trypsin. Examination of the primary sequence of MetRS_F (16) shows that most of the potential cross-linked peptides containing more than 20 amino acids are found in the N-terminal half of the protein.

The present results allow previous cross-linking experiments to be interpreted in light of the known three-dimensional structures of the tRNA (17) and the protein fragment (4). The 2.5Å resolution map of MetRS_F reveals an elongated molecule (90Å x 52Å x 44Å) organized in a biglobular structure composed of N-terminal and C-terminal domains (4). The crystal structure has recently been refined to 1.8Å resolution and the three lysine residues cross-linked to MetRS_F have been located in the structure (5).

Brunie, personal communication). Lysine 465 is found at the extreme periphery of the molecule at a distance of 52Å from the active site. This indicates that the anticodon, containing nucleotide bases critical for recognition of the tRNA (19,20), is positioned at one end of the C-terminal domain, while the 3' terminus is directed towards the catalytic site in the N-terminal domain. Lysines 402 and 439 are located between the anticodon binding site and the active-site cleft. These residues are cross-linked to the D-loop of tRNA^{fMet} (9). This region of the tRNA does not make contacts with the enzyme which are important for substrate recognition (19). Earlier studies have shown, however, that Lys 402 is protected from acetylation by acetic anhydride following noncovalent binding of either the initiator or elongator methionine tRNAs to native MetRS (21), suggesting a direct or indirect effect of tRNA substrates on the accessibility of this lysine residue to solvent. The data presented here indicate that the D-loop of the bound tRNA is within 14Å (the length of the cross-linker side chain) of Lys 402 and Lys 439. Others have found that the periodate-oxidized 3' terminus of tRNA^{fMet} cross-links to Lys 335 and Lys 61, on each side of the active site cleft of the enzyme (22). All of these cross-linking sites provide landmarks for construction of a detailed model of the tRNA-synthetase interaction, and identify particular regions of the protein which may contain amino acid residues required for tRNA binding.

ACKNOWLEDGEMENTS

We are indebted to Simone Brunie for helpful discussions and communication of results prior to publication. We thank Codjo Hountondji, Sylvain Blanquet, and Paul Sigler for helpful suggestions on purification of MetRS_F. We also thank Rita Romita for expert typing. This work was supported by research grant GM-16995 from the National Institutes of Health.

REFERENCES

1. Koch, G.L.E. and Bruton, C.J. (1974) FEBS Letters **40**, 180-182.
2. Cassio, D. and Waller, J.P. (1971) Eur. J. Biochem. **20**, 283-300.
3. Waller, J.P., Risler, J.L., Monteilhet, C. and Zelwer, C. (1971) FEBS Letters **16**, 186-188.
4. Zelwer, C., Risler, J.L. and Brunie, S. (1982). J. Mol. Biol. **155**, 63-81.
5. Schulman, L.H., Valenzuela, D. and Pelka, H. (1981) Biochem. **20**, 6018-6023.
6. Valenzuela, D., Leon, O. and Schulman, L.H., (1984) Biochem. Biophys. Res. Commun. **119**, 677-684.
7. Valenzuela, D. and Schulman, L.H. (1986) Biochem. **25**, 4555-4561.
8. Leon, O. and Schulman, L.H. (1987) Biochem. **26**, 1933-1940.
9. Leon, O. and Schulman, L.H. (1987) Biochem. **26**, 5416-5422.
10. Leon, O. and Schulman, L.H. (1987) Biochem. **26**, in press.
11. Schulman, L.H. and Pelka, H. (1977) J. Biol. Chem. **252**, 814-819.
12. Lomant, A.J. and Fairbanks, G. (1976) J. Mol. Biol. **104**, 243-261.
13. Schulman, L.H., Pelka, H. and Reines, S. (1981) Nucleic Acids Res. **9**, 1203-1217.
14. Blanquet, S., Iwatsubo, M. and Waller, J.P. (1973) Eur. J. Biochem. **36**, 213-226.

15. Dessen, P., Fayat, G., Zaccari, G. and Blanquet, S. (1982) *J. Mol. Biol.* 154, 603-613.
16. Barker, D.G., Ebel, J.P., Jakes, R. and Bruton, C.J. (1982) *Eur. J. Biochem.* 127, 449-457.
17. Woo, N.H., Roe, B.A. and Rich, A. (1980) *Nature* 286, 346-351.
18. Risler, J.L., Zelwer, C. and Brunie, S. (1981) *Nature* 292, 383-384.
19. Schulman, L.H. and Pelka, H. (1977) *Biochem.* 16, 4256-4265.
20. Schulman, L.H. and Pelka, H. (1983) *Proc. Natl. Acad. Sci.* 80, 6755-6759.
21. Bruton, C.J. (1979) in *Nonsense Mutation and tRNA Suppressors*, Celis, J.E. and Smith, J.D. Eds., pp47-68, Academic Press, N.Y.
22. Hountondji, C., Blanquet, S. and Lederer, F. (1985) *Biochem.* 24, 1175-1180.