Identification of the tRNA Anticodon Recognition Site of *Escherichia coli* Methionyl-tRNA Synthetase†

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ABSTRACT: We have previously shown that the anticodon of methionine tRNAs contains most, if not all, of the nucleotides required for specific recognition of tRNA substrates by *Escherichia coli* methionyl-tRNA synthetase [Schulman, L. H., & Pelka, H. (1988) *Science 242*, 765–768]. Previous cross-linking experiments have also identified a site in the synthetase that lies within 14 Å of the anticodon binding domain [Leon, O., & Schulman, L. H. (1987) *Biochemistry 26*, 5416–5422]. In the present work, we have carried out site-directed mutagenesis of this domain, creating conservative amino acid changes at residues that contain side chains having potential hydrogen-bond donors or acceptors. Only one of these changes, converting Trp461 → Phe, had a significant effect on aminoacylation. The mutant enzyme showed an approximately 60–100-fold increase in K_m for methionine tRNAs, with little or no change in the K_m for methionine or ATP or in the maximal velocity of the aminoacylation reaction. Conversion of the adjacent Pro460 to Leu resulted in a smaller increase in K_m for $tRNA^{Met}$s, with no change in the other kinetic parameters. Examination of the interaction of the mutant enzymes with a series of $tRNA^{Met}$ derivatives containing base substitutions in the anticodon revealed sequence-specific interactions between the Phe461 mutant and different anticodons. K_m values were highest for $tRNA_m^{Met}$ derivatives containing the normal anticodon wobble base C. Base substitutions at this site decreased the K_m for aminoacylation by the Phe461 mutant, while increasing the K_m for the wild-type enzyme and for the Leu460 mutant to values greater than 100 μM. In addition, decreased K_m values were observed in aminoacylation of several noncognate tRNAs by the enzyme containing Phe461. Reductions in K_m's were accompanied by decreased turnover numbers, however, resulting in low overall efficiency of aminoacylation by the Phe461 derivative and indicating improper positioning of the bound tRNAs on the surface of the mutant enzyme. The data suggest that Trp461 may directly interact with the C of the methionine anticodon CAU and show that the presence of this amino acid effectively prevents interaction of the synthetase with tRNAs containing other nucleotides at the wobble position.

Elucidation of the molecular basis for the highly specific selection of tRNA substrates by aminoacyl-tRNA synthetases is an intriguing problem in RNA–protein recognition. Synthetases specific for each of the 20 amino acids encounter a pool of tRNAs in the cell having similar overall structures. Selection of the appropriate tRNAs for attachment of each amino acid occurs by formation of RNA–protein contacts unique to each cognate tRNA–synthetase pair. Recent studies from a number of laboratories have begun to reveal the nucleotide bases in tRNAs which specify their amino acid acceptor identity (Normanly & Abelson, 1989). Little is known, however, about the corresponding amino acid sequences in synthetases which determine the tRNA substrate specificity of these enzymes (Schimmel, 1987). The best-characterized interaction to date is that of *Escherichia coli* glutaminyl-tRNA synthetase (GlnRS)[1] and $tRNA^{Gln}$. Mutants of GlnRS containing single amino acid substitutions have been isolated which show relaxed tRNA specificity (Inokuchi et al., 1984; Hoben et al., 1984; Perona et al., 1989), and a high-resolution X-ray structure of the GlnRS–$tRNA^{Gln}$ complex has recently been solved which indicates a role for these and other specific amino acid residues in discrimination of tRNAs by this enzyme (Rould et al., 1989). Data on a new crystal form of yeast AspRS and $tRNA^{Asp}$ is also expected to yield details of the interaction of this tRNA–synthetase complex (Ruff et al., 1988). High-resolution crystal structures of two uncomplexed aminoacyl-tRNA synthetases have also been reported for *Bacillus stearothermophilus* TyrRS (Bhat et al., 1982) and for a biologically active proteolytic fragment of *E. coli* MetRS (Zelwer et al., 1982; Brunie et al., 1987). Site-directed mutagenesis of amino acids on the surface of the tyrosine enzyme has identified specific residues involved in binding $tRNA^{Tyr}$ and allowed modeling of the interaction of the active site of the enzyme with the tRNA acceptor arm (Bedouelle & Winter, 1986; Labouze & Bedouelle, 1989).

Native *E. coli* MetRS is a dimeric enzyme containing identical subunits of molecular weight 76000 (Koch & Bruton, 1974). Controlled proteolysis of the native enzyme releases amino acids from the carboxy terminus of each protomer and yields a monomeric fragment of molecular weight 64000 which retains full biological activity (Cassio & Waller, 1971). The crystal structure of this form of the enzyme complexed to ATP has recently been refined to 1.8-Å resolution (S. Brunie, personal communication). The crystal structures of two tRNA substrates of MetRS have also been determined (Woo et al., 1980; Schevitz et al., 1979; Basavappa and Sigler, personal communication); thus, three-dimensional structures suitable

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[1] Abbreviations: GlnRS, *Escherichia coli* glutaminyl-tRNA synthetase; MetRS, native, dimeric *E. coli* methionyl-tRNA synthetase; MetRS547, truncated, monomeric MetRS that terminates at amino acid 547; $tRNA^{fMet}$, *E. coli* initiator methionine tRNA; $tRNA_m^{Met}$, *E. coli* elongator methionine tRNA; $tRNA^{Thr}$(GGU), major *E. coli* threonine tRNA containing the anticodon GGU; $tRNA^{Val}$(UAC), major *E. coli* valine tRNA having the anticodon UAC; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate.

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for modeling the MetRS–tRNA^{Met} interaction are available. This is an especially attractive system for study since the nucleotides required for tRNA recognition by the enzyme have been elucidated and preliminary identification of the tRNA binding site on the protein has already been made. Earlier structure–function studies showed that specific recognition of methionine tRNAs by MetRS requires interaction of the enzyme with nucleotide bases in the anticodon sequence (Schulman & Goddard, 1973; Schulman et al., 1983; Schulman & Pelka, 1983, 1984, 1985; Pelka & Schulman, 1986). More recently, wild-type methionine acceptor activity has been conferred on a valine tRNA by substitution of the valine anticodon UAC by the methionine anticodon CAU (Schulman & Pelka, 1988), indicating that this sequence alone allows discrimination between cognate and noncognate tRNAs by MetRS. Cross-linking experiments have identified a site (Lys465) in both the native synthetase and the monomeric fragment which lies within 14 Å of the anticodon binding domain (Valenzuela & Schulman, 1986; Leon & Schulman, 1987; Schulman et al., 1987). In this paper, we describe the results of site-directed mutagenesis studies of this region of the protein to locate the amino acid residues involved in specific recognition of tRNAs by MetRS.

Experimental Procedures

Materials

Native tRNA^{fMet} was purchased from Boehringer-Mannheim. Other tRNAs were prepared by in vitro transcription as described below. [^{35}S]Methionine was purchased from Amersham and sodium [^{32}P]pyrophosphate from New England Nuclear. Deoxyoligonucleotides were synthesized on an Applied Biosystems DNA synthesizer. T7 RNA polymerase was purified from an overproducing strain (Davanloo et al., 1984). Other enzymes for plasmid constructions were purchased from New England Biolabs. M13K07 bacteriophage and *E. coli* strain RZ1032 were obtained from Jo Messing.

Methods

Construction of MetRS Mutants. We obtained the plasmid pBR EcoMTS-a5 encoding the N-terminal 564 amino acids of MetRS from David Barker (Barker et al., 1982). A 2.15-kb *Bam*HI fragment from this plasmid was subcloned in the *Bam*HI site of the phagemid vector pTZ18R (Pharmacia) with the endogenous promoter of the MetRS gene oriented opposite the *lac* promoter of the vector, to yield plasmid pGG3. Transformation of *E. coli* cells with this plasmid resulted in a 40–50-fold increase in MetRS activity in crude cell extracts. *E. coli* RZ1032 (*dut*$^-$ *ung*$^-$ F′) cells harboring pGG3 were infected with the helper phage M13K07 (Mead et al., 1986), to yield uracil-containing single-stranded DNA for high-efficiency oligonucleotide-directed mutagenesis by the method of Kunkel (1985). Positive clones were picked following transformation of *ung*$^+$ cells by direct DNA sequencing of the gene.

