Protein Engineering of Homodimeric Tyrosyl-tRNA Synthetase to Produce Active Heterodimers

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Heterodimers of tyrosyl-tRNA synthetase from Bacillus stearothermophilus have been produced by mutagenesis at the subunit interface. Oppositely charged subunits have been engineered into the subunits so that they can form a complementary pair. Wild-type tyrosyl-tRNA synthetase is a symmetrical dimer in which the side chains of the 2 Phe-164 residues interact at the subunit interface. Phe-164 was mutated to Asp in tyrosyl-tRNA synthetase and to Lys in a truncated enzyme (des-(321-419)tyrosyl-tRNA synthetase) which lacks the two tRNA-binding sites, but which can catalyze pyrophosphate exchange. The size difference allows subunit association to be studied by gel filtration chromatography. These changes induce reversible dissociation from active dimers into inactive monomers at pH values which favor ionization at position 164. A mixture of the two mutants near neutral pH is apparently fully active in pyrophosphate exchange and consists of a heterodimer of [Asp'64]tyrosyl-tRNA synthetase and [Lys'64]des-(321-419)tyrosyl-tRNA synthetase. Despite having only one binding site for tRNA, heterodimer has full aminoacylation activity at high concentrations of tyrosine. We have therefore produced a family of dimers that differ in stability near neutral pH. This novel approach using protein engineering allows specific dimerization of subunits of the same size that have different defined mutations, each subunit being tagged by the charge. Such hybrid proteins can be used to study subunit interaction.

Wild-type tyrosyl-tRNA synthetase is a homodimer (\(M_r = 2 \times 47,300\)) (1) with each subunit having one active site (2). In common with several other enzymes, only one active site appears functional per dimer (3). Neither the mechanism nor the role of this half-of-the-sites activity is known. The enzyme (E) catalyzes the aminoacylation of tRNA as a two-step reaction (Equations 1 and 2).

\[
\begin{align*}
E + ATP + Tyr & \rightarrow E \cdot Tyr \cdot AMP + PP, \\
E \cdot Tyr \cdot AMP + tRNA & \rightarrow E + Tyr \cdot tRNA + AMP
\end{align*}
\]

Each monomer has two domains. The subunits interact through hydrophobic regions on their amino-terminal domains. The carboxyl-terminal domains are not involved in the contacts between the monomers (2). The side chains of the 2 Phe-164 residues interact at the subunit interface and lie on the axis of symmetry (2). Mutation of Phe-164 to Asp in tyrosyl-tRNA synthetase introduces potential negative charges at the hydrophobic interface and induces pH-dependent reversible dissociation from inactive monomers (4). Deletion of the carboxyl-terminal domains of wild-type tyrosyl-tRNA synthetase (producing des-(321-419)tyrosyl-tRNA synthetase, \(M_r = 2 \times 36,300\)) abolishes tRNA-binding (Reaction 2), but the formation of tyrosyl adenylate is unaffected (Reaction 1) (5). In this study Phe-164 is mutated to Lys-164 in des-(321-419)tyrosyl-tRNA synthetase to introduce potential positive charge into the hydrophobic subunit interface and so induce activity and dissociation analogous to that of [Asp'64]tyrosyl-tRNA synthetase but with the opposite pH dependency. We mixed monomeric [Asp'64]tyrosyl-tRNA synthetase with monomeric [Lys'64]des-(321-419)tyrosyl-tRNA synthetase to produce heterodimers (\(M_r = 83,600\)) of size intermediate between those of parent homodimers (see Fig. 1). We show that such heterodimers are much more stable at neutral pH than are the parent homodimers, and that they are apparently fully active.

