Two Substrate Binding Sites on Tryptophanyl Transfer Ribonucleic Acid Synthetase of Escherichia coli*

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Tryptophanyl-tRNA synthetase of Escherichia coli has 1.8 binding sites for L-tryptophan with $K_{diss}$ of $12 \times 10^{-5} \text{M}$ as shown by equilibrium dialysis. The results are in accord with the known structure of the enzyme, an $a_2$ dimer of 74,000 molecular weight, and with 2 binding sites for tryptophanyl-ATP ester. Ordinary sucrose density gradient centrifugation reveals a complex composed of one tRNA$^{Trp}$ bound per enzyme dimer. When tRNA$^{Trp}$ is mixed throughout the gradient at concentrations from $5.4 \times 10^{-6} \text{M}$ to $2.0 \times 10^{-5} \text{M}$, a new peak appears in the position expected for a complex with two tRNA$^{Trp}$ molecules bound per enzyme dimer. Sedimentation through gradients lacking tRNA$^{Trp}$ favors dissociation of the 1:2 complex but not of the 1:1 complex. The data indicate 2 binding sites for tRNA$^{Trp}$ on tryptophanyl-tRNA synthetase.

The aminoacyl-tRNA synthetases are a family of enzymes which catalyze analogous reactions. At least one pair, the tryptophanyl-tRNA synthetases of Escherichia coli and of human placenta, contain a sequence homology (1). The substrate tRNAs have evolved with the synthetases and are uniform in structure (2). However, the synthetases exhibit diverse structures and may be single polypeptide chains ($a_2$) with molecular weights from 40,000 to 112,000, $a_2$ dimers with subunit molecular weights from 35,000 to 90,000, or $a_3$ dimers and $a_3b_2$ tetramers with subunit molecular weights from 33,000 to 80,000 (3, 4).

Although diverse quaternary structures and protomer molecular weights are unexpected for homologous enzymes, recent structural data indicate intramers or repeating sequences in the methionyl-, valyl-, and leucyl-tRNA synthetases of Bacillus stearothermophilus, with protomer molecular weights in the range 66,000 to 110,000 (5). The smaller protomers (44,000) of tyrosyl-tRNA synthetase from the same organism lack evidence of intramers (5). E. coli leucyl-tRNA synthetase, a single polypeptide chain of molecular weight 100,000, gives evidence of repeating sequences, whereas seryl-tRNA synthetase, an $a_2$ enzyme of the same molecular weight, does not (6). Methionyl-tRNA synthetase, an $a_3$ enzyme of molecular weight 180,000, and isoleucyl-tRNA synthetase, a single polypeptide chain of molecular weight 114,000, also give evidence of repeating sequences (7, 8).

To fully understand the diversity of quaternary structure accurate information on the number of substrate-binding sites of the various synthetases is clearly necessary. In accordance with the repeating sequences already mentioned, 2 binding sites for valine (in presence of ATP) and 2 binding sites for ATP (in presence of valine) have been found (9) on the monomeric valyl-tRNA synthetase of B. stearothermophilus. However, only single binding sites for amino acids, for ATP, and for tRNA have been found on the other single polypeptide chain synthetases. Multiple binding sites for tRNA have not ordinarily been demonstrable even on the $a_3$ synthetases (3, 4).

Sucrose density gradient sedimentation is widely used for binding studies, but the method has not demonstrated more than one binding site for tRNA on any synthetase, including those with multiple subunits. For example, yeast lysyl-tRNA synthetase is an $a_2$ structure with 2 binding sites for ATP and for L-lysine by equilibrium dialysis (10) but only 1 binding site for tRNA$^{Lys}$ by sucrose density gradient sedimentation (11). Again, E. coli seryl-tRNA synthetase is an $a_3$ structure (12, 13) with 2 binding sites for ATP and for L-serine (14, 15) and 2 binding sites for tRNA$^{Ser}$ by equilibrium gel filtration (14), but only 1 binding site for tRNA$^{Ser}$ by sucrose density gradient sedimentation (16).

E. coli tryptophanyl-tRNA synthetase is an $a_2$ enzyme with subunits of 37,000 molecular weight (17). The dimer has 2 binding sites for tryptophanyl-ATP ester as shown in nonequilibrium gel filtration (17). Earlier studies with sucrose density gradient sedimentation detected only 1 binding site for tryptophanyl-tRNA (17). The present work demonstrates two L-tryptophan binding sites by equilibrium dialysis and two tRNA$^{Trp}$ binding sites by use of sucrose density gradient sedimentation with tRNA$^{Trp}$ present throughout the gradient.

EXPERIMENTAL PROCEDURE

Materials—Visking dialysis tubing was obtained from Union Carbide. Ribonuclease—free sucrose was from Schwarz/Mann. Trypto-
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Tryptophanyl-tRNA synthetase and tRNA<sub>Trp</sub> was prepared as previously described (18). Tryptophanyl-tRNA synthetase mass was 91% (18) of that determined by the method of Lowry et al. (19) with bovine serum albumin as standard. Enzyme and tRNA<sub>Trp</sub> activities were measured as previously described (18, 20). In some instances tRNA consisting principally of tRNA<sub>Trp</sub> is designated tRNA<sub>Trp</sub><sup>a</sup> and the specific activity is given. A specific activity of 1800 pmol of tryptophanyl-tRNA per A<sub>260</sub> unit is taken as pure tryptophanyl-tRNA (18). One A<sub>260</sub> unit of tRNA has an A<sub>260</sub> of 1.0 when dissolved in 1.0 ml of 5 mM KH<sub>2</sub>PO<sub>4</sub>, 0.5 mM K<sub>2</sub>HPO<sub>4</sub> buffer (pH 6.9) in a 1.0-cm optical path. Transfer RNA with no detectable tRNA<sub>Trp</sub> contained less than 0.5 pmol of tRNA<sub>Trp</sub> per A<sub>260</sub> unit and was the material recovered from the NaCl gradient containing no ethanol on BD-cellulose chromatography (18). Valyl-tRNA synthetase was purified by chromatography on DEAE-cellulose and on hydroxylapatite as previously described (20). All other materials were of the highest grade commercially available or were obtained as previously described (18).

Preparation of Dialysis Membranes—Visking no. 20 tubing was used. The general procedures of Martin and Ames (21) were used but applied to the SW56 Beckman rotor with six tubes, each containing 3.8 ml of 50 mM potassium phosphate buffer, pH 5.5, 5.5 mM MgCl<sub>2</sub>, 1.0 mM dithiothreitol, 0.2 mM EDTA, 50 μg/ml of bovine serum albumin, a linear gradient from 5 to 26% sucrose, and tRNA<sub>Trp</sub> as indicated in the figure legends. Experiments with the SW39 rotor (17, 22) gave similar results. The gradients were formed at 23° with a Buchler universal density gradient mixer, then placed at 23° and allowed to stand for 4 to 12 h. The samples to be sedimented were layered on the gradients in volumes of 50 to 100 μl. After sedimentation for 90 h at 51,000 rpm in a Beckman ultracentrifuge set at 0°, the polyallomer tube bottoms were punctured in a Buchler piercing unit, and 5-drop fractions (0.12 ml) were collected by hand.

Results and Discussion

Binding of t-Tryptophan—The Scatchard plot (23) shown in Fig. 1 indicates that tryptophanyl-tRNA synthetase binds 2 mol of t-trypophan per mol of dimeric enzyme. Molecular weight 74,000. The calculated dissociation constant is 12 × 10<sup>-5</sup> M. The apparent K<sub>a</sub> is 3 × 10<sup>-5</sup> M under similar but not identical conditions (18). The results are in accord with the 2 binding sites on each enzyme dimer for tryptophanyl-ATP as determined by nonequilibrium gel filtration (17). In parallel experiments with radioactive ATP as the ligand valid data were unobtainable because of the degradation of the ATP to ADP and AMP during equilibration. Either the ATPase activity is intrinsic to tryptophanyl-tRNA synthetase, or the ATPase is a trace contaminant in the preparation. Others (10) have encountered the same problem in studies of the lysyl-tRNA synthetases of both yeast and Escherichia coli. Further attempts to study ATP binding by the rapid measurement of dialysis rate (24) were unsuccessful because of the high dissociation constant of ATP and tryptophanyl-tRNA synthetase position in the presence of tRNA<sub>P</sub> is readily induced by tRNA<sub>P</sub> is specific for tryptophanyl-tRNA synthetase, and tRNA<sub>P</sub> having no effect on valyl-tRNA synthetase function as an inhibitor in kinetic studies (25). Part of the shift in the enzyme peak seen in the experiment with tRNA<sub>P</sub> may be explained by the slow conversion of tRNA<sub>Trp</sub> into tRNA<sub>Trp</sub><sup>a</sup>. (In a separate experiment tRNA<sub>Trp</sub> was placed throughout a gradient and allowed to remain for 3 days at 0°, the time including a 9-h centrifugation at 51,000 rpm was 82% converted into tRNA<sub>Trp</sub><sup>a</sup> with a 96% recovery of activity.) Since tRNA<sub>Trp</sub>, tRNA<sub>P</sub>, and free tryptophanyl-tRNA synthetase sediment with similar velocity, any tRNA<sub>Trp</sub> formed could bind to tryptophanyl-tRNA synthetase to give the complex. The shift induced by tRNA<sub>P</sub> is specific for tryptophanyl-tRNA synthetase, tRNA<sub>P</sub> having no effect on valyl-tRNA synthetase present in the same centrifuge tube.

When the same amount of tRNA, lacking tRNA<sub>P</sub> and consisting of the other specific amino acid acceptors, is substituted for tRNA<sub>P</sub> there is a slight shift of the enzyme peak as shown in the bottom panel of Fig. 2, and this shift is a measure of the nonspecific binding (with high dissociation constant) of tryptophanyl-tRNA synthetase to tRNA at the low pH (5.8) employed. Although the shift in tryptophanyl-tRNA synthetase position in the presence of tRNA<sub>P</sub> is readily

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1 tRNA<sub>P</sub> is the active and tRNA<sub>P</sub> is the inactive form of tRNA<sub>P</sub>.
We can calculate the expected sedimentation coefficients for enzyme·tRNA (1:1 complex) and for enzyme·(tRNA)$_2$ (1:2 complex) if we assume the tRNA$_{Trp}$ and the enzyme to be enzyme·tRNA (1:1 complex) and for enzyme·(tRNA)$_2$ (1:2 complex) as shown by assays with and without chloroquine (25).

Because the tryptophanyl-tRNA synthetase binds 2 molecules of L-tryptophan and 2 molecules of tryptophanyl-ATP ester (17), the enzyme was expected to bind 2 molecules of tRNA$_{Trp}$. However, as shown in Fig. 3 an increase in the tRNA$_{Trp}$ concentration 5-fold above that required to form the 1:1 complex produced no indication of a 1:2 complex.

The tRNA$_{Trp}$, even when present in an amount only equimolar to the tryptophanyl-tRNA synthetase at 7 x 10$^{-4}$ M, converted all of the enzyme into the 1:1 complex, as shown in Fig. 3. When tRNA$_{Trp}$ was less than stoichiometric to tryptophanyl-tRNA synthetase, the enzyme sedimented as two peaks, one representing free enzyme and the other representing the 1:1 complex peak. A 7.4 S complex with a partial specific volume of 0.66 would sediment as 7.2 S according to the correction for partial specific volume provided by Martin and Ames in Fig. 3 of Ref. 21 and would center between Fractions 14 and 15 in Fig. 2 (panel 3). The 1:1 complex actually centers between Fractions 13 and 14, in good agreement with the calculation. If present, the 9.6 S complex with a partial specific volume of 0.63 would sediment as 9.0 S (21) and would center between Fractions 9 and 10 in Fig. 2. No peak exists in this position.

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The tRNA$_{Trp}$, even when present in an amount only equimolar to the tryptophanyl-tRNA synthetase at 7 x 10$^{-4}$ M, converted all of the enzyme into the 1:1 complex, as shown in Fig. 3. When tRNA$_{Trp}$ was less than stoichiometric to tryptophanyl-tRNA synthetase, the enzyme sedimented as two peaks, one representing free enzyme and the other representing the 1:1 complex, as shown in panel 2 of Fig. 4. The position of the 1:1 complex peak was almost constant in Figs. 3 and 4, even over the 5- to 8-fold range of tRNA$_{Trp}$ concentrations present, although the slightly diminished sedimentation velocity of the 1:1 complex peak in the presence of limiting tRNA$_{Trp}$ (panel 9, Fig. 4) indicates the reversibility of the binding equilibrium.

If we consider the dissociation sequence:

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\text{Enzyme·(tRNA)$_2$} \rightleftharpoons \text{enzyme·tRNA} + \text{tRNA} \quad (a)
\]

\[
\text{Enzyme·tRNA} \rightleftharpoons \text{enzyme} + \text{tRNA} \quad (b)
\]

demonstrated at pH 6.9, the change is greater at pH 5.8. The enhanced binding of tRNA$_{As}$ to their specific aminoaacyl-tRNA synthetases at lower pH values is a general finding (26, 27) especially in phosphate buffer (28). During sedimentation at either pH the tRNA$_{Trp}$ is stable and does not become tRNA$^{70}_{Trp}$, as shown by assays with and without chloroquine (25).

We can calculate the expected sedimentation coefficients for enzyme·tRNA (1:1 complex) and for enzyme·(tRNA)$_2$ (1:2 complex) if we assume the tRNA$_{Trp}$ and the enzyme to be globular and to undergo no major changes in conformations or
Fig. 3. Effect of increasing tRNA<sup>Trp</sup> concentration on formation of the enzyme-tRNA<sup>Trp</sup> complex. Samples, 50 µl, were layered on the gradients and centrifuged as described under "Experimental Procedure." The first (top) panel shows the position of 26 µg (350 pmol) of free tryptophanyl-tRNA synthetase. The second panel shows the new position of the enzyme (●) now in a complex with tRNA<sup>Trp</sup> (○) after mixing immediately before sedimentation the 350 pmol of enzyme (7.0 x 10⁻⁶ M) with 0.188 A₂₆₀ unit of tRNA<sup>Trp</sup> (1360 pmol/A₂₆₀ unit, 256 pmol, 5.1 x 10⁻⁴ M), which had been converted into the active conformation immediately before mixing as described in the legend of Fig. 2. In panels 3, 4, and 5 the same 7.0 x 10⁻⁶ M enzyme (●) was mixed with 10 x 10⁻⁴ M, 15 x 10⁻⁴ M, and 25 x 10⁻⁴ M tRNA<sup>Trp</sup> (○), respectively, immediately before sedimentation. In panels 2, 3, 4, and 5 there were 144, 137, 143, and 148 pmol, respectively, of tRNA<sup>Trp</sup> in the peaks associated with the enzyme peaks, and total recoveries of tRNA<sup>Trp</sup> acceptor activities were 84, 87, 80, and 79%, respectively. Recovery of enzyme activity is at least 46, 46, 44, 47, and 49% in gradients 1 through 5, respectively. True recovery is higher, because the tRNA<sup>Trp</sup> in the enzyme assays was limiting for peak fractions.

The peak appears in a position invariant over a 4-fold range of tRNA<sup>Trp</sup> concentration in the sucrose gradient. When a concentration of mixed tRNA equivalent to the higher concentration of tRNA<sup>Trp</sup> but containing no detectable tRNA<sup>Trp</sup> is used instead (gradient 6), the new peak does not appear, and the enzyme sediments almost in the position of free enzyme. Therefore, the new peak is the result of a specific interaction of the enzyme with tRNA<sup>Trp</sup>. On the basis of the position of free tRNA<sup>Trp</sup> (4.6 S) in gradient 3, the 1:1 complex (7.4 S corrected to 7.2 S for a partial specific volume of 0.66) in gradient 3 should be centered between Fractions 11 and 12, as actually found. A 1:2 complex of 9.6 S corrected to 9.0 S for a partial specific volume of 0.63 (21) should be centered between Fractions 6 and 7. The new peak actually centers on Fraction 7, in good agreement with the calculation. For the 1:2 complex the calculation of enzyme-tRNA<sup>Trp</sup> stoichiometry is impossible because of the high concentration of tRNA<sup>Trp</sup> in the plateau.

The recoveries of enzyme activities in the various gradients of this experiment reveal that free tryptophanyl-tRNA synthetase is not entirely stable to the sedimentation conditions. When the enzyme in gradient 1 (Fig. 4) was allowed to stand a further 24 h at 0°, it lost 40% of its activity. The enzyme-tRNA<sup>Trp</sup> complex is more stable than the enzyme-tRNA<sup>Trp</sup> complex, which in turn is more stable than free enzyme. Presence of nonspecific tRNAs does not stabilize the enzyme.

The simple placement of tRNA<sup>Trp</sup> throughout the gradient on complex formation. Samples, 50 µl, were layered on gradients and centrifuged as described under "Experimental Procedure." The first (top) panel shows the position of 26 µg (350 pmol) of free tryptophanyl-tRNA synthetase. Gradient 2 shows the enzyme (●) in two peaks, one representing a complex with tRNA<sup>Trp</sup> (○) formed by mixing immediately before sedimentation the 350 pmol of enzyme (7.0 x 10⁻⁶ M) with 0.094 A₂₆₀ unit of tRNA<sup>Trp</sup> (1360 pmol/A₂₆₀ unit, 128 pmol, 2.5 x 10⁻⁶ M) which had been converted into the active conformation immediately before mixing as described in the legend of Fig. 2. In gradient 3 the same 7.0 x 10⁻⁶ M enzyme (●) was mixed immediately before sedimentation with 2.0 x 10⁻⁵ M (0.75 A₂₆₀ unit) tRNA<sup>Trp</sup> (○), which sediments in the complex peak with all of the enzyme and in a free tRNA<sup>Trp</sup> peak. In gradient 4 the 7.0 x 10⁻⁶ M enzyme (●) was mixed with 5.1 x 10⁻⁴ M tRNA<sup>Trp</sup> (○) immediately before sedimentation through the sucrose gradient, now containing 5.4 x 10⁻⁴ M tRNA<sup>Trp</sup> (4.0 A₂₆₀ units/ml) in addition to the usual ingredients. In gradient 5 the 7.0 x 10⁻⁶ M enzyme (●) was mixed with 2.0 x 10⁻⁴ M tRNA<sup>Trp</sup> (○) immediately before sedimentation through the sucrose gradient, now containing 2.0 x 10⁻⁵ M tRNA<sup>Trp</sup> (14.8 A₂₆₀ units/ml) in addition to the usual ingredients. In gradient 6 the 7.0 x 10⁻⁶ M enzyme (●) was mixed with 0.79 A₂₆₀ unit of <i>E. coli</i> mixed tRNA (○) containing no detectable tRNA<sup>Trp</sup> immediately before sedimentation through the sucrose gradient now containing that tRNA, 16.1 A₂₆₀ units/ml, in addition to the usual ingredients. Recoveries of enzyme activities were 42, 57, 56, 77, 82, and 52% in gradients 1 through 6, respectively. Recoveries of tRNA<sup>Trp</sup> were 88, 74, 58, and 62% in gradients 2 through 5, respectively, and recovery of tRNA<sub>Trp</sub> units was 69% in gradient 6. The input tRNA values are based on the tRNA mixed with the 5% and 20% sucrose solutions prior to formation of the gradients.
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synthetase activity to a position consistent with a 1:2 complex. The number of binding sites for tRNA<sub>TP</sub> thus becomes one per subunit, in accord with the binding sites for L-tryptophan and for tryptophanyl ATP ester (17). The method may be applicable to other synthetases for which only one tRNA binding site has been demonstrable even in the face of multiple binding sites for the other substrates.

Each subunit of tryptophanyl-tRNA synthetase has a single binding site for each substrate and a single, required thiol group. The role of the thiol in enzyme function and the structures of thiol peptides are discussed elsewhere (31).

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