Demonstration of Two Active Sites on a Monomeric Aminoacyl-tRNA Synthetase. Possible Roles of Negative Cooperativity and Half-of-the-Sites Reactivity in Oligomeric Enzymes†

Alan R. Fersht

With the technical assistance of Jeremy Ashford

ABSTRACT: The dimeric tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* which binds (tightly) only one tyrosyl adenylate or tyrosine per dimer is shown from kinetic, equilibrium dialysis, and gel filtration methods to have a second active site. ATP and tyrosine bind strongly and synergistically to the tyrosyl-tRNA synthetase tyrosyl adenylate complex, [E · