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Ligand Binding Stoichiometries, Subunit Structure, and Slow Transitions in Aminoacyl-tRNA Synthetases†

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ABSTRACT: The binding of various combinations of ^{14}C-labeled amino acid, ^{14}C-labeled ATP, and γ-^{32}P-labeled ATP to representative monomeric and dimeric aminoacyl-tRNA synthetases has been studied by equilibrium dialysis, equilibrium and nonequilibrium gel filtration, nitrocellulose disc filtration, and active site titration. The valyl-tRNA synthetase from *Escherichia coli,* a monomer belonging to the class containing regions of duplicated amino acid sequence, forms 1 mol of bound valyl adenylate rapidly. This is followed by the slow binding ($t_{1/2} \sim 11$ min) of an additional mole of chemically unreacted valine, showing that there is a second binding site. Similar evidence has not been obtained for other monomeric enzymes investigated. Another slow process is found in the reactions of the dimeric tyrosyl-tRNA synthetases from *E. coli* and *Bacillus stearothermophilus.* It is known from previous studies that these bind only 1 mol of tyrosine and form only 1 mol of tyrosyl adenylate rapidly per mol of dimeric enzyme. It is now found that a second mole of tyrosyl adenylate is formed and bound with a half-life of several minutes. Although the physiological importance of these slow processes is not known, they provide information on molecular symmetry which is of importance in interpreting the results of x-ray diffraction studies and also provide evidence for the induced fit model of Koshland.

The aminoacyl-tRNA synthetases are a structurally diverse set of enzymes. They may be divided into three main classes with further subdivisions: monomers with molecular weights ranging from 55 000 to 120 000, although typically 110 000; dimers of the form α_2 of molecular weight 2 × 35 000 to 2 × 85 000; and tetramers of the form $\alpha_2\beta_2$ (Soll and Schimmel, 1974). There is the further complication that the monomeric enzymes of molecular weight 110 000 have extensive regions of duplicated primary structure (Koch et al., 1974; Kula, 1973; Waterson and Konigsberg, 1974; Bruton, 1975). One possibility is that these monomers are functionally similar to the dimers of molecular weight about 2 × 50 000; that is, they are effectively "covalently linked" dimers. We have been conducting a comparative survey of the dimeric and monomeric enzymes to see if this is so and to obtain information about the

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reaction mechanism, if there is a common mechanism, by gleaning different aspects from the different enzymes.

We have shown so far that the tyrosyl-tRNA synthetase from *Bacillus stearothermophilus,* a dimer of 2 × 45 000 daltons, binds only 1 mol of tyrosine per mol of dimer in the absence of ATP but 2 in its presence (Fersht, 1975), and that only 1 mol of tyrosine binds under the conditions of the pyrophosphate exchange reaction (Fersht et al., 1975b). It was also shown that only 1 mol of tyrosyl adenylate was rapidly formed, but the presence of a second slowly formed aminoacyl adenylate was detected indirectly (Fersht, 1975). However, an extensive search failed to isolate the complex containing two bound adenylates (Bosshard et al., 1975). Similar behavior was found for the methionyl-tRNA synthetase (2 × 82 000 daltons) from *B. stearothermophilus,* although the complex of two methionyl adenylates was isolated and their rates of formation at the two sites directly measured and found to be greatly different (Mulvey and Fersht, 1976). Furthermore, evidence was presented that the monomeric valyl-tRNA synthetase (110 000 daltons) from *B. stearothermophilus* also binds a second mole of valine in the presence of ATP (Fersht, 1975).

We have now isolated these and other enzymes in larger quantities and have been able to delineate more precisely the nature of the binding sites and the bound species in the amino acid activation reaction.

Experimental Section

Materials. The tyrosyl-tRNA synthetases from *Escherichia coli* and *B. stearothermophilus* and the isoleucyl-tRNA synthetase of *E. coli* were obtained as described previously (Jakes and Fersht, 1975; Koch, 1974; Fersht and Kaethner, 1976a).

Partially purified valyl-tRNA synthetases of *B. stearothermophilus* and *E. coli* were obtained from the Microbiological Research Establishment, Porton Down, Wiltshire, England. The enzyme from *B. stearothermophilus* was further purified by the following chromatographic steps: (1) DEAE[1]-Sephadex, pH 7.5 Tris-Cl , 100–300 mM (the enzyme being eluted at 250 mM Tris); (2) hydroxylapatite, phosphate buffer (pH 6.8), 50–200 mM (the enzyme being eluted at 120 mM phosphate); (3) phosphocellulose, phosphate buffer (pH 6.8), 30 mM (the enzyme is not retarded); (4) Sephadex G-150, pH 7.5–Tris-Cl, 75 mM.

The valyl-tRNA synthetase from *E. coli,* EM 20031 (a K12 strain), was purified in a similar series of chromatographic steps: (1) DEAE-Sephadex, pH 6.9 phosphate buffer, 50–300 mM (the enzyme being eluted at 250 mM phosphate); (2) hydroxylapatite, pH 6.8 phosphate buffer, 50–250 mM (the enzyme being eluted at 180 mM phosphate); (3) DEAE-Sephadex, pH 7.8 phosphate buffer, 150–300 mM (the enzyme being eluted at 250 mM phosphate); (4) Sephadex G-150, pH 7.5 Tris-Cl, 75 mM.

All enzymes were purified to homogeneity. Each preparation ran as a single band in polyacrylamide gel electrophoresis with sodium dodecyl sulfate, and when assayed by active site titration (Fersht et al., 1975a) they gave 96% purity or better.

All enzymes were stored at −20 °C in 50% glycerol, 10 mM mercaptoethanol, 0.1 mM phenylmethanesulfonyl fluoride, and 0.144 M Tris-Cl (pH 7.78). Activity was routinely checked by the nitrocellulose filter assay and active site titration, and proteolysis by polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

Enzyme Molarities. The concentrations of the enzyme solutions were determined by active site titration (Fersht et al., 1975a) monitoring the burst of [^{32}P]orthophosphate from [γ-^{32}P]ATP (Fersht and Kaethner, 1976a) or by nitrocellulose filtration (Yarus and Berg, 1970). Concentrations were also estimated using the following molecular weights and A_{280} values (cm^{-1} mg^{-1} mL^{-1}): (for the synthetases from *E. coli*) isoleucyl, 110 000, 1.8 (Fersht and Kaethner, 1976a); valyl, 110 000, 1.8 (see below); tyrosyl 95 000, 1.11 (Jakes and Fersht, 1975, where the inverse of the A_{280} was reported); (for the synthetases from *B. stearothermophilus* (Koch et al., 1974)) valyl, 110 000, 2.2; tyrosyl, 95 000, 1.39; and methionyl (Mulvey and Fersht, 1976), 164 000, 1.55.

The A_{280} of the valyl-tRNA synthetase from *E. coli* was determined by amino acid analysis. Protein samples from a solution of known optical density were hydrolyzed in 6 N HCl in vacuo for 24, 40, and 80 h with and without prior oxidation in performic acid. Analyses were performed on a Durrum amino acid analyzer D-500.

Standard Conditions. All experiments were performed at 25 ± 0.1 °C in buffers containing 10 mM $MgCl_2$, 10 mM mercaptoethanol, 0.1 mM phenylmethanesulfonyl fluoride, and 144 mM Tris-Cl at pH 7.78 or, where stated, 13 mM Bistris-Cl at pH 5.87.

Equilibrium dialysis and equilibrium gel filtration were conducted as described by Jakes and Fersht (1975). The details of enzyme and substrate concentrations used in the equilibrium gel filtration experiments are given in Table III. Where ATP binding was studied with a mixture of [γ-^{32}P]ATP and [^{14}C]ATP, the isotopes were monitored using Cerenkov radiation and scintillation counting, as described below.

Differential Mobilities of [^{32}P]Orthophosphate and [^{14}C]ATP on G-25 Sephadex Determined by Sequential Cerenkov and Scintillation Counting. The column used for the gel filtration experiments (a tuberculin syringe, 6 × 0.46 cm, with a siliconized record tip, containing Sephadex G-25 fine) was equilibrated with ATP (100 μM) in the Tris-Cl buffer. A solution (100 μL) containing [^{14}C]ATP (10 nmol, 0.3 μCi) and [^{32}P]orthophosphate (10 nmol, 0.04 μCi) in the Tris-Cl buffer was added to the column and eluted with the buffer used for the equilibration. The individual drops were collected in polythene scintillation vials and assayed for their radioactivity initially by Cerenkov radiation, which detects the ^{32}P only (40% efficiency), and then after the addition of a water-miscible scintillant (to detect both ^{32}P and ^{14}c).

Time Dependence of Filter Assays. Aliquots (20 μL) of the solution of enzyme, incubated with either labeled amino acid or ATP at 25 °C, were spotted onto presoaked nitrocellulose filters (Schleicher and Schüll, BA 85) and washed with cold Bistris-Cl buffer (3.0 mL), and the radioactivity was monitored after drying.

Preparation and Hydrolysis of Aminoacyl Adenylates. The hydrolysis rates of the enzyme-bound aminoacyl adenylates were measured as previously described (Fersht, 1975). The enzyme-free aminoacyl adenylates were prepared by a modification of the method of Baldwin and Berg (1966). The enzyme-bound adenylate was stripped of excess ligands by gel filtration (Sephadex G-25) in Bistris-Cl buffer, and, as a further precaution, alkaline phosphatase was added to remove any residual ATP. The protein was precipitated by the addition of 5% trichloroacetic acid (20 μL) to aliquots (100 μL). After centrifugation, aliquots (10 μL) of the supernatant were added to the Tris-Cl buffer (1.0 mL) and the hydrolysis rate was measured using the cognate aminoacyl-tRNA synthetase to

[1] Abbreviations used: DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; Bistris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane.

TABLE I: Amino Acid Composition of Valyl-tRNA Synthetase from *E. coli* K12.

Amino acid	Composition	
	EM 20031[a]	HfrC[b]
Cys	9	11
Asp	102	140
Thr	51	32
Ser	36	35
Glu	129	150
Pro	40	28
Gly	67	93
Ala	100	145
Val	72	78
Met	34	17
Ile	60	58
Leu	81	67
Tyr	18	39
Phe	28	22
His	14	5
Lys	57	47
Arg	48	37
Trp	[c]	11

[a] This study, based on a mol wt of 110 000. [b] Yaniv and Gros (1969). [c] Not determined directly; the high value of the A_{280} suggests 30–35 Trp.

TABLE II: Active Site Titration by the Burst Method.[a]

Enzyme (source)	$[Enz]_0$ (μM)	$[ATP]_0$ (μM)	n	k_h[b] $\times 10^3$ (s^{-1})
VRS (*E.c.*)	1–2	5–8	0.97	0.94
VRS (*B.s.*)	1–3	6	0.96	0.9
IRS (*E.c.*)[c]	1–2	10	1.0	1.67
MRS (*E.c.*)	1–1.6	5.5	2.18	4.4
MRS (*B.s.*)[d]	1–2	10	2.0	4.9
PRS (yeast)[e]	1.5–6	25	2.05	10
TRS (*E.c.*)	5.5	46	1.18	6.5
	106	948	1.15	
TRS (*B.s.*)	2.8	11	1.18	0.8

[a] All results obtained at pH 7.78 and 10 mM $MgCl_2$ (*E.c., Escherichia coli; B.s., Bacillus stearothermophilus*). [b] Steady-state turnover of ATP after burst (mol of ATP hydrolyzed/mol of enzyme—not per active site). [c] Fersht and Kaethner (1976a). [d] Mulvey and Fersht (1976). [e] Fasiolo et al. (1977).

transfer the amino acid residue from the remaining aminoacyl adenylate to tRNA.

Results

The valyl-tRNA synthetase of *E. coli* is known to be a monomer with a molecular weight of 110 000 (Yaniv and Gros, 1969). These results have been confirmed by gel filtration chromatography and electrophoresis on denaturing polyacrylamide gels. However, the amino acid composition of the enzyme purified in this study (Table I) differs extensively from that reported by Yaniv and Gros (1969) even though they investigated the enzyme from a similar *E. coli* K12 strain. The A_{280} determined in this study, 1.8 cm^{-1} mg^{-1} mL^{-1}, is considerably higher than the value reported by Yaniv and Gros.

