Lewis, E. N., Bittman, R., & Levin, I. W. (1986) *Biochim. Biophys. Acta 861*, 44-52.
McMurchie, E. J. (1988) in *Advances in Membrane Fluidity, Vol. 3, Physiological Regulation of Membrane Fluidity* (Aloia, R. C., Curtain, C. C., & Gordon, L. M., Eds.) pp 189-237, Alan, R. Liss, New York.
Mendelsohn, R., & Koch, C. C. (1980) *Biochim. Biophys. Acta 598*, 260-271.
Miljanich, G. P., Sklar, L. A., White, D. L., & Dratz, E. A. (1979) *Biochim. Biophys. Acta 552*, 294-306.
Mitchell, D. C., Straume, M., Miller, J. L., & Litman, B. J. (1990) *Biochemistry 29*, 9143-9149.
Mushayakarara, E., Albon, N., & Levin, I. W. (1982) *Biochim. Biophys. Acta 686*, 153-159.
Nielsen, N. C., Fleischer, S., & McConnell, D. G. (1970) *Biochim. Biophys. Acta 211*, 10-19.
Poincelot, R. P., & Abrahamson, E. W. (1970) *Biochemistry 9*, 1820-1825.
Seelig, A., & Seelig, J. (1977) *Biochemistry 16*, 47-50.
Snyder, R. G., & Schachtschneider, J. B. (1963) *Spectrochim. Acta 19*, 85-116.
Snyder, R. G., & Scherer, J. R. (1979) *J. Chem. Phys. 71*, 3221-3228.
Snyder, R. G., & Strauss, H. L. (1982) *J. Phys. Chem. 86*, 5145-5150.
Snyder, R. G., Hsu, S. L., & Krimm, S. (1978) *Spectrochim. Acta 34A*, 395-406.
Straume, M., & Litman, B. J. (1987) *Biochemistry 26*, 5113-5120.
Straume, M., & Litman, B. J. (1988) *Biochemistry 27*, 7723-7733.
Thompson, T. E., & Huang, C. (1986) in *Physiology of Membrane Disorder*, 2nd ed. (Andrioli, T. E., Hoffman, J. F., Fanestil, D. D., & Schultz, S. G., Eds.) pp 25-44, Plenum, New York.
White, D. A. (1973) in *Phospholipid Composition of Mammalian Tissues* (Ansell, G. B., Hawthorne, J. N., & Dawson, R. M. C., Eds.) p 441, Elsevier Scientific, New York.

Assembly of a Class I tRNA Synthetase from Products of an Artificially Split Gene†

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ABSTRACT: The aminoacyl-tRNA synthetases arose early in evolution and established the rules of the genetic code through their specific interactions with amino acids and RNA molecules. About half of these tRNA charging enzymes are class I synthetases, which contain similar N-terminal nucleotide-fold-like structures that are joined to variable domains implicated in specific protein–tRNA contacts. Here, we show that a bacterial synthetase gene can be split into two nonoverlapping segments. We split the gene for *Escherichia coli* methionyl-tRNA synthetase (a class I synthetase) at several sites near the interdomain junction, such that one segment codes for the nucleotide-fold-containing domain and the other provides determinants for tRNA recognition. When the segments are folded together, they can recognize and charge tRNA, both in vivo and in vitro. We postulate that an early step in the assembly of systems to attach amino acids to specific RNA molecules may have involved specific interactions between discrete proteins that is reflected in the interdomain contacts of modern synthetases.

Because of their role in interpreting the genetic code, the aminoacyl-tRNA synthetases were among the earliest proteins (Rich, 1962; Woese, 1970). The three-dimensional structures of three class I enzymes, the tyrosyl- (from *Bacillus stearothermophilus*; Bhat et al., 1982; Blow & Brick, 1985), methionyl- (from *Escherichia coli*; Brunie et al., 1990), and glutaminyl-tRNA synthetases (from *E. coli*; Rould et al., 1989), show that these enzymes adopt similar N-terminal nucleotide-fold motifs, even though their primary sequences show only limited similarity (Schimmel & Söll, 1979; Schimmel, 1987). Structural modeling and sequence comparisons suggest a similar N-terminal nucleotide fold for the remaining class I synthetases (Burbaum et al., 1990). [In contrast, the class II synthetases contain no nucleotide binding fold (Eriani et al., 1990; Cusack et al., 1990).] In the class I synthetases, the site of amino acid binding and activation is found within the nucleotide binding fold (Brick & Blow, 1987), and as shown in the cocrystal of $tRNA^{Gln}$ with the glutaminyl-tRNA synthetase (Rould et al., 1989), the 3′-end of the tRNA substrate can be docked near the active site through interactions with a structural element that is inserted into this fold. In contrast, interactions with other portions of bound tRNA are facilitated through separate domains that are structurally unrelated to one another.