Purification of Enzymes. Wild-type MetRS547 and the Phe461 and Leu460 mutant enzymes were purified from overproducing strains carrying the corresponding MetRS genes in pGG3. Cells from overnight cultures grown in enriched medium were sonicated in 20 mM potassium phosphate, pH 7.0, 0.5 mM EDTA, and 0.1 mM PMSF and centrifuged (100000*g*). Crude extracts were adjusted to 0.2% polymin P and centrifuged, and the supernatant was extracted with ammonium sulfate. The 35–70% fraction was dialyzed and loaded onto a column of DEAE-Sepharose (Pharmacia) equilibrated with buffer A (20 mM Tris-HCl, pH 7.5, 0.1 mM PMSF, 10% glycerol, and 10 mM 2-mercaptoethanol) containing 20 mM KCl. The protein was eluted with the same buffer containing 80 mM KCl and further purified on a Mono-Q column using a gradient of 0–600 mM NaCl in 20 mM Tris-HCl, pH 8.1, 1 mM DTT, and 10% glycerol. The protein eluted at 350 mM NaCl, was concentrated, and was chromatographed on a Superose-12 column (Pharmacia) equilibrated with the same buffer minus NaCl in order to remove a small amount of contaminating native MetRS.

The Asn456 mutant of MetRS547 was expressed in *E. coli* strain BL21 from a T7 promoter plasmid by induction of a chromosomally integrated T7 RNA polymerase gene (Studier & Moffatt, 1986). The ammonium sulfate fractionated crude cell extract was step eluted from a DE-52 column with buffer A containing 100 mM KCl and further purified by chromatography on a G-100 column in buffer A, followed by gradient elution (20–250 mM KCl) from DE-52 in the same buffer and gradient elution from Bio-Gel HTP (Bio-Rad) in potassium phosphate, pH 6.8, 1 mM DTT, and 10% glycerol. The truncated protein eluted at 40–50 mM salt and was well separated from traces of native MetRS.

Enzyme Assays. Crude cell extracts from late-log cultures were prepared for assay of methionine acceptor activity as described above. Reaction mixtures (75–150 μL) contained 5–10 A_{260} of crude *E. coli* tRNA, 17 μM [^{35}S]methionine, 2 mM ATP, 4 mM free magnesium chloride, 150 mM ammonium chloride, 20 mM imidazole hydrochloride, pH 7.5, 0.1 mM EDTA, and various amounts of crude extract. Incubations were at 37 °C, and aliquots were withdrawn at various times for measurement of cold trichloroacetic acid insoluble radioactivity as described before (Schulman & Pelka, 1983). Activity was calculated from initial rates, which were linear with time and proportional to protein concentration.

Kinetic parameters for methionine acceptance by native tRNA^{fMet} were measured as described above with purified enzymes and 0.5–40 μM tRNA. K_m values for methionine were determined with 12–24 μM tRNA^{fMet}, 2 mM ATP, and 0.5–200 μM methionine. K_m values for ATP were determined with 17 μM methionine, 12–24 μM tRNA^{fMet}, and 10–1000 μM ATP. Kinetic parameters for the tRNA transcripts were determined as described for tRNA^{fMet} except that reaction mixtures contained 10 mM free $MgCl_2$ and 0.5–60 μM pure tRNA.

[^{32}P]PP$_i$–ATP isotopic exchange was assayed in reaction mixtures containing 2 mM methionine, 2 mM ATP, 7 mM $MgCl_2$, 20 mM imidazole hydrochloride, pH 7.5, 0.1 mM EDTA, 0.1 mg/mL BSA, and 2 mM sodium [^{32}P]pyrophosphate as described by Lawrence et al. (1973), except that the reaction was stopped by addition of aliquots to 200 mM EDTA–20 mM sodium pyrophosphate, pH 6.0, followed by filtration through glass fiber filter disks (Whatman GF/C) and washing with 30 mL of 10 mM sodium pyrophosphate, pH 6.0.

Kinetic constants were calculated from the nonlinear regression analysis program Enzfitter (Biosoft). When K_m values for aminoacylation of tRNAs were greater than 100 μM, individual kinetic parameters could not be accurately determined, and k_{cat}/K_m values were obtained from the slope of the linear plot of initial velocity vs tRNA concentration.

In Vitro Transcription. Synthetic tRNA genes having a T7 RNA polymerase promoter adjacent to the 5′ end and a *Bst*NI restriction enzyme site at the 3′ end (Sampson & Uhlenbeck, 1988) were constructed from overlapping deoxyoligonucleotides and inserted into the *Eco*RI/*Sst*I site of the phagemid vector pUC119 as described before (Schulman & Pelka, 1988). Plasmids linearized with *Bst*NI were transcribed

Table I: Aminoacylation Activity of MetRS547 Mutants[a]

Val_{455}-Asp-Glu-Gln-Ala-Pro_{460}-Trp-Val-Val-Ala-Lys_{465}-Gln-Glu-Gly-Arg-Asp_{470}-Ala-Asp[b]

position	amino acid: wild type	amino acid: mutant	rel act. mutant/WT × 100
456	Asp	Asn	50
457	Glu	Asp, Gln	100, 33
458	Gln	His	100
460	Pro	Leu	10
461	Trp	Phe	<5
465	Lys	Asn	50
467	Glu	Gln	100
469	Arg	Gln	33
470	Asp	Asn	50
472	Asp	Asn	50

[a] Crude extracts of cells overproducing wild-type and MetRS547 mutants were assayed for methionine acceptor activity as described under Experimental Procedures. Analysis of the extracts by SDS–polyacrylamide gel electrophoresis showed that most of the mutants were overproduced to a similar extent, and no correction was made for small variations in yield. The activity of the Asn456 mutant was determined as described under Methods. The activity of the Gln457 mutant was estimated from the apparent protein concentration in the crude extract. The activities of the other extracts were compared with that of MetRS547 and rounded off to the nearest-fold difference, e.g., none, 2-fold, 3-fold, etc. [b] The sequence of amino acids near Lys465 in MetRS547.

with purified T7 RNA polymerase and the transcripts purified by electrophoresis on denaturing polyacrylamide gels followed by HPLC chromatography (Schulman & Pelka, 1990).

In Vivo Complementation. *E. coli* strain LS50 was constructed from strain CS50 (*metG146*) by phage P1 mediated transduction of *recA*:*Tn*10, followed by episome transfer from JM101. The strain carries a defective chromosomal gene for MetRS encoding a protein that requires elevated concentrations of methionine for activity (Somerville & Ahmed, 1977). Wild-type and mutant MetRS547 genes on pGG3 were tested for their ability to complement strain LS50 by growth of the transformed cells in minimal medium in the absence of added methionine.

Results

Synthesis and Activity of Wild-Type and Mutant MetRS547. Site-directed mutagenesis was used to insert a UAA stop codon following Lys547 of the MetRS gene cloned in a phagemid pUC-type vector. Expression of the protein from the endogenous MetRS promoter of the altered gene yielded overproduction of a truncated protein, MetRS547, corresponding in length to the biologically active crystallized monomeric fragment of the methionine synthetase (Mellot et al., 1989). This protein could be readily distinguished from the native dimeric enzyme. Additional mutations were then introduced into the gene at sites encoding amino acids containing potential hydrogen-bonding side chains located within a distance of 14 Å from Lys465, the residue previously coupled to the anticodon of $tRNA^{fMet}$ via a cross-linker of this length (Leon & Schulman, 1987).

The methionine acceptor activity of crude extracts prepared from cells overproducing each MetRS547 derivative was determined in order to screen for defective synthetase mutants (Table I). Small (2–3-fold) or negligible changes in activity were observed for all of the mutations in the region between residues 456 and 472 except for a Trp → Phe substitution at residue 461, which resulted in a large loss of aminoacylation activity. An additional mutation at the adjacent Pro460 residue was then introduced and found to produce a smaller, but significant, loss of activity.

Table II: Kinetic Parameters for Aminoacylation of Native $tRNA^{fMet}$ by Wild-Type and Mutant MetRS547

enzyme	K_m (μM)	k_{cat} (s^{-1})	relative act.[a]	ΔG_{app} (kcal mol^{-1})[b]
wild-type MetRS547	1.2 ± 0.2	3.4 ± 0.2	100	
Trp461 → Phe	75.0 ± 15	4.3 ± 0.6	2	2.4
Pro460 → Leu	7.2 ± 1.2	3.3 ± 0.3	16	1.1

[a] Relative activity = $[(k_{cat}/K_m)_{mutant}/(k_{cat}/K_m)_{wild\ type}] \times 100$. [b] $\Delta G_{app} = -RT \ln [(k_{cat}/K_m)_{mutant}/(k_{cat}/K_m)_{wild\ type}]$ (Wilkinson et al., 1983).

Table III: K_m Values for Methionine and ATP in the Aminoacylation Reaction Catalyzed by Wild-Type and Mutant MetRS547

enzyme	K_m (μM): Met	K_m (μM): ATP
wild-type MetRS547	5.8 ± 1.6	607 ± 245
Trp461 → Phe	5.8 ± 1.3	435 ± 43
Pro460 → Leu	7.0 ± 1.8	458 ± 66

Characterization of the Kinetic Parameters for Aminoacylation of Native $tRNA^{fMet}$ by the Defective MetRS547 Mutants. The Phe461 and Leu460 MetRS547 mutants were purified in order to characterize the effect of the amino acid substitutions on enzyme activity. Table II compares the kinetic parameters for aminoacylation of *E. coli* $tRNA^{fMet}$ by the wild-type and mutant enzymes. A large increase in K_m for the tRNA was observed for the Phe461 mutant and a smaller increase for the Leu460 mutant, with no significant change in k_{cat} for either enzyme. Comparison of K_m values for methionine and ATP showed that the interaction of these substrates with the mutant and wild-type enzymes was the same (Table III). In addition, no change was observed in kinetic parameters of the ATP–PP_i exchange reaction catalyzed by the MetRS547 derivatives (data not shown), confirming that the mutations at positions 460 and 461 have little or no effect on the active site of the enzyme. These results show that Phe461 and Leu460 specifically reduce initial complex formation between MetRS547 and $tRNA^{fMet}$.

Effect of Anticodon Base Changes on Recognition of tRNAs by MetRS547 Mutants. In order to assess the effect of the amino acid substitutions on interaction of MetRS547 with tRNA anticodon nucleotides, a series of $tRNA_m^{Met}$ derivatives containing normal and altered anticodon sequences were prepared by in vitro transcription and assayed with the wild-type, Phe461, and Leu460 enzymes. The tRNA transcript containing the normal methionine anticodon CAU has previously been shown to be a good substrate for native MetRS (Schulman & Pelka, 1988) and was found here to exhibit kinetic parameters with MetRS547 which were the same as those observed with native $tRNA^{fMet}$ (Table IV). Alterations in the anticodon sequence of $tRNA_m^{Met}$ affected both the K_m and k_{cat} for aminoacylation by the truncated wild-type enzyme. Base changes at the wobble position of the anticodon produced the largest negative effect on interaction of the tRNA with MetRS547, increasing K_m values to immeasurably high levels (Table IV), in keeping with previous results obtained with native MetRS (Schulman & Pelka, 1983, 1984, 1985, 1988). In contrast, base changes at the wobble position were found to facilitate initial complex formation between the Phe461 mutant and $tRNA_m^{Met}$, as reflected by a decrease in K_m values for these tRNA derivatives (Table IV). G at the wobble position was more favorable than U, and the presence of an additional G residue in the anticodon GGU further reduced the K_m for aminoacylation by the Phe461 mutant. This behavior was not observed with the Leu460 mutant, which

Table IV: Kinetic Parameters for Aminoacylation of tRNA Transcripts by Wild-Type and Mutant MetRS547

tRNA	anti-codon	wild-type MetRS547		Trp461 → Phe		Pro460 → Leu		relative k_{cat}/K_m	
		K_m (μM)	k_{cat}/K_m (s^{-1} μM^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} μM^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} μM^{-1})	Trp461/ Phe	Pro460/ Leu
$tRNA_m^{Met}$	CAU	1.2 ± 0.2	2.7 ± 0.2	>100	$(1.8 \pm 0.1) \times 10^{-2}$	23 ± 10	0.14 ± 0.05	150	19
	CAG	63 ± 16	$(4.9 \pm 1.0) \times 10^{-3}$	>100	$(3.7 \pm 0.4) \times 10^{-5}$	>100	$(2.1 \pm 0.1) \times 10^{-4}$	132	23
	UAU	>100	$(2.2 \pm 0.2) \times 10^{-4}$	134 ± 56	$(1.6 \pm 0.5) \times 10^{-5}$	>100	$(5.2 \pm 0.5) \times 10^{-5}$	14	4
	GAU	>100	$(3.2 \pm 0.3) \times 10^{-5}$	27 ± 4	$(1.7 \pm 0.2) \times 10^{-5}$	>100	$(2.2 \pm 0.2) \times 10^{-5}$	2	1.5
	GGU	>100	$(4.1 \pm 0.8) \times 10^{-5}$	17 ± 1	$(7.6 \pm 0.4) \times 10^{-6}$	ND[a]		5	
$tRNA^{Thr}$	GGU	>100	$(7.1 \pm 1.5) \times 10^{-5}$	10 ± 4	$(3.4 \pm 0.8) \times 10^{-6}$	ND		21	
$tRNA^{Val}$	UAC	>100	$(4.6 \pm 0.5) \times 10^{-6}$	71 ± 34	$(7.7 \pm 2.9) \times 10^{-7}$	ND		6	

[a] ND = not determined.

showed immeasurably high K_m values with all of the non-methionine anticodon derivatives tested. Enhanced initial complex formation between tRNA substrates and the Phe461 mutant was accompanied by a decrease in k_{cat} for aminoacylation, however, resulting in lower k_{cat}/K_m values for all of the $tRNA_m^{Met}$ derivatives than those observed with wild-type MetRS547. The largest difference in substrate specificity (relative k_{cat}/K_m) between MetRS547 and the Phe461 mutant was observed with $tRNA_m^{Met}$ derivatives containing the normal C wobble base, suggesting a specific role for Trp461 in interaction with this nucleotide.

Specificity of Aminoacylation by the Phe461 Mutant of MetRS547. The interaction of two noncognate tRNAs, $tRNA^{Thr}$(GGU) and $tRNA^{Val}$(UAC), with MetRS547 and the Phe461 mutant was examined in detail. In vitro transcripts of the tRNAs were used in order to completely eliminate the low level of methionine tRNAs which contaminate preparations of native tRNAs isolated from cells. As expected, both tRNAs were poor substrates for the wild-type enzyme (Table IV). Kinetic parameters for aminoacylation of $tRNA^{Thr}$-(GGU) by the Phe461 mutant were similar to those for aminoacylation of $tRNA_m^{Met}$(GGU), indicating that the anticodon plays a dominant role in the enzyme–tRNA interaction and reduces both K_m and k_{cat}. K_m for aminoacylation of $tRNA^{Val}$(UAC) by Phe461 MetRS547 was also significantly lower than that observed with the wild-type enzyme, in keeping with the lower K_m seen on aminoacylation of $tRNA_m^{Met}$(UAU) by the mutant synthetase. As observed before, the low k_{cat} values exhibited by the Phe461 mutant prevented any significant mischarging of the noncognate tRNAs, despite the enhanced interaction with the non-methionine anticodons. In addition, aminoacylation of crude *E. coli* tRNA by high levels of the Phe461 mutant showed no mischarging above the low level exhibited by the wild-type synthetase, indicating no loss of specificity of the mutant enzyme (data not shown). Approximately 40-fold overproduction of the Phe461 derivative had no adverse effect on the growth of *E. coli* cells and allowed complementation of a strain of *E. coli* containing a defective chromosomal MetRS gene. These results further indicate that the Trp461 → Phe mutation leads only to reduced activity for aminoacylation of methionine tRNAs with no significant change in specificity.

Discussion

We have undertaken the present study in order to locate the amino acid residues in MetRS which specifically recognize the anticodon of methionine tRNAs. Previous cross-linking experiments indicated that the anticodon binding domain was located close to Lys465 (Valenzuela & Schulman, 1986; Leon & Schulman, 1987), near the extreme periphery of the MetRS molecule at a maximal distance from the active site (Brunie et al., 1987). Previous structure–function studies on methionine tRNAs had suggested that specific hydrogen bonds were likely to be formed between the protein and functional groups on each of the anticodon nucleotide bases, with the dominant interaction at the C wobble base (Schulman et al., 1983; Schulman & Pelka, 1983, 1984, 1985, 1988). We therefore altered hydrophilic amino acid residues close to Lys465 by site-directed mutagenesis and screened for proteins defective in aminoacylation. Lys465 itself was not expected to play a major role in anticodon recognition since reaction with the cross-linker leaves a covalently modified lysine residue on release of the tRNA but has little effect on enzyme activity (Schulman et al., 1981). More surprising was the small effect of substitutions at the majority of the hydrophilic residues surrounding Lys465. Only one change, Trp461 → Phe, significantly altered aminoacylation of $tRNA^{fMet}$. The data show that the effect of this substitution is only on initial complex formation between the protein and the tRNA. The maximal rate of methionine acceptance remains unchanged, suggesting that specific contacts with the anticodon are lost but no overall change in orientation of the two molecules has occurred, leaving the 3′ end of the tRNA correctly positioned to receive the activated methionine in the active site of the enzyme. In contrast, base changes in the anticodon of methionine tRNAs affect both K_m and k_{cat} for aminoacylation by MetRS [Schulman and Pelka (1985, 1988, 1989, 1990) and Table IV]. This is likely to result from loss of specific contacts with anticodon bases and improper fit induced by negative contacts with the altered anticodon sequence, leading to incorrect positioning of the 3′ terminus at the active site. Mellot et al. (1989) have shown that sequences close to the active site of MetRS play an important role in tRNA complex formation. Transfer RNAs which are efficient substrates of MetRS do not have uniquely conserved nucleotides near the 3′ end of the tRNA, which would allow specific recognition of this region of the molecule; however, proper binding to the anticodon nucleotides could lead to unique contacts between the protein and the acceptor end of methionine tRNAs.

It is clear that Trp461 plays a major role in recognition of tRNAs by MetRS since a conservative amino acid substitution at this site dramatically increases the K_m for aminoacylation of methionine tRNAs. The data suggest that Trp461 stabilizes the interaction with the methionine anticodon, while blocking binding to non-methionine anticodons. The results further suggest that the interaction between Trp461 and the tRNA occurs at the 5′ end of the anticodon, possibly directly with the C wobble base. Substitution of this base with a G residue dramatically increases K_m for the wild-type enzyme containing Trp461 but decreases K_m for the corresponding Phe461 mutant. In contrast, substitution of a G residue at the 3′ end of the anticodon fails to facilitate initial complex formation with the mutant enzyme and has a lesser effect on the wild-type synthetase. The N3 position of the anticodon cytidine base has previously been shown to be protected from methylation by binding to MetRS (Pelka & Schulman, 1986). This ring nitrogen and the exocyclic NH_2 at C4 are the only functional groups which differ between C and U, yet U at the wobble

position has a dramatic negative effect on interaction of the tRNA with MetRS. Substitution of an acetyl group at the C4 position of cytidine has no effect on the K_m for aminoacylation (Stern & Schulman, 1977), suggesting that this functional group may be oriented away from the protein. We suggest that Trp461 may make a direct hydrogen bond with the ring nitrogen of the C wobble base. Such an interaction would be excluded by substitution of U at this site and be replaced by a negative interaction with the N–H group present at the corresponding position in uridine. The presence of Trp461 also strongly excludes anticodons containing G or A at the wobble position [Table IV and Schulman and Pelka (1983)]. The apparent change in free energy on substitution of Phe461 for Trp461 is consistent with formation of a single strong hydrogen bond (Table II). Conversion of Pro460 to Leu460 is expected to result in some structural alteration of the α-helix containing Trp461 and may alter the position of this amino acid residue, increasing K_m to a lesser extent.

Tryptophan has previously been implicated in binding of proteins to single-stranded regions of nucleic acids (Helene & Lancelot, 1982; Chase & Williams, 1986; Casas-Finet et al., 1988). Stacking of the aromatic side chain between nucleotide bases has been postulated to be involved in the nonspecific interaction of nucleic acids with single-strand binding proteins such as *E. coli* SSB and bacteriophage T4 gene 32 protein (Casas-Finet et al., 1987; Khamis et al., 1987; Prigodich et al., 1984). Stacking or partial stacking may also play a role in interaction of Trp461 with the methionine anticodon. Intercalation of phenylalanine residues between nucleic acid bases has also been reported (O'Connor & Coleman, 1983; Chase & Williams, 1986; Prigodich et al., 1984). Substitution of Phe for Trp461 eliminates the possibility of hydrogen bonding between the amino acid side chain and an anticodon base yet enhances interaction of MetRS547 with non-methionine anticodons, particularly those containing G at the wobble position. This indicates that negative interactions with G and U are weaker in the mutant enzyme and suggests that the lack of hydrogen bonding may facilitate intercalation of Phe between the anticodon nucleotides. Any energy gained through this type of hydrophobic interaction does not result in increased methionine acceptance by tRNAs containing non-methionine anticodons, however, presumably because the interaction interfers with proper positioning of the tRNAs on the enzyme surface, greatly reducing the maximal rate of aminoacylation. It is possible that second-site mutations in the Phe461 enzyme might allow aminoacylation of anticodon wobble base derivatives of methionine tRNAs, or of certain noncognate tRNAs such as $tRNA^{Thr}$(GGU), and provide additional clues as to the mechanism of selection of tRNA substrates by MetRS.

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Articles

Cytosolic Factors in Bovine Neutrophil Oxidase Activation. Partial Purification and Demonstration of Translocation to a Membrane Fraction†

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ABSTRACT: The $O_2^{\cdot-}$-generating oxidase of bovine neutrophils is activated in a cell-free system consisting of a particulate fraction enriched in plasma membrane and containing the dormant oxidase, a high-speed supernatant from neutrophil homogenate (cytosol), Mg ions, GTPγS, and arachidonic acid [Ligeti, E., Doussiere, J., & Vignais, P. V. (1988) *Biochemistry 27*, 193–200]. The cytosolic components participating in the activation of the membrane-bound oxidase have been investigated. These components were resolved into several active peaks by Q Sepharose chromatography. The oxidase-activating potency of these peaks was synergistically enhanced by combining samples from separate peaks, or by supplying them with a threshold amount of crude cytosol. Partial purification of two active fractions containing a limited number of proteins of 65, 56, 53, and 45 kDa was achieved by gel filtration of cytosol on Ultrogel AcA44, followed by chromatography on hydroxylapatite and Mono Q. The specific oxidase-activating potency of these partially purified fractions (activating potency per milligram of soluble protein) was 6–8-fold higher than that of crude cytosol; it was enhanced up to 75-fold by complementation with a minute amount of crude cytosol, which per se had a limited efficiency. These data indicate that oxidase activation requires more than one cytosolic component to be activated. To check whether translocation of cytosolic proteins to the membrane occurred concomitantly with oxidase activation, use was made of radiolabeled cytosolic proteins. Cytosol was treated with phenyl[^{14}C]isothiocyanate ([^{14}C]PITC], such that 60% of its activation potency was still present. Translocation was studied under conditions in which production of $O_2^{\cdot-}$ was largely modulated by varying the amount of arachidonic acid added to the cell-free system. Maximal oxidase activation with optimal concentration of arachidonic acid resulted in the selective translocation of labeled cytosolic proteins of 65, 53, 45, and 17 kDa to the membrane.

The plasma membrane bound oxidase of neutrophils, which is responsible for the production of large quantities of superoxide (O_2^-) during phagocytosis, is dormant in circulating neutrophils. It becomes activated upon binding of particulate or soluble ligands to specific receptors of the plasma membrane [see Rossi (1986) and Bellavite (1988)]. Elaboration of a cell-free system for oxidase activation in guinea pig macrophages (Bromberg & Pick, 1984, 1985) and in neutrophils from different species (Heyneman & Vercauteren, 1984; Curnutte, 1985; Mc Phail et al., 1985; Clark et al., 1987; Gabig et al., 1987; Seifert & Schultz, 1987; Ligeti et al., 1988; Tanaka et al., 1988) has led to the conclusion that a cytosolic factor of protein nature is required for activation of the membrane-bound oxidase. The search for the isolation and identification of this factor has been intensive. So far, attempts to purify this cytosolic factor by size-exclusion chromatography (Gabig et al., 1987; Curnutte et al., 1987; Fujita et al., 1987; Ishida et al., 1989) and affinity chromatography on 2′,5′-ADP–agarose (Sha'ag & Pick, 1988) or GTP–agarose (Volpp et al., 1988) have resulted in the recovery of fractions only partially enriched in cytosolic factor activity. It was recently recognized that neutrophils of patients suffering from an autosomal recessive form of chronic granulomatous disease (CGD)[1] are deficient in cytosolic proteins (Curnutte et al.,

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