During purification of the valyl-tRNA synthetase from *B. stearothermophilus*, the enzyme elutes in two poorly resolved peaks from hydroxylapatite (step 2 in the purification). This result is reproducible and has been found by other workers (G. L. E. Koch, personal communication). We have purified the material from both fractions separately but have worked

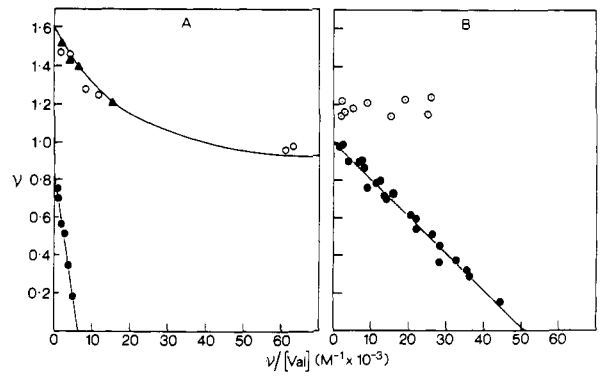

FIGURE 1: Stoichiometry of valine binding measured by equilibrium dialysis. (A) Valyl-tRNA synthetase from *E. coli* (79 μM), yeast inorganic pyrophosphatase (1 unit/mL), [^{14}C]Val (40–1000 μM, 11 Ci/mol); (○) 5 mM ATP; (●) absence of ATP; (▲) stoichiometries measured by nitrocellulose disk filtration (see Figure 6). (B) Valyl-tRNA synthetase from *B. stearothermophilus* (9–64 μM), yeast inorganic pyrophosphate (1 unit/mL), [^{14}C]Val (5–900 μM, 12–64 Ci/mol); (○) 10 mM ATP; (●) no ATP added.

mainly with the slower running fraction which constitutes about two-thirds of the activity. The two forms are probably very similar: N-terminal sequences of reduced and carboxymethylated valyl-tRNA synthetase from both fractions were determined to about residue 20 using a Beckman 890 B sequencer and were found to be identical; both have a molecular weight of 110 000 on polyacrylamide gel electrophoresis under reducing and denaturing conditions; both bind valine with the same association constant and stoichiometry.

Active Site Titration–Detection of Rapidly Formed Aminoacyl Adenylate. The concentration of enzyme active sites can be estimated by measuring a burst of ATP depletion or phosphate release during the formation of aminoacyl adenylate (Fersht et al., 1975a; Fersht and Kaethner, 1976a). This procedure detects only those adenylates which accumulate rapidly compared with the rate of hydrolysis or dissociation of the enzyme-bound aminoacyl adenylate. The monomeric synthetases give a burst corresponding to a single rapidly formed aminoacyl adenylate (Table II). It was previously reported that the rate of valyl adenylate formation with valyl-tRNA synthetase (*B. stearothermophilus*) is slow, perhaps because of a high K_M for ATP. Since then we have found that this enzyme loses activity reversibly when stored at −20 °C in glycerol. Full activity may be regained by incubating the enzyme for an hour at 37 °C in buffer containing 10 mM mercaptoethanol. The pre-steady-state formation of adenylate on the "reactivated" enzyme is then too fast to be observed in this titration procedure.

The phenylalanyl-tRNA synthetase of yeast and the methionine activating enzymes of *E. coli* and *B. stearothermophilus* each accumulate two adenylates. Though in the last case it has been shown that the two adenylates are formed at very different rates (Mulvey and Fersht, 1976) and the same appears true with the phenylalanyl-tRNA synthetase (Fasiolo et al., 1977). The dimeric tyrosyl-tRNA synthetases give a burst corresponding to just over one adenylate per mole of enzyme. Titrations of 106 μM tyrosyl-tRNA synthetase (*E. coli*) with 948 μM ATP or 5.5 μM enzyme with 46 μM ATP were performed using the rapid quenched-flow technique (Fersht and Jakes, 1975) and gave a stoichiometry of 1.15 mol of rapidly formed adenylate per dimer.

Equilibrium Dialysis and Amino Acid Binding. (a) Absence of Other Ligands. In the absence of other ligands, the monomeric synthetases bind only 1 mol of amino acid per mol of enzyme at experimentally accessible concentrations of the

TABLE III: Experimental Conditions for Equilibrium Gel Filtration.[a]

Expt No.	Aminoacyl-tRNA synthetase		Amino acid		ATP	
	Source	Concn (μM)	Label	Concn (μM)	Label	Concn (μM)
1	IRS (*E.c.*)	44	[^{14}C]Ile	204		
2		44	[^{14}C]Ile	204	ATP	4×10^3
3		40	Ile	200	[^{14}C]ATP	200
4		39	Ile	200	[γ-^{32}P]ATP	190
5	VRS (*E.c.*)	65	[^{14}C]Val	205		
6		65	[^{14}C]Val	205	ATP	4×10^3
7		44	[^{14}C]Val	170	ATP	5×10^3
8		35	Val	2×10^3	[^{14}C], [γ-^{32}P]ATP[b]	120
9	VRS (*B.s.*)	46	[^{14}C]Val	218		
10		46	[^{14}C]Val	218	ATP	4×10^3
11		19	Val	2×10^3	[^{14}C]ATP	119, 152[c]
12		19	Val	2×10^3	[^{14}C], [γ-^{32}P]ATP[b]	122
13		19	Val	2×10^3	[γ-^{32}P]ATP	110
14		26	[^{14}C]Thr	110, 205[c]	ATP	4×10^3
15	TRS (*E.c.*)	33	[^{14}C]Tyr	135		
16		33	[^{14}C]Tyr	135	ATP	4×10^3
17		30	Tyr	250	[^{14}C]ATP	86
18		30	Tyr	250	[γ-^{32}P]ATP	95
19	TRS (*B.s.*)	15	[^{14}C]Tyr	56		
20		15	[^{14}C]Tyr	56	ATP	5×10^3
21		15	Tyr	200	[^{14}C]ATP	70, 100[c]
22		15	Tyr	200	[γ-^{32}P]ATP	70, 100[c]

[a] pH 7.78, 25 °C. [b] Mixture of ^{14}C-labeled and γ-^{32}P-labeled ATP. [c] Results in Table IV average of experiments at two ligand concentrations.

amino acid (Table IV). This is also true of the tyrosyl-tRNA synthetases (Fersht, 1975; Jakes and Fersht, 1975), the methionyl-tRNA synthetase from *B. stearothermophilus* (Mulvey and Fersht, 1976), and phenylalanine-tRNA synthetase from yeast (Fasiolo et al., 1977).

(b) Presence of ATP. When the monomeric synthetases are incubated with excess ATP and inorganic pyrophosphatase, the amino acid binding increases, often to give a stoichiometry above 1. Thus the valyl-tRNA synthetases from *E. coli* and *B. stearothermophilus* bind respectively 1.5 and 1.2 mol of valine under these conditions (Figure 1). Similar experiments with isoleucyl-tRNA synthetase from *E. coli* gave stoichiometries close to 1.0 and occasionally higher so that it is difficult to decide conclusively whether this enzyme has a second binding site for amino acid. The dimeric synthetases all bind 2 mol of amino acid in the presence of ATP and pyrophosphatase, as does the phenylalanyl-tRNA synthetase from yeast (Fasiolo et al., 1977).

The stoichiometries reported previously for ligand binding to tyrosyl-tRNA synthetase (*E. coli*) (Jakes and Fersht, 1975) are about 26% too low because an incorrect value of A_{280} had been used to calculate the enzyme concentration (in fact, the inverse of the real value). The correct value for the A_{280} is 1.11 mg^{-1} mL^{-1} cm^{-1}. The stoichiometry of tyrosine binding should be adjusted to 0.83 in the absence and 1.9 in the presence of ATP and pyrophosphatase. This same correction should be made for other stoichiometries reported in that paper.

Does Aminoacyl Adenylate Accumulate in Solution? Equilibrium dialysis experiments where a chemical reaction is occurring, such as the formation and hydrolysis of aminoacyl adenylate in the present study, may be complicated by the formation of reaction products and the accumulation of intermediates free in solution. It is possible to calculate the maximum concentration of free aminoacyl adenylate that can accumulate in the equilibrium dialysis chamber containing the enzyme and to give an upper limit on the error this could introduce into the measured stoichiometries. The steady-state concentration of free aminoacyl adenylate is given by the ratio of its rate of formation from the enzyme-bound complex to the rate of its destruction (i.e., its destruction by hydrolysis, diffusion into the other dialysis chamber, and recombination with the enzyme). The rate of production of free aminoacyl adenylate cannot be any faster than the apparent rate of hydrolysis of the enzyme-bound aminoacyl adenylate under the conditions of the active site titration experiments (eq 1).

$$E \xrightarrow[\text{fast}]{ATP,AA} E{\cdot}AA{\sim}AMP \xrightarrow[\text{slow}]{k_h} E + AA + AMP \quad (1)$$
$$\uparrow \qquad\qquad + \qquad\qquad \downarrow$$
$$\leftarrow\!\!\!- \qquad PP_i \qquad\!\!\!-\!\!\!\rightarrow$$

The destruction of the E·AA~AMP complex with the rate constant k_h occurs by either the direct hydrolysis of the enzyme-bound intermediate or by the rate-determining dissociation of the complex followed by the hydrolysis of the aminoacyl adenylate in solution. Thus k_h (listed in Table II) gives an upper limit for the rate constant for dissociation. The first-order rate constants for the hydrolysis of isoleucyl, valyl, and tyrosyl adenylates free in solution at pH 7.78 and 25 °C were measured in this study to be 2.9×10^{-3}, 3.7×10^{-3}, and 7.6×10^{-3} s^{-1}, respectively. Assuming that the maximum rate of formation of the free aminoacyl adenylate is k_h[E] (where [E] is the concentration of enzyme), the *upper limits* on the concentrations of the free aminoacyl adenylates are given by [Ile~AMP] = 0.6[IRS], [Val~AMP] = 0.24[VRS] (*E. coli* and *B. stearothermophilus*), and [Tyr~AMP] = 0.1[TRS] (*B. stearothermophilus*), and [Tyr~AMP] = 0.86[TRS] (*E. coli*). If the aminoacyl adenylates accumulate to these levels in the chamber of the equilibrium dialysis cell containing enzyme, and not in the other because of slow passage across the membrane, the measured stoichiometry of amino acid binding will be artefactually high by these amounts. The above values

TABLE IV: Summary of Data from Equilibrium Dialysis and Equilibrium Gel Filtration.[a]

Enzyme (source)	Expt No.	Equilibrium gel filtration: Ligand labeled (unlabeled)	ν	ν_∞[b]	Equilibrium dialysis: ν	Equilibrium dialysis: K_S (μM)
IRS (*E.c.*)	1	[^{14}C]Ile	1.00	1.03	1.0	6.7[c]
	2	[^{14}C]Ile (ATP)	1.06			
	3	[^{14}C]ATP (Ile)	1.04			
	4	[γ-^{32}P]ATP (Ile)	0			
VRS (*E.c.*)	5	[^{14}C]Val	0.61	0.98	0.9	128
	6	[^{14}C]Val (ATP)	0.99			
	7	[^{14}C]Val (ATP)	1.25[d]		1.6	
	8[e]	[^{14}C]ATP (Val)	1.08			
		[γ-^{32}P]ATP	<0.05			
VRS (*B.s.*)	9	[^{14}C]Val	0.93	1.02	1.0	19.6
	10	[^{14}C]Val (ATP)	1.05		1.2	
	11	[^{14}C]ATP (Val)	0.99			
	12[e]	[^{14}C]ATP (Val)	1.06			
		[γ-^{32}P]ATP	<0.08			
	13	[γ-^{32}P]ATP (Val)	<0.07			
	14	[^{14}C]Thr (ATP)	0.96			
TRS (*E.c.*)	15	[^{14}C]Tyr	0.67	0.80	0.82	24[f]
	16	[^{14}C]Tyr (ATP)	1.45			
	17	[^{14}C]ATP (Tyr)	1.37			
	18	[γ-^{32}P]ATP (Tyr)	0			
TRS (*B.s.*)	19	[^{14}C]Tyr	0.96	1.15	0.92	11.6
	20	[^{14}C]Tyr (ATP)	1.73			
	21	[^{14}C]ATP (Tyr)	1.66			
	22	[γ-^{32}P]ATP (Tyr)	0			

[a] pH 7.78, 25 °C. [b] Extrapolated to infinite ligand concentration using the value of K_S determined by equilibrium dialysis and concentrations in Table IV. [c] Fersht and Kaethner (1976). [d] Reaction mixture incubated 40 min before loading onto column. [e] Mixture of ^{14}C- and ^{32}P-labeled ATP. [f] Jakes and Fersht (1976).

for the tyrosyl-tRNA synthetase from *B. stearothermophilus* and the valyl-tRNA synthetase from *E. coli* are too low to account for the increase in stoichiometry above 1. The high stoichiometries observed for the binding of amino acid in the presence of ATP to these enzymes are thus real. However, the high binding to the other enzymes could be artefactual. Because of this, we turned to equilibrium gel-filtration experiments to check the results. In this procedure, the enzyme-bound complexes are separated from the reaction products and intermediates that dissociate into solution.