E. coli methionyl-tRNA synthetase is an α_2 dimer containing identical polypeptides of 676 amino acids (Dardel et al., 1984). Limited proteolysis generates an active monomeric fragment of about 550 amino acids (Cassio & Waller, 1971), whose crystal structure has been reported (Brunie et al., 1990). The N-terminal domain (360 amino acids) of the monomer is organized as a nucleotide fold with alternating α-helices and β-strands, while the C-terminal domain ($\sim$190 amino acids)

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is predominantly α-helical. The anticodon of the tRNA substrate interacts with this helical region, where Trp461[1] plays a critical role in recognition (Ghosh et al., 1990), while the reactive 3′-end is docked into the nucleotide fold some 75 Å away (Brunie et al., 1990; Hountondji et al., 1985). To accommodate these geometrical requirements, the tRNA must bridge the two domains, with its 3′-end docked into the nucleotide fold where it can be positioned to react with an activated amino acid.

Even though these structural domains seem apparent, they are not released jointly by conventional proteolysis experiments (Cassio & Waller, 1971). The failure of these experiments raised doubts that the structural domains in methionyl-tRNA synthetase could be separated or recombined, despite their apparent three-dimensional segregation. These results contrast with other reconstitution experiments for many diverse proteins (Wetlaufer & Ristow, 1973), including ribonuclease A (Kato & Anfinsen, 1969, Richards & Wyckoff, 1971), staphylococcal nuclease (Anfinsen et al., 1971), cytochrome b_5 reductase (Strittmatter et al., 1972), human pituitary growth hormone (Li & Bewley, 1976), thioredoxin C (Holmgren & Slaby, 1979), adenylate kinase (Saint Girons et al., 1987), and alanine racemase (Galakatos & Walsh, 1987). Because of ongoing interest in the evolution and assembly of coding systems in general and aminoacyl-tRNA synthetases in particular, we have pursued a rational, genetic approach to split a protein as it is being translated. This approach involves the introduction of a translation stop and restart signal at variable positions within the *metG* mRNA, resulting in a protein that is split into two pieces during its synthesis.

Experimental Procedures

Materials. Oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer in the MIT Center for Protein Structure and Engineering. [^{35}S]Methionine was obtained from Amersham, and tRNA^{fMet} was obtained fom Boehringer Mannheim Biochemicals. The background strain for the preparation of all protein extracts was MJR [K12 1EA *metG*(K_m)*rec*A$^-$] (Starzyk et al., 1989), where the chromosomal *metG* allele confers methionine auxotrophy because the K_m for methionine of the encoded enzyme is elevated about 350-fold (Barker et al., 1982).

Insertional Mutagenesis. Mutagenesis was performed with the Amersham Eckstein mutagenesis kit. Insertion of a 12-nucleotide sequence from *glyS*, consisting of a stop codon (TAA), a ribosome binding (Shine-Dalgarno) sequence (Keng et al., 1982), and an ATG start codon was accomplished by use of oligonucleotides of 45–49 residues on the single-stranded form of a phagemid (pJB103), which contains a full-length *metG* inserted between the *Kpn*I and *Sac*I sites of pBlueScript KS(+) (Stratagene, Inc.), under the control of the endogenous *lac* promoter. (Further details are available upon request.) Screening for mutant genes was achieved by direct sequencing of the single-stranded form of the phagemid, with Sequenase (U.S. Biochemicals). Mutations were obtained at 50–65% efficiency.

Western Blotting. French press lysates in 50 mM potassium phosphate (pH 7.5) containing 50 mM 2-mercaptoethanol were prepared, and the protein concentration of each extract was measured (Bradford, 1976). Proteins were separated on a 12% SDS–PAGE gel and then transferred to Immobilon PVDF membranes with a Milliblot-SDE apparatus according to the directions of the manufacturer (Millipore). Antiserum against methionyl-tRNA synthetase was raised in rabbits immunized with purified truncated *E. coli* methionyl-tRNA synthetase [a kind gift of P. Rosevear, purified by the method of Rosevear (1988)]. Visualization of methionyl-tRNA synthetase derived bands was achieved with ^{125}I-protein A, followed by autoradiography.

Activity Measurements. Complementation was tested in the host strain MJR, with positives scored by growth on M9 glucose media. Charging activity was measured by a modification of published methods (Schulman & Pelka, 1983). (Further details are available upon request.)

Purification of Wild-Type and Split Methionyl-tRNA Synthetases. The cells from a 3-L culture of strain MJR (containing the plasmid of interest), harvested in late-log phase (approximately 18 h), were washed once with sterile 0.85% NaCl equilibrated to 4 °C and then lysed (French press, 15000 psi) in 40 mL of potassium phosphate buffer (50 mM, pH 7.5) containing 0.1 M NaCl and 50 mM 2-mercaptoethanol. Phenylmethanesulfonyl fluoride was added to the lysate, and the extract was clarified by centrifugation. This extract was fractioned on a MonoQ HR10/10 column (Pharmacia), with a linear gradient (0–350 mM, 135 mL × 135 mL) of NaCl in Tris-HCl (25 mM, pH 7.5) containing 1 mM 2-mercaptoethanol. Fractions were assayed by Western blot and enzymatic activity, and methionyl-tRNA synthetase was found to elute at about 125 mM salt. Active fractions were concentrated and then fractionated on a Superose-12 column (Pharmacia) equilibrated in Tris-HCl (25 mM, pH 7.5) containing 1 mM 2-mercaptoethanol and 1 M NaCl.

N-Terminal Sequence Determination. The protein from pJB103-R367//M[2] was taken through the above purification, fractionated on a 12% SDS–PAGE gel, and transferred to a PVDF membrane. The N-terminal sequence of both fragments was determined in the protein microsequenator facility of the Whitehead Institute for Biomedical Research, following Matsudaira (1987).

Expression and Partial Purification of α and β Fragments. The α fragment was expressed from a plasmid from which all but the first four codons of the βR367//M segment had been deleted. The β fragment was expressed from a plasmid from which the AUG initiator codon and 277 codons from the αR367//M segment had been deleted. [Further details of these constructions are available upon request.] Extracts, prepared as described above, were equilibrated to 4 °C and then fractionated by addition of solid $(NH_4)_2SO_4$.

Reconstitution of Methionyl-tRNA Synthetase Activity. Extracts were fractionated as above, and the pellets from αR367//M (insoluble) and βR367//M [30–40% $(NH_4)_2SO_4$] were redissolved in 6 M guanidinium chloride–50 mM sodium phosphate (pH 7) containing 0.5 mM dithiothreitol, 30 μM $ZnCl_2$, and 10 μM EDTA. Protein refolding was initiated by rapid dilution (10 μL → 1 mL) into the same buffer, containing no guanidinium chloride, with 1 mM ATP and 1 mM $MgCl_2$ added. Refolding was allowed to proceed for 2 h at 25 °C, and the insoluble material was removed by passage through a Millex-GV membrane. The filtrate was concen-

[1] Abbreviations: Trp, tryptophan; Lys, lysine; A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isolcucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, (ethylenedinitrilo)tetraacetate; ATP, adenosine 5′-triphosphate.

[2] This nomenclature, designating the absence of a covalent bond between the segments with "//", is adapted from Knowles (1987).

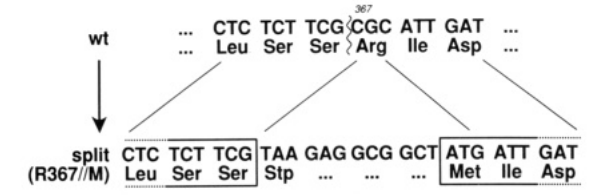


FIGURE 1: Scheme for construction of split genes.

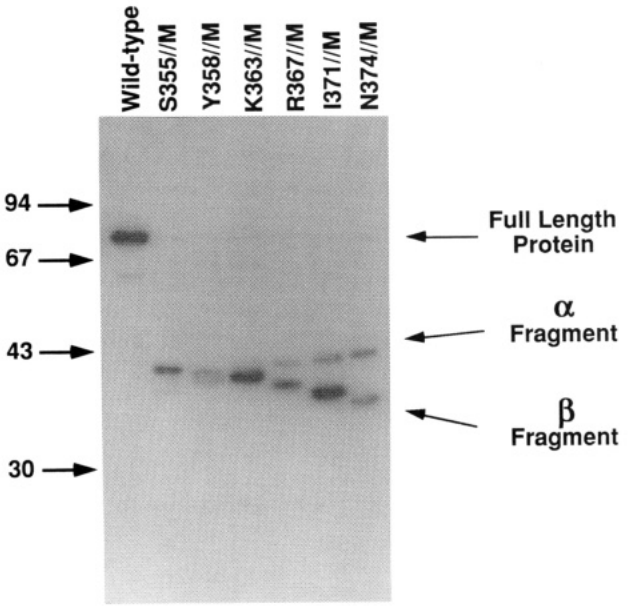


FIGURE 2: Western blot of protein extracts resolved by 12% SDS–PAGE. The identities of the N-terminal (α) and C-terminal (β) fragments are apparent from their sizes: As the location of the split point moves along the gene, the α fragment increases and the β fragment decreases in size.

trated to 50 μL in a Centricon-30 microconcentrator and then assayed for activity.

Results and Discussion

We split the coding sequence of cloned *metG* into sequences that code for two separate polypeptide chains by inserting just 12 nucleotides derived from *glyS*. Initially, to allow for the possibility that the two chains may need to be synthesized near one another to fold into an active conformation, the two domains of the protein were expressed in tandem from the same mRNA. For this purpose, a 15-nucleotide sequence (the intersubunit region) from the gene for *E. coli* glycyl-tRNA synthetase (Keng et al., 1982; Toth & Schimmel, 1986), which consists of a TAA stop codon, followed by a 12-nucleotide sequence that includes a ribosome-binding site and a start codon, was inserted by oligonucleotide-directed mutagenesis (Figure 1). To express the domains of the enzyme separately, we targeted our insertions to the methionyl-tRNA synthetase interdomain region, which extends from the last β-strand of the nucleotide fold to within the first α-helix of the helical domain. Specifically, we inserted this sequence at six sites in the interdomain region, to replace the codons for S355, Y358, K363, R367, I371, and N374, respectively. For each of these constructions, the first amino acid of the C-terminal segment was changed to methionine, so that the designation of the protein split at S355, for example, is S355//M.

Expression of the proteins from these split genes gave an N-terminal (α) fragment and a C-terminal (β) fragment which could be distinguished on Western blots of SDS–polyacrylamide gels with polyclonal antibodies against methionyl-tRNA synthetase (Figure 2). In all cases, the β fragment is observed, but the signal from the α fragment is either weak or nonexistent when the split is at S355, Y358, or K363. To confirm our assignments, we purified the R367//M mutant and sequenced both the upper (α) band and lower (β) bands. The α band has the same N-terminal sequence as wild-type methionyl-tRNA synthetase (TQVA...) while the β band has a sequence consistent with the retention of methionine (MIDDIDL...). This methionine is unblocked, as demonstrated by its ability to be cleaved in the Edman degradation.

In the three cases where two stable fragments are observed (R367//M, I371//M, and N374//M), the split proteins complement the chromosomal defect (Figure 4). Complementation does not depend on dimerization of the enzyme, because introduction of the R367//M mutation into the monomeric protein also complements the *metG*(K_m) allele (Figure 3). [The monomer was constructed by introducing tandem stop codons after Lys548 in the cloned gene, which as been shown to remove the dimerization domain and result in a monomeric synthetase (Mellot et al., 1989).] In Western blots, this shorter protein gives the same α fragment but a shorter β' fragment (data not shown), as expected.

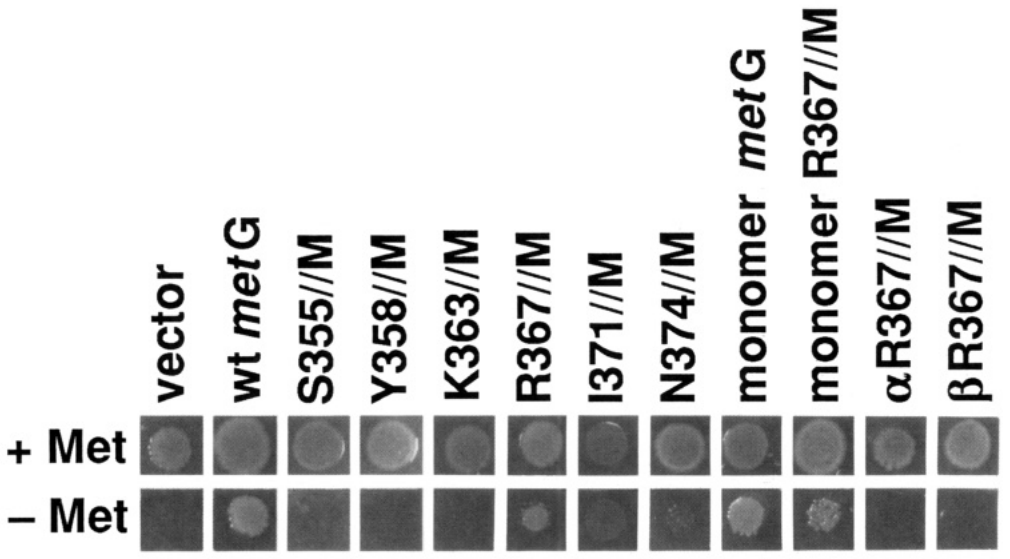


FIGURE 3: Growth of strain MJR containing plasmids that encode split versions of methionyl-tRNA synthetase. Top row: Growth on M9 glucose containing 20 μg/mL methionine. Bottom row: Growth on M9 glucose in the absence of methionine. Under these growth conditions, strain MJR pJB103-N374//M grows slowly.

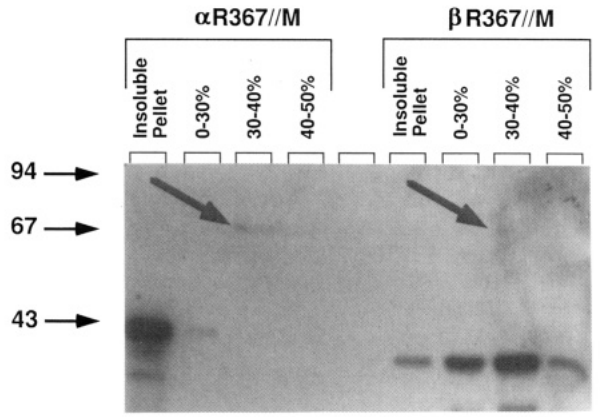
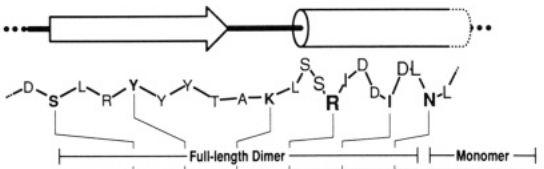


FIGURE 4: Fractionation of separately expressed α and β fragments. Arrows on the autoradiogram indicate the position of the *metG*(K_m) synthetase, which is precipitated in the 30–40% $(NH_4)_2SO_4$ fraction.

Table I: Correlation of in Vivo and in Vitro Properties of Split Methionyl-tRNA Synthetase Mutants



Junction at Residue No.[a]	–	S355	Y358	K363	R367	I371	N374	–	R367
Full-length Protein	+	–	–	–	–	–	–	+	–
N-terminal (α)[b] Fragment	–	–	(+)	(+)	+	+	+	–	+
C-terminal (β) Fragment	–	+	+	+	+	+	+	–	+
Complementation (in vivo)	+	–	–	–	+	+	+	+	+
Enzymatic Activity[c] (in vitro)	100	0	0	0	31	38	12	400	42

[a] Numbering of the amino acids begins with the initiator methionine. [b] The protein species detected on the Western blot of Figure 3 is assigned + = clear, strong signal, – = no signal observed, and + in parentheses = signal near the limits of detection. [c] Units of activity are given relative to the wild-type extract = 100 and are normalized to constant protein concentration.

Split genes that complement the *metG*(K_m) defect also raise the level of methionyl-tRNA synthetase activity in extracts (Table I), with specific activities in the R367//M, I371//M, and N374//M constructs from 12 to 38% of similarly prepared wild-type extracts. In contrast, split genes that fail to complement the *metG*(K_m) have no methionyl-tRNA synthetase activity and are missing or have reduced amounts of at least one of the fragments. Possibly, the substitution of methionine for one of the amino acids may also be a factor that prevents complementation and/or reduces protein stability. We infer from this correlation that complementation results from the combination of the two plasmid-encoded fragments to form an active enzyme.

We selected the mutant R367//M for further study, because the *metG*(K_m) strain with the plasmid-encoded mutant grows well and the split protein is stable enough to be isolated. A purification procedure identical with that for the wild-type enzyme was effective, demonstrating that the physical properties of the split and wild-type proteins are similar: Both proteins elute at the same ionic strength (250 mM NaCl) upon ion-exchange chromatography, and both have identical elution volumes on nondenaturing gel-filtration chromatography. Thus, it is likely that the split and unsplit proteins have a comparable distribution of surface charges and a roughly similar size and shape. The split Met-tRNA synthetase (R367//M) thus appears to be an $\alpha_2\beta_2$ tetramer that contains both the α and β polypeptides. Kinetic studies of both split and wild-type proteins, purified in parallel, uncovered only small differences in catalytic parameters [for the split versus the wild-type enzyme (shown in parentheses): K_m^{app}(Met) = 50 μM (40 μM); K_m^{app}(ATP) = 0.8 mM (0.2 mM); K_m^{app}($tRNA^{fMet}$) = 8 μM (7 μM)]. [Because of the small differences in the specific activities of crude extracts (Table I) and in the Michaelis constants (above), large differences in k_{cat} are unlikely.] Other than the quaternary structure differences engineered into the enzyme, the split protein appears to differ little from wild type in either physical or catalytic properties.

To investigate the properties of the separate domains of R367//M, the components of the split protein [αR367//M and βR367//M] were expressed separately. Steady-state levels of expression of each fragment, as judged by Western blot analysis, were comparable to that of the parent split protein (data not shown). As expected, neither the α nor the β fragment alone confers complementation (Figure 3). Combinations of the separately expressed components in crude extracts, however, also gave no enhancement of enzymatic activity.