RESULTS

Previous work (4) has shown that the monomeric form of the mutant [Asp'64]tyrosyl-tRNA synthetase is inactive and has only weak, if any, affinities for tyrosine and ATP. The dimer is active and has higher affinities for these ligands. Association of subunits is therefore favored by high concentrations of the substrates. [Asp'64]Tyrosyl-tRNA synthetase has the same value of \(K_m\) in pyrophosphate exchange at pH 7.8 as does wild-type enzyme, but the value of \(K_m\) for tyrosine is greatly increased (4). [Asp'64]Tyrosyl-tRNA synthetase is predominantly monomeric above pH 6 and so forms enzyme-bound tyrosyl adenylate only slowly and with a low stoichiometry which falls with increasing pH as the Asp-164 ionizes (4) (Fig. 2). [Lys'64]des-(321-419)Tyrosyl-tRNA synthetase behaves in an analogous manner, having a reversible change of activity with pH except that activity decreases at low pH as Lys-164 becomes protonated. But, on mixing the two mutants at pH 7.8, enzyme-bound tyrosyl adenylate is formed with a stoichiometry approaching 1 mol/mol of dimeric enzyme, as found with the wild type (Fig. 2). As the mixture of the two mutants is more active than the constituents, there must be a functional interaction between the two types of subunit.

The composition of the active heterodimer was confirmed by determining its apparent \(M_r\) by gel filtration using FPLC. Both mutants at position 164 are predominantly monomers in the absence of tyrosine (Table I). Addition of tyrosine causes a reversible association at certain values of pH as found previously for [Asp'64]tyrosyl-tRNA synthetase (4). At low pH where the carboxylates of [Asp'64]tyrosyl-tRNA synthetase have a greater tendency to be protonated, this mutant dimerizes. Conversely, [Lys'64]des-(321-419)tyrosyl-tRNA synthetase has a greater tendency to be protonated, this mutant dimerizes.
Protein Engineering of Homodimers to Produce Heterodimers

**FIG. 1. Strategy for protein engineering to produce a heterodimer from homodimeric tyrosyl-tRNA synthetase.** This strategy is explained in the text. TyrTS, tyrosyl-tRNA synthetase; ΔTyrTS, des-(321-419)tyrosyl-tRNA synthetase.

Tyrosyl-tRNA synthetase is predominantly dimeric at high pH when the ε-amino groups are more readily deprotonated. At pH 7.8, both mutants are mainly monomeric even in the presence of tyrosine (Table I). A mixture of the two mutants at pH 7.8 in the absence of tyrosine elutes as a single broad peak of apparent Mr = 57,000, indicative of weak dimerization. In the presence of tyrosine, the mixture elutes as a single sharper peak of apparent Mr = 77,000 which is much greater than that for either of the constituents under the same conditions and is close to that expected for the heterodimer (Table I). This value suggests that heterodimer is made from [Asp164]tyrosyl-tRNA synthetase and [Lys164]des-(321-419)tyrosyl-tRNA synthetase and that it is the predominant form. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of fractions across this peak shows coelution of the differently sized subunits.

In the presence of low concentrations of tyrosine at pH 7.8, the individual mutants have low activity in pyrophosphate exchange (Reaction 1) and aminoacylation of tRNA (Reactions 1 and 2) (Table II), showing that inactive monomers predominate under these conditions. On mixing the two mutants they have identical activity to wild-type enzyme in pyrophosphate exchange and low activity in tRNA charging (Table II). Adding tRNA before tyrosine and ATP, compared with adding tRNA after the other substrates, gives lower rates of charging or pyrophosphate exchange by [Asp164]tyrosyl-tRNA synthetase or by heterodimer. This is because tRNA binds to catalytically inactive monomeric [Asp164]tyrosyl-tRNA synthetase and apparently inhibits formation of active dimer (4). At high tyrosine concentrations all of the enzymes have similar activities in both assays (except truncated dimers which do not charge tRNA because they lack the tRNA-binding domain), suggesting that they all have similar values of kcat. Individual mutant homodimers, however, have increased values of Km for tyrosine compared with heterodimer and wild-type enzymes.

**DISCUSSION**

Heterodimers of [Phe164]tyrosyl-tRNA synthetase and [Phe164]des-(321-419)tyrosyl-tRNA synthetase have recently been made by dissociating a mixture of the enzymes in urea, removing the denaturant, and purifying dimers of intermediate Mr, by gel electrophoresis (9, 10). This method has an absolute requirement for a size difference between parent homodimers and heterodimers. The technique of using polar mutations is not limited in this way. The difference in sizes was used only to facilitate analysis of production of heterodimers. Production of heterodimers using urea resulted in a significant irreversible loss of activity (10), which did not occur in the current work when polar mutations were used. Employing polar mutations rather than reversible denaturation, heterodimers can be prepared in much larger quantities. We have shown that heterodimers of polar mutants of position 164 predominate over parent monomers or dimers so that a mixture of full-length [Asp164]tyrosyl-tRNA synthetase and [Phe164]des-(321-419)tyrosyl-tRNA synthetase were essentially identical. TyrTS(TyrTS, des-(321-419)tyrosyl-tRNA synthetase; ΔTyrTS(TyrTS, [Phe164]tyrosyl-tRNA synthetase; ΔTyrTS(Lys164), [Lys164]des-(321-419)tyrosyl-tRNA synthetase

**FIG. 2. Effects of pH on active site titers of tyrosyl-tRNA synthetase.** The construction of genes coding for [Asp164]tyrosyl-tRNA synthetase and [Phe164]des-(321-419)tyrosyl-tRNA synthetase has been published (4, 5). The [Lys164]des-(321-419)tyrosyl-tRNA synthetase gene was made by site-directed mutagenesis of that coding for [Phe164]des-(321-419)tyrosyl-tRNA synthetase by the method of Winter et al. (6) using an oligonucleotide of sequence 5′-AACTCGGTCTTGAATA3′ (where * is a mismatched base). The mutation was verified by dideoxy DNA sequencing (7). All enzymes were expressed and purified as described by Lowe et al. (8). Each preparation was homogeneous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Active site titrations were performed by measuring accumulation of the stable enzyme-tyrosyl adenylate complex in the presence of pyrophosphatase (Reaction 1) (4). Enzyme (60 μl) was added to 40 μl of assay mixture and incubated for 5 min. Means of triplicate determinations (which agreed to within 5%) were plotted. Buffers were made to constant ionic strength at different pH values. Their final concentrations were as follows: pH 6.0, 128 mM Bistris·Cl; pH 6.5 and 7.0, 100 mM KH2PO4/K2HPO4; pH 7.5, 100 mM Tris·Cl; pH 8.5, 329 mM Tris·Cl; and pH 9.3, 60.4 mM glycine sodium. Enzyme concentration was estimated spectrophotometrically (based on A280 = 1.05 for 1 mg/ml solution). Enzyme concentration was 1.0 μM except for the mixture which was 0.5 μM in each component. The values obtained for [Phe164]tyrosyl-tRNA synthetase and [Phe164]des-(321-419)tyrosyl-tRNA synthetase were essentially identical. TyrTS(TyrTS, [Phe164]tyrosyl-tRNA synthetase; ΔTyrTS(Lys164), [Lys164]des-(321-419)tyrosyl-tRNA synthetase...
**Table I**

Determination of apparent \( M_r \) by FPLC gel filtration

FPLC gel filtration columns were calibrated using proteins of known \( M_r \) and the apparent \( M_r \) values of tyrosyl-tRNA synthetase mutants were estimated by comparison of elution volumes (4). It should be noted that values of \( M_r \) for oligomers determined by gel filtration where there is rapid dissociation and association of subunits underestimate the absolute values. The apparent values of \( M_r \) lie between the absolute values for the monomer and oligomer and are functions of concentration and various rate constants. Apparent values tend to the absolute values as association becomes tighter.

In this study do weaken the association of subunits, they do not appear to change the functional interactions across the subunit interface as heterodimers are apparently fully active and show properties similar to wild type rather than parent mutant homodimer.

**Heterodimer** and wild type have similar activities in pyrophosphate exchange at low substrate concentrations and both show half-of-the-sites reactivity. Detailed analysis of these mutant enzymes will indicate if changes at position 164 alter only the association of subunits or whether they also affect the activity of the dimers after they are formed. If the absolute properties of the dimers can be measured, this system can be used to investigate the basis of subunit association. The kinetic behavior of the dissociating enzymes would provide a means of analysing functional consequences of structural changes. By making two mutations at position 164 of tyrosyl-tRNA synthetase we have produced a family of dimers which decrease in stability near neutral pH in the order: wild type > heterodimer > [Asp\(^{164}\)]tyrosyl-tRNA synthetase = [Lys\(^{164}\)]des-(321-419)tyrosyl-tRNA synthetase. Heterodimers produced both by urea denaturation (9, 10) and by salt bridging (Table II) have charging activity despite having only one tRNA-binding site. Thus, the model for aminoacyl-tRNA synthetase and tRNA interaction where pseudosymmetrical regions of tRNA are recognized by symmetrical regions on two tRNA-binding domains of the enzyme (11) does not apply in this case.

Formation of hybrid oligomeric proteins by the classical technique of reversible denaturation and reassociation has been valuable in the study of subunit interactions, especially where the subunits are of differing composition (12). Many enzymes cannot regain significant activity after denaturation. Our novel application of protein engineering to change quaternary structure allows hybrids to be formed where subunits are of the same size and, possibly, in cases where enzymes may not be reversibly denatured.

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**Table II**

Activity of tyrosyl-tRNA synthetases at pH 7.8

Pyrophosphate exchange was measured by incorporation of \([\text{PP}^\text{P}]\text{PP}\), into ATP. All assays were performed in 144 mM Tris-Cl (pH 7.8) containing 10 mM MgCl\(_2\), 10 mM 2-mercaptoethanol, and 0.1 mM phenylmethanesulfonyl fluoride (4). Enzyme concentration was 0.3 μM, into ATP. All assays were performed in 144 mM Tris-C1 (pH 7.8) containing 200 mM NaCl; pH 8.0, 20 mM Tris-Cl containing 100 mM NaCl; and pH 9.3, 36.2 mM glycine sodium containing 100 mM NaCl. Each buffer also contained 10 mM 2-mercaptoethanol and 0.1 mM phenylmethanesulfonyl fluoride. In each case the sample loaded was 50 μl of 10 μM enzyme (in monomer) except for the mixture which was 10 μM in each mutant monomer. Tyrosine, when added, was at 100 μM in both the sample and the elution buffer. The absolute \( M_r \) values for the expected monomers and dimers are calculated from the published sequence data (1, 5) and are shown for comparison. Asp-164, [Asp\(^{164}\)]tyrosyl-tRNA synthetase: Lys-164, [Lys\(^{164}\)]des-(321-419)tyrosyl-tRNA synthetase.

| Conditions | Apparent \( M_r \) |
|------------|------------------|
|            | Asp-164 | Lys-164 | Asp-164 + Lys-164 |
| pH 6.0     |         |         |                   |
| No Tyr     | 53,000  | 36,000  | 47,000 + 36,000   |
| 100 μM Tyr | 96,000  |         |                   |
| pH 7.8     |         |         |                   |
| No Tyr     | 55,000  | 46,000  | 57,000            |
| 100 μM Tyr | 58,000  | 49,000  | 77,000            |
| pH 9.3     |         |         |                   |
| No Tyr     | 44,000  |         |                   |
| 100 μM Tyr | 65,000  |         |                   |
| Absolute \( M_r \) |         |         |                   |
| Monomers   | 47,000  | 36,000  | 47,000 + 36,000   |
| Dimers     | 95,000  | 73,000  | 83,000            |

**Pyrophosphate exchange**

| \( \text{tRNA} \) charging | Initial rate | \( \text{mol product/mol enzyme/s} \) |
|---------------------------|-------------|-------------------------------------|
| Tyr                       |             |                                     |
| Wild type                 |             |                                     |
| \( \Delta \text{wild type} \) |             |                                     |
| Pyrophosphate exchange    |             |                                     |
| 10 μM Tyr                 | 0.1         | 0.2                                 |
| 300 μM Tyr                | 5.4         | 6.1                                 |
| tRNA charging             |             |                                     |
| 137 μM Tyr                | 0.06        | 0.12                                |
| 300 μM Tyr                | 0.54        | 0.53                                |