Binding Stoichiometries from Equilibrium Gel Filtration. The conditions for several equilibrium gel filtration experiments are described in Table III and the results for each experiment are summarized in Table IV. In this procedure, the length of incubation of enzyme and substrates is short (10–15 min) compared with equilibrium dialysis (90–120 min) and the binding of aminoacyl adenylate formed in situ may be estimated using either labeled amino acid or labeled ATP. Further, by using [γ-^{32}P]ATP, the binding of unreacted ATP can be detected under reaction conditions since the label is lost on forming aminoacyl adenylate or AMP. However, results of experiments using [γ-^{32}P]ATP are sometimes complicated because Sephadex G-25 retards the passage of ATP relative to inorganic phosphate. Any $^{32}P_i$ produced on the hydrolysis of the ATP travels faster than the parent ATP and gives rise to a peak and trough. This is mimicked in a control experiment, shown in Figure 2, where equimolar quantities of $^{32}P_i$ and [^{14}C]ATP were passed through the 1-mL Sephadex column. A peak of $^{32}P_i$ appears where the "trough" would be in a normal equilibrium gel-filtration experiment where there is no retardation of small ligands. An additional trough follows this. The binding stoichiometries in experiments where this complicating phenomenon occurs may be calculated from the area

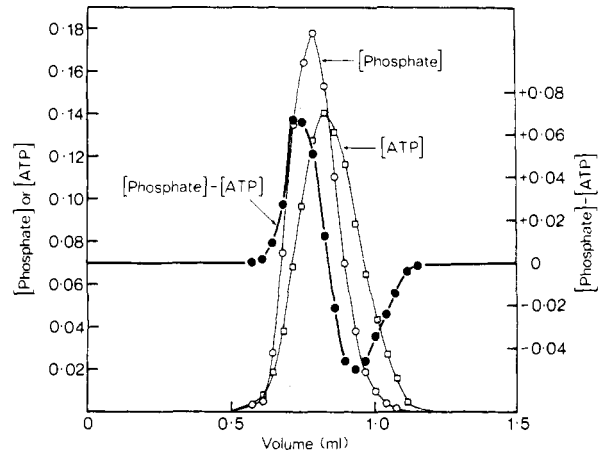

FIGURE 2: The separation of [^{32}P]orthophosphate and [^{14}C]ATP on Sephadex G-25 that gives rise to artefactual peaks and troughs in equilibrium gel filtration (ordinate = fraction of total material).

under the peak containing the enzyme, since this is not affected by the rate of elution of small enzyme-free ligands.

Figure 3 shows the elution patterns of the equilibrium gel filtration experiments with the isoleucyl-tRNA synthetase of *E. coli* (Table IV, experiments 1–4). At 204 μM isoleucine, one site on the enzyme is saturated. When isoleucine, ATP, and pyrophosphate are incubated with the enzyme, the stoichiometry remains close to one. The absence of any bound [γ-^{32}P]ATP under these conditions suggests that the ligands are bound as isoleucyl adenylate. The position of the small peak and trough in the elution profile of this experiment (see Figure 3) shows that it is due to the retardation of ATP relative to

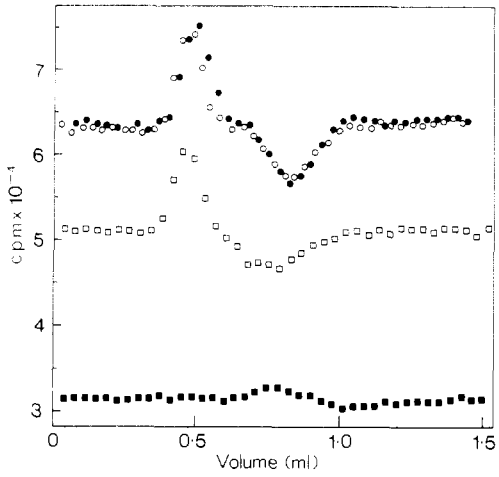

FIGURE 3: Equilibrium gel-filtration experiments with the isoleucyl-tRNA synthetase from *E. coli* (40 μM). The upper curves show the binding of [^{14}C]Ile (204 μM, 6.0 Ci/mol) in the absence (○) and presence (●) of 4.0 mM ATP. (The latter points have been displaced to the right for greater clarity.) The lower curves show the binding of [^{14}C]ATP (□, 200 μM, 5.0 Ci/mol) and [γ-^{32}P]ATP (■, 190 μM, 3.2 Ci/mol) in the presence of 200 μM isoleucine. All solutions contain 1 unit/mL inorganic pyrophosphatase. See Tables III and IV, experiments 1-4.

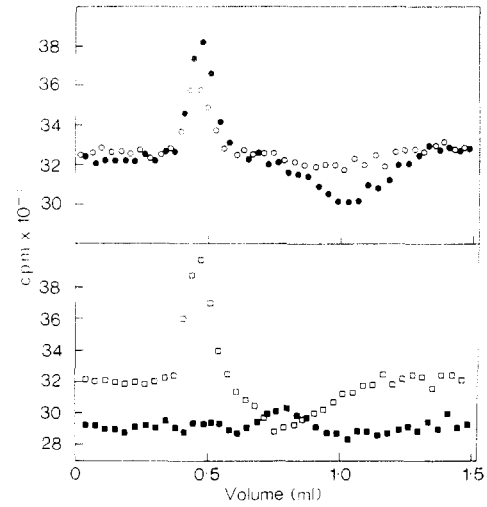

FIGURE 5: Equilibrium gel-filtration experiments with the tyrosyl-tRNA synthetase from *E. coli* (30 μM). Top: the binding of [^{14}C]Tyr (135 μM, 3.6 Ci/mol) in the absence (○) and presence (●) of 4.0 mM ATP. Bottom: the binding of [^{14}C]ATP (□, 86 μM, 5.8 Ci/mol) and [γ-^{32}P]ATP (■, 95 μM, 4.2 Ci/mol) in the presence of 250 μM tyrosine. All solutions contain 1 unit/mL inorganic pyrophosphatase. See Tables III and IV, experiments 15-18.

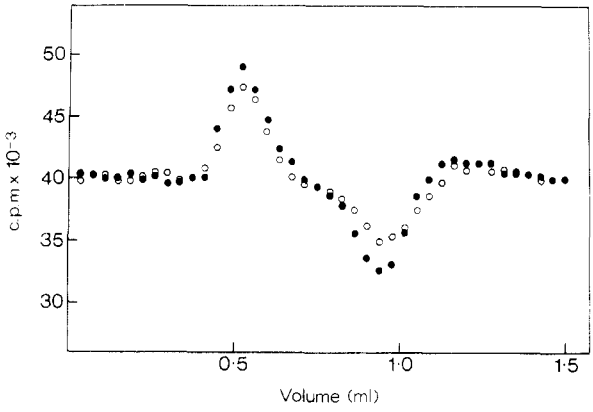

FIGURE 4: Time-dependent change in the binding of valine to the valyl-tRNA synthetase (44 μM) from *E. coli*. The enzyme and the column of Sephadex G-25 were equilibrated with [^{14}C]Val (170 μM, 4.9 Ci/mol), ATP (5.0 mM), and inorganic pyrophosphatase. Samples were loaded onto the column after 1-min incubation (○), and after 40-min incubation (●). See Tables III and IV, experiment 7.

orthophosphate on Sephadex G-25 and not to the binding of unreacted [γ-^{32}P]ATP.

Valine only partially saturates the valyl-tRNA synthetase (*E. coli*) at a concentration of 205 μM (Table IV, experiment 5). The presence of 4 mM ATP and pyrophosphatase increases this stoichiometry to 1. However, if the enzyme and its substrates are incubated at 25 °C for 40 min before loading on the column, the stoichiometry increases to 1.25. The effect of preincubation is shown in Figure 4 where samples from the same reaction mixture were gel filtered at 1 min and 40 min after mixing. This slow increase in stoichiometry was studied in more detail by nitrocellulose filtration (see below).

The valyl-tRNA synthetase from *B. stearothermophilus* gives a similar pattern of results (see Table IV, experiments 9-14). From these it is not clear that the presence of ATP alters the stoichiometry of valine binding. Though the K_S for threonine is probably above 10^{-3} M (Fersht and Kaethner, 1976b), it is possible to observe a stoichiometry of one threonine bound in the presence of ATP and pyrophosphatase, presumably as the threonyl adenylate.

The results obtained with the tyrosyl-tRNA synthetase (Table IV, experiments 15-22 and Figure 5) are similar with the enzymes from both *E. coli* and *B. stearothermophilus*. Tyrosine will bind to one site in the absence of ATP, but, when ATP, pyrophosphatase, and tyrosine are present, the stoichiometry is as high as 1.5 or more. The lack of bound [γ-^{32}P]-ATP suggests that the ligands are bound at both sites as tyrosyl adenylate. (The failure to observe saturation of the second binding site was subsequently found to have been caused by not incubating the reaction mixture for a sufficient period before loading on to the column—see below.)

Active Site Titration by Nitrocellulose Disc Filtration. The accumulation of enzyme-bound aminoacyl adenylate may be measured by trapping the ^{14}C-labeled complex on a nitrocellulose filter (Yarus and Berg, 1970). This assay has the advantage of being rapid and was used primarily to monitor changes in the stoichiometry of aminoacyl adenylate or amino acid binding. The monomeric synthetases examined in this study accumulate close to one aminoacyl adenylate per mole of enzyme when the complex is filtered within a minute of mixing the reactants (Table V). The tyrosine activating enzymes give stoichiometries between 1.2 and 2.0 depending on the precise conditions and the methionyl-tRNA synthetase binds 2.0 mol of methionyl adenylate per mol of enzyme.

Prolonged incubation of the isoleucyl-tRNA synthetase (*E. coli*) with its substrates before nitrocellulose filtration does not alter the stoichiometry of adenylate binding. However, the number of moles of [^{14}C]valine bound per mole of valyl-tRNA synthetase (*E. coli*) increases from 1 to plateau at a value which is dependent on the concentration of free amino acid (Figure 6). The half-life for this increase is about 11 min. If ^{14}C-labeled ATP is used, the incubation does not lead to any increase in stoichiometry (Figure 6) which suggests that only 1 mol of valine is bound as valyl adenylate and any additional valine is bound as unreacted amino acid. In Table V it may be seen that the final stoichiometry of valine binding is dependent on the ATP concentration over the range 0.2-1.9 mM. The final stoichiometries of valine binding are compared in Figure 1 with those observed by equilibrium dialysis. The two experiments, though very different in procedure, show excellent agreement.

TABLE V: Active Site Titration by Nitrocellulose Disc Filtration.[a]

Enzyme (μM)	Amino acid (μM)	ATP (μM)	ν[b]	ν_∞[c]	$t_{1/2}$[d] (min)
IRS (*E.c.*) (6.9)	[^{14}C]Ile (115)	ATP (5×10^3)	1.0	1.04	
	[^{14}C]Ile (315)	ATP (5×10^3)	1.0	1.07	
VRS (*E.c.*) (5.2)	[^{14}C]Val (78)	ATP (5×10^3)	1.03	1.20	10–12
	[^{14}C]Val (206)	ATP (5×10^3)	1.05	1.33	
	[^{14}C]Val (350)	ATP (5×10^3)	0.97	1.39	
	[^{14}C]Val (408)	ATP (5×10^3)	0.99	1.44	
	[^{14}C]Val (760)	ATP (5×10^3)	1.02	1.50	
(2.6)	[^{14}C]Val (455)	ATP (186)	1.03	1.25	
	[^{14}C]Val (455)	ATP (372)	1.01	1.33	
	[^{14}C]Val (455)	ATP (1.9×10^3)	1.05	1.43	
(5.0)	Val (2.5×10^3)	[^{14}C]ATP (219)	1.0	1.02	
	Val (1.8×10^3)	[^{14}C]ATP (673)	0.95	0.99	
VRS (*B.s.*) (3.7)	[^{14}C]Val (204)	ATP (5×10^3)	1.0	1.14	
	[^{14}C]Val (450)	ATP (5×10^3)	1.02	1.17	5
TRS (*E.c.*) (1.3)	[^{14}C]Tyr (7.3)	ATP (60)	1.2	1.29	8
	[^{14}C]Tyr (20)	ATP (5×10^3)	1.3	1.68	
TRS (*B.s.*) (1.7)	[^{14}C]Tyr (7.2)	ATP (60)	1.5	2.0	3.9
	Tyr (7.2)	[^{14}C]ATP (60)	1.2	1.6	
MRS (*B.s.*)	[^{14}C]Met (260)	ATP (2×10^3)	2.0	2.0	

[a] pH 7.78, 10 mM $MgCl_2$, incubation at 25 °C, filtered at room temperature, filter washed with 3 mL of 0.01 μM Bistris (pH 5.9). [b] Stoichiometry measured within 1 min after mixing reactants. [c] Stoichiometry after prolonged incubation of reactants. [d] Half-life for slow increase in stoichiometry.

The possibility that the protein becomes covalently labeled during the lengthy incubation through acylation of residues by the aminoacyl adenylate was ruled out by showing that no radioactivity is bound to the protein which has been precipitated by trichloroacetic acid. The slow increase in the binding of valine is not altered by preincubation of the enzyme at 25 °C for 1 h, nor is it changed by the addition of 50 μM AMP and 100 μM inorganic phosphate to the reaction mix nor by preincubation of the isolated enzyme–valyl adenylate complex at 25 °C before adding free substrates. These controls demonstrate that the slow step cannot be the result of a heat-induced conformational change which occurs in either the free enzyme or the complex with valyl adenylate in the absence of free substrates nor is it an artefact arising from the accumulation of AMP and phosphate in the reaction mix. The stoichiometry of the binding of valine showed a small variation with the batch of enzyme. For instance, a batch of the valyl-tRNA synthetase which had been stored for a longer period of time gave lower stoichiometries than those reported here (i.e., 1.3 mol of valine per mol of enzyme at the highest concentrations of free valine) but the agreement between the results of nitrocellulose disc filtration and equilibrium dialysis was still maintained. Interestingly enough, it was found that prolonged storage of the enzyme at 4 °C (i.e., for 1 month) followed by its incubation with ATP and [^{14}C]Val led to the covalent incorporation of [^{14}C]Val into the protein, as shown by retention of the radioactivity on acid precipitation.

The stoichiometry of valine binding to the valyl-tRNA synthetase from *B. stearothermophilus* showed a small increase with time (see Table V) but the magnitude of this change is only twice the margin of error in this filtration technique.

Similar experiments with the tyrosyl-tRNA synthetase also show a slow increase in the binding of [^{14}C]tyrosine (Table V). The equilibrium gel-filtration study suggests that all the ligands are bound as tyrosyl adenylate.

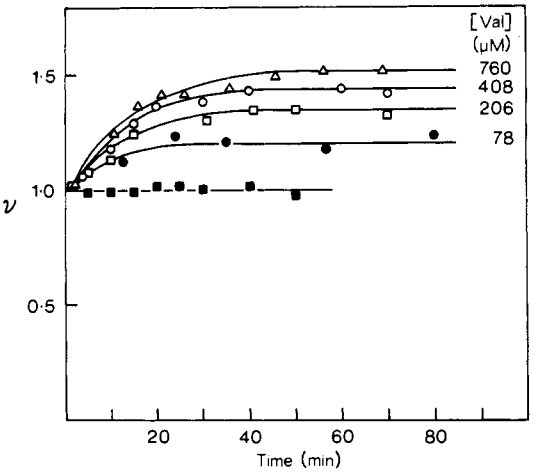

FIGURE 6: The time-dependent increase in the binding of valine to the valyl-tRNA synthetase from *E. coli* assayed by nitrocellulose disc filtration. The enzyme (5.2 μM) was incubated with [^{14}C]Val (9.6–30.0 Ci/mol), 4.0 mM ATP, and inorganic pyrophosphatase (2 units/mL). The control (■) contains [^{14}C]ATP (670 μM, 12.4 Ci/mol) and valine (1.8 mM).

At pH 7.78 the formation of the second methionyl adenylate on methionyl-tRNA synthetase (*B. stearothermophilus*) is too fast to be followed by nitrocellulose disc filtration. However, at 0 °C and pH 5.8, the half-life for formation of the second adenylate is over 0.5 min and its accumulation can be followed by this procedure (Mulvey and Fersht, 1976).

Isolation of the Enzyme-Bound Aminoacyl Adenylate

TABLE VI: Isolation of Enzyme-Aminoacyl Adenylate Complex by Gel Filtration.[a,b]

Enzyme	ν
IRS (*E.c.*)	1.0
VRS (*E.c.*)	1.03
	0.5[c]
VRS (*B.s.*)	0.9
	0.9[c]
TRS (*E.c.*)	1.4[d]
TRS (*B.s.*)	1.0
	1.85[e]
MRS (*B.s.*)	1.8

[a] Enzyme and substrates incubated for 1–2 min before application to column and elution in Bistris (pH 5.85) at 4 °C. [b] Enzyme concentration measured by A_{280} after elution from column. [c] VRS-threonyl adenylate complex. [d] Preincubation: 35 min. [e] Preincubation: 20 min.

Complexes by Gel Filtration. Enzyme-bound aminoacyl adenylate can be separated from excess substrates by gel filtration on Sephadex G-25. Using ^{14}C-labeled amino acids, the stoichiometries observed with the monomeric synthetases are close to 1.0 and did not vary with the length of incubation prior to gel filtration (Table VI). The stoichiometry observed with the tyrosyl-tRNA synthetases depended on the period of incubation as might be expected from previous studies with the nitrocellulose disc filtration. In contrast to the results of Bosshard et al. (1975), the tyrosyl-tRNA synthetase (*B. stearothermophilus*) in this study gave a complex of 1.85 mol of tyrosyl adenylate bound per mol of enzyme when preincubated with substrates for 20 min. Similarly, it was possible to isolate the methionyl-tRNA synthetase (*B. stearothermophilus*)-methionyl adenylate complex with stoichiometries as high as 1.8.

Discussion

Dimeric Aminoacyl-tRNA Synthetases. It was found previously (Fersht, 1975) that the tyrosyl-tRNA synthetase from *B. stearothermophilus* binds only 1 mol of tyrosine and (rapidly) forms only 1 mol of tyrosyl adenylate per mol of dimeric enzyme. In the presence of ATP and inorganic pyrophosphatase to allow the accumulation of tyrosyl adenylate, it was found that 2 mol of tyrosine bind to the enzyme. It was also inferred from indirect kinetic measurements, comparing first-order and second-order rate constants for the hydrolysis of enzyme-bound tyrosyl adenylate, that 2 mol of tyrosyl adenylate simultaneously bind to the enzyme to give a possibly symmetrical E·(Tyr~AMP)$_2$ complex. However, as an extensive search failed to detect the accumulation of this complex (Bosshard et al., 1975), it was assumed that the E·(Tyr~AMP)$_2$ complex was slowly formed and rapidly hydrolyzed so that it did not accumulate but was present at low steady-state concentrations only. Instead it was suggested that the predominant complex in solution was E·Tyr~AMP·ATP·Tyr. That is, the second moles of tyrosine and ATP in the E·Tyr~AMP complex were the tightly bound but chemically unreacted molecules. This description has to be revised in the light of two observations found in this study. Contrary to the findings of Bosshard et al. (1975), we find that the complex E·(Tyr~AMP)$_2$ is formed and may be isolated by nonequilibrium gel filtration and may be trapped by nitrocellulose filtration. Further, equilibrium gel-filtration experiments measuring the binding of [^{14}C]Tyr, [^{14}C]ATP, and [γ-^{32}P]-ATP show that two tyrosyl and two adenosyl moieties are bound to the enzyme and no unreacted [γ-^{32}P]ATP (Table IV). The second tyrosyl adenylate accumulates with a very slow half-life of about 3.8 min compared with 39 ms for the first (at saturating reagent concentrations). The accumulation of the second mole of tyrosyl adenylate is of course too slow to be of importance in the stepwise formation of tyrosyl-tRNA (eq 2) but it does give structural information about the enzyme.

$$\begin{array}{ccccccc} E + Tyr + ATP & \rightleftharpoons & E\cdot(Tyr\sim AMP)_2 & & & & \\ & & PP & \downarrow 2tRNA & & & (2) \\ & & & E + 2Tyr\text{-}tRNA & & & \end{array}$$

Thus, the main conclusions and hypotheses of the previous study have been confirmed: the enzyme exhibits negative cooperativity of binding of tyrosine with only 1 mol of tyrosine bound per mol of dimer; the enzyme exhibits (virtual) half-of-the-sites reactivity in forming only 1 mol of tyrosyl adenylate rapidly (k = 17.8 s^{-1}); the enzyme does form a second mole of tyrosyl adenylate to give an E·(Tyr~AMP)$_2$ complex (at $k = 3 \times 10^{-3}$ s^{-1}) which has now been isolated.

The reason for the failure to detect the E·(Tyr~AMP)$_2$ complex previously is not clear. One possibility is that the failure was an artefact of the preparation of enzyme used in those studies. We used the same preparation as Bosshard et al. (1975) and also were not able to detect the second mole of bound tyrosyl adenylate using the identical nitrocellulose filter assays used in the present study. The results presented in this study are reproducible with three different, fresh enzyme preparations.

Similar results are found with the tyrosyl-tRNA synthetase from *E. coli.* One mole of enzyme-bound tyrosyl adenylate is formed rapidly whilst a second (partly) accumulates with a half-time for formation of about 8 min. However, as pointed out previously (Jakes and Fersht, 1975), the experiments with this enzyme are not as clear-cut as those with the thermophilic enzyme because nonintegral stoichiometries are often found.

The tyrosyl activating enzymes are similar to the methionyl-tRNA synthetase from *B. stearothermophilus* which we have previously shown to form 1 mol of methionyl adenylate rapidly and one very slowly, and to bind only 1 mol of methionine in the absence of ATP (Mulvey and Fersht, 1976). In contrast, the methionyl activating enzyme from *E. coli* exhibits full site reactivity and binds 2 mol of methionine (Fayat and Waller, 1974).

The crystal structure of the tyrosyl-tRNA synthetase from *B. stearothermophilus* has been solved at high resolution and found to be a symmetrical dimer with no direct interactions between the sites (Irwin et al., 1976). The half-of-the-sites reactivity and negative cooperativity of binding are therefore caused by an induced fit mechanism (Koshland et al., 1966) rather than the Monod-Wyman-Changeux (1965) mechanism. The latter model requires that symmetry is maintained throughout the reaction, and it is clearly lost in this example.

Monomeric Aminoacyl-tRNA Synthetases. The binding experiments are designed to detect whether or not the repeated sequences in the monomeric enzymes form additional binding sites for ligands. The previous evidence on the valyl-tRNA synthetase from *B. stearothermophilus* appeared to demonstrate that there are two binding sites for valine. About 0.8 to 0.85 mol of valine per mol of enzyme was found to bind in the absence of other ligands, but 1.4 to 1.5 in the presence of ATP. The more extensive survey using larger quantities of enzyme in the present study indicates that the former values are respectively too low and too high by about 20%: 1.0 mol of valine binds in the absence of ATP and 1.2 in its presence. Although

this is now clearly inadequate evidence for a second binding site, the binding studies on the valyl-tRNA synthetase from *E. coli* give more positive results: 1.0 mol of valine is bound in the absence of ATP but up to 1.6 mol in its presence (Table IV and Figures 1 and 6). In the presence of ATP, the first mole of valine binds as a rapidly formed mole of valyl adenylate, but the second binds extremely slowly as free valine.

The physiological significance of the binding of the second mole of valine is not known; it is far too slow to be of importance in the stepwise aminoacylation of tRNA. However, it is good evidence for a second binding site. There is no direct evidence for a second binding site on the isoleucyl-tRNA synthetase from binding studies, but the presence of a second site could explain some anomalies in the kinetics of charging (Fersht and Kaethner, 1976a). There is evidence for a distinct and separate active site on the valyl- and isoleucyl-tRNA synthetases which catalyzes the hydrolysis of mischarged tRNAs (Fersht and Kaethner, 1976b; Fersht, 1977). Perhaps the second site found in the binding studies is related to this hydrolytic site?

Conformational States of the Tyrosyl-tRNA Synthetase. The solution studies on the tyrosyl-tRNA synthetase from *B. stearothermophilus* are being performed in parallel with crystallographic studies on the enzyme in this laboratory. One aim of the present work is to assist in the design and interpretation of crystallographic experiments in which the structures of various enzyme–substrate complexes are solved. The solution work suggests that the enzyme may take up different conformations of varying symmetry when different ligands are bound. Elucidation of structural aspects of the reaction mechanism would thus appear to be a formidable task. The following is a summary of the conformational changes involved (eq 3).

$$E_{(sym)} \rightarrow E_{(asym)}' \cdot Tyr \rightarrow E_{(asym)}''Tyr \sim AMP \rightarrow E_{(sym?)}'''(Tyr \sim AMP)_2 \quad (3)$$

The crystalline tyrosyl-tRNA synthetase is a symmetrical dimer (Irwin et al., 1976). Binding of tyrosine to the enzyme in solution induces a conformational change that destroys this symmetry: only 1 mol of tyrosine is bound per mol of dimer. The observed rate constant for the binding step is slow and almost certainly results from a two-step process in which there is a rapid binding step followed by a rearrangement (Fersht et al., 1975b). A two-step process has been directly observed for the binding of isoleucine to the monomeric isoleucyl-tRNA synthetase (Holler and Calvin, 1972). The enzyme is also asymmetric in the presence of ATP and pyrophosphate since only 1 mol of tyrosine binds under these conditions. However, in the presence of tRNA, ATP, and pyrophosphate, 2 mol of tyrosine is bound (Jakes and Fersht, 1975). The formation of aminoacyl adenylate probably involves a second conformational change in most aminoacyl-tRNA synthetases: there appears in general to be changes in the fluorescence of the protein or the fluorescence of noncovalently bound probes such as toluidinylnaphthalenesulfonic acid (Holler et al., 1971). Formation of the second mole of aminoacyl adenylate may also involve a further conformational change (there is some evidence for this for the methionyl-tRNA synthetase from *B. stearothermophilus;* Mulvey and Fersht, 1976). Kinetic studies on the $E\cdot(Tyr\sim AMP)_2$ complex suggest that this may be symmetrical (Fersht, 1975).

Acknowledgment

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