Ammonium sulfate fractionation of extracts (Figure 4) showed that the αR367//M fragment is localized to the insoluble pellet after lysis, while the βR367//M fragment precipitates between 30 and 40% $(NH_4)_2SO_4$ and cofractionates with the full-length *metG*(K_m) enzyme. Thus, the α fragment is activated and solubilized when coexpressed with the β fragment. We conclude that, unlike other protein fragments (Wetlaufer, 1981), the α fragment does not fold properly when expressed alone. Sequences in the C-terminal domain appear to stabilize the nucleotide-fold conformation of the α domain of the wild-type protein and may be necessary to generate an enzymatically active species. This conformational instability may partly explain why C-terminal deletions in *E. coli* methionyl-tRNA synthetase, unlike other tRNA synthetases, are defective in both charging and adenylate synthesis (Mellot et al., 1989). It would be interesting to extend these studies to the enzyme from the thermophile *Thermus thermophilus* HB8, which has been investigated by limited proteolysis in vitro (Kohda et al., 1987).

To generate an active species in vitro, the separately expressed components of R367//M may need to fold together, as in the refolding of naturally occurring oligomeric proteins (Fischer & Schmid, 1990; Taniuchi et al., 1986; Jaenicke & Rudolph, 1986; Gerschitz et al., 1978; Gerschitz & Jaenicke, 1978). To test this hypothesis, the two enriched fractions, the αR367//M insoluble pellet and the βR367//M 30–40% $(NH_4)_2SO_4$ fraction, were dissolved and unfolded in 6 M guanidinium chloride. The proteins, both together and separately, were refolded under conditions that had been optimized for reconstitution of the activity of the purified, full-length protein (see Experimental Procedures). Only when both proteins were present in an unfolded form could enzymatic activity be reconstituted (Figure 5). Under these conditions, the reconstitution yield (based on the soluble β fragment) was less than 1% of the theoretical maximum, because most of the α fragment becomes insoluble during these experiments. Nevertheless, the background activity in these experiments is quite low, and we conclude that the observed enzymatic activity is the result of interaction of the inactive polypeptides.

The results establish that noncovalent packing interactions alone are sufficient to bind together two domains of a tRNA synthetase. More significantly, the two domains—one that

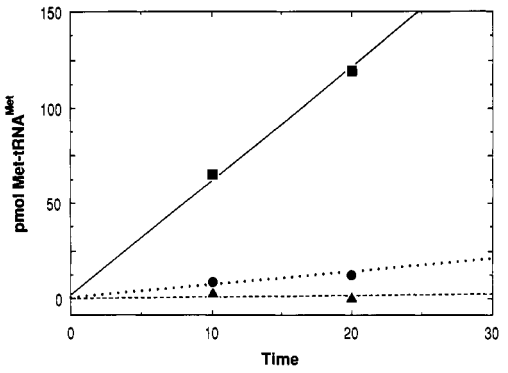


FIGURE 5: Measurement of reconstituted activity. Solid line (■): The αR367//M and βR367//M fractions were mixed before refolding. Dotted line (●): The βR367//M fraction alone was refolded. Because this fraction is not completely free of the host cell methionyl-tRNA synthetase, it is designated as β-alone. Dashed line (▲): The αR367//M fraction alone was refolded.

contains residues for amino acid activation and one that has critical determinants for tRNA recognition—can bind together, both in vivo and in vitro, in a way that mimics the unsplit enzyme. The reconstitution of activity is particularly significant because tRNA binding to its cognate synthetase, although necessary, is not alone sufficient for charging. Sequence-specific influences on the catalytic parameter k_{cat} can trigger aminoacylation of bound tRNA (Ebel et al., 1973; Roe et al., 1973; Bare & Uhlenbeck, 1985; Schulman & Pelka, 1985; Park et al., 1989). Thus, tRNA specificity is not determined solely by binding energies but may require functional communication between two discrete regions: one that interacts with tRNA, and one that transfers the activated amino acid.

While these two domains cannot be released directly by proteolysis, they can be synthesized as stable split proteins by recombinant DNA methods. Further, the exact location of the inserted stop codon determines the stability of the split protein (or, at least, its N-terminal fragment). Cleavage by proteases within the intersubunit region may give unstable products, explaining the limited success of proteolysis studies of methionyl-tRNA synthetase. Using molecular genetics, we have directed cleavage of the protein to specific sites that may not be recognized by proteases, because of either sequence-specific incompatibilities or steric constraints.

The results also suggest possibilities for the engineering and early evolution of aminoacyl-tRNA synthetases. One possibility is the engineering of chimeric aminoacyl-tRNA synthetases in which the nucleotide fold of one enzyme is noncovalently associated with a tRNA-recognizing domain of another synthetase. Possibly, a bound RNA could bridge these domains and stabilize their association. Alternatively, residues that strengthen packing interactions between the domains of a noncovalently linked chimeric protein might be introduced by site-directed mutagenesis.

The evolution of primitive aminoacyl-tRNA synthetases might have occurred analogously through noncovalent associations of protein fragments. In modern synthetases, the segments associated with tRNA binding are highly diversified—for example, *E. coli* glutaminyl-tRNA synthetase interacts with the anticodon of $tRNA^{Gln}$ through a domain rich in β-structure (Rould et al., 1989), while its counterpart in *E. coli* methionyl-tRNA synthetase is α-helical (Brunie et al., 1990). In contrast, the amino acid activation domains for the class I synthetases are structurally similar (Bhat et al., 1982; Blow & Brick, 1985; Brunie et al., 1990; Rould et al., 1989; Brick & Blow, 1987; Burbaum et al., 1989). This dichotomy suggests that the domains of these enzymes may have evolved independently, before they combined into a single gene product. In this view, similar domains responsible for amino acid activation would have noncovalently associated with unrelated RNA binding proteins.

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REFERENCES

Anfinsen, C. B., Cuatrecasas, P., & Taniuchi, H. (1971) *Enzymes (3rd Ed.) 4*, 177–204.

Bare, L., & Uhlenbeck, O. C. (1985) *Biochemistry 24*, 2354–2360.

Barker, D. G., Ebel, J.-P., Jakes, R. C., & Bruton, C. J. (1982) *Eur. J. Biochem. 127*, 449–457.

Bhat, T. N., Blow, D. M., Brick, P., & Nyborg, J. (1982) *J. Mol. Biol. 158*, 699–709.

Blow, D. M., & Brick, P. (1985) in *Biological Macromolecules & Assemblies* (Jurnak, F. A., & MacPherson, A., Eds.) Vol. 2, pp 441–469, John Wiley and Sons, New York.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Brick, P., & Blow, D. M. (1987) *J. Mol. Biol. 194*, 287–297.

Brunie, S., Zelwer, C., & Risler, J.-L. (1990) *J. Mol. Biol. 216*, 411–424.

Burbaum, J. J., Starzyk, R. M., & Schimmel, P. (1990) *Proteins 7*, 99–111.

Cassio, D., & Waller, J. P. (1971) *Eur. J. Biochem. 20*, 283–300.

Cusack, S., Berthetcolominas, C., Härtlein, M., Nassar, N., & Leberman, R. (1990) *Nature 347*, 249–255.

Dardel, F., Fayat, G., & Blanquet, S. (1984) *J. Bacteriol. 160*, 1115–1122.

Ebel, J., Giege, R., Bonnet, J., Kern, D., Befort, N., Bollack, C., Fasiolo, F., Gangloff, J., & Dirheimer, G. (1973) *Biochimie 55*, 547–557.

Eriani, G., Delarue, M., Poch, O., Gangloff, J., & Moras, D. (1990) *Nature 347*, 203–206.

Fischer, G., & Schmid, F. X. (1990) *Biochemistry 29*, 2205–2212.

Galakatos, N. G., & Walsh, C. T. (1987) *Biochemistry 26*, 8475–8480.

Gerschitz, J., Rudolph, R., & Jaenicke, R. (1978) *Eur. J. Biochem. 87*, 591–599.

Ghosh, G., Pelka, H., & Schulman, L. H. (1990) *Biochemistry 29*, 2220–2225.

Holmgren, A., & Slabý, I. (1979) *Biochemistry 18*, 5591–5599.

Hountondji, C., Blanquet, S., & Lederer, F. (1985) *Biochemistry 24*, 1175–1180.

Jaenicke, R., & Rudolph, R. (1986) *Methods Enzymol. 131*, 218–250.

Kato, I., & Anfinsen, C. B. (1969) *J. Biol. Chem. 244*, 1004–1007.

Keng, T., Webster, T. A., Sauer, R. T., & Schimmel, P. (1982) *J. Biol. Chem. 257*, 12503–12508.

Knowles, J. R. (1987) *Science 236*, 1252–1258.

Kohda, D., Yokoyama, S., & Miyazawa, T. (1987) *J. Biol. Chem. 262*, 558-563.
Li, C. H., & Bewley, T. A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1476-1479.
Matsudaira, P. (1987) *J. Biol. Chem. 262*, 10035-10038.
Mellot, P., Mechulam, Y., Le Corre, D., Blanquet, S., & Fayat, G. (1989) *J. Mol. Biol. 208*, 429-443.
Park, S. J., Hou, Y.-M., & Schimmel, P. (1989) *Biochemistry 28*, 8479-8484.
Rich, A. (1962) in *Horizons in Biochemistry* (Kasha, M., & Pullman, B., Eds.) pp 103-126, Academic Press, New York.
Richards, F. M., & Wyckoff, H. W. (1971) *Enzymes (3rd Ed.) 4*, 647-806.
Roe, B., Sirover, M., & Dudock, B. (1973) *Biochemistry 12*, 4146-4154.
Rosevear, P. (1988) *Biochemistry 27*, 7931-7939.
Rould, M. A., Perona, J. J., Söll, D., & Steitz, T. A. (1989) *Science 246*, 1135-1142.
Rudolph, R., Gerschitz, J., & Jaenicke, R. (1978) *Eur. J. Biochem. 87*, 601-606.
Saint Girons, I., Gilles, A.-M., Margarita, D., Michelson, S., Monnot, M., Fermandjian, S., Danchin, A., & Bârzu, O. (1987) *J. Biol. Chem. 262*, 622-629.
Schimmel, P. (1987) *Annu. Rev. Biochem. 56*, 125-158.
Schimmel, P., & Söll, D. (1979) *Annu. Rev. Biochem. 48*, 601-648.
Schulman, L. H., & Pelka, H. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6755-6759.
Schulman, L. H., & Pelka, H. (1985) *Biochemistry 24*, 7309-7314.
Starzyk, R. M., Burbaum, J. J., & Schimmel, P. (1989) *Biochemistry 28*, 8479-8484.
Strittmatter, P., Barry, R. E., & Corcoran, D. (1972) *J. Biol. Chem. 247*, 2768-2775.
Taniuchi, H., Parr, G. R., & Juillerat, M. A. (1986) *Methods Enzymol. 131*, 185-217.
Toth, M. J., & Schimmel P. (1986) *J. Biol. Chem. 261*, 6643-6646.
Wetlaufer, D. B. (1981) *Adv. Protein Chem. 34*, 61-92.
Wetlaufer, D. B., & Ristow, S. (1973) *Annu. Rev. Biochem. 42*, 135-158.
Woese, C. R. (1970) in *Organization and Control of Prokaryotic and Eukaryotic Cells: 20th Symposium of the Society for General Microbiology* (Charles, H. P., & Knight, B. C. J. G., Eds.) pp 39-54, Cambridge University Press, London.

A New Role for the Transferrin Receptor in the Release of Iron from Transferrin†

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ABSTRACT: Iron removal by pyrophosphate from human serum diferric transferrin and the complex of transferrin with its receptor was studied in 0.05 M HEPES or MES buffers containing 0.1 M NaCl and 0.01 M CHAPS at 25 °C at pH 7.4, 6.4, and 5.6. At each pH, the concentration of pyrophosphate was adjusted to achieve rates of release amenable to study over a reasonable time course. Released iron was separated from protein-bound iron by poly(ethylene glycol) precipitation of aliquots drawn from the reaction mixture at various times during the course of a kinetic run. The amount of ^{59}Fe label associated with the protein and pyrophosphate was determined from the radioactivity of precipitate and supernatant, respectively, in each aliquot. Iron removal of 0.05 M pyrophosphate at pH 7.4 from diferric transferrin bound to the receptor is considerably slower than that from free diferric transferrin, with observed pseudo-first-order rate constants of 0.020 and 0.191 min^{-1}, respectively. For iron removal by 0.01 M pyrophosphate at pH 6.4, corresponding rate constants are 0.031 and 0.644 min^{-1}. However, at pH 5.6, iron removal by 0.001 M pyrophosphate is faster from diferric transferrin bound to its receptor than from free transferrin (observed rate constants of 0.819 and 0.160 min^{-1}, respectively). Thus, the transferrin receptor not only facilitates the removal of iron from diferric transferrin at the low pH that prevails in endocytic vesicles but may also reduce its accessibility to iron acceptors at extracellular pH, thereby minimizing the likelihood of nonspecific release of iron from transferrin at the cell surface.

Transferrin, the iron-binding protein of plasma, is the principal or only source of iron for the metabolic needs of most vertebrate cell types [for recent reviews of the physical chemistry of transferrin and the transferrin-cell interaction, see Brock (1985), Baldwin and Egan (1987), and Aisen (1989), and for the structure and function of the transferrin receptor, see Kühn (1989) and Forsbeck (1990)]. The transferrin molecule consists of a single 80-kDa polypeptide chain disposed in two lobes of highly homologous amino acid sequence. Each lobe is arranged in two domains surrounding a cleft bearing its iron-binding site (Anderson et al., 1987). Ligands of each iron-binding site are identical: two phenolic oxygen atoms from tyrosyl residues, one histidyl nitrogen atom, a single aspartyl oxygen atom, and two oxygen atoms from a carbonate anion (Anderson et al., 1987). Without carbonate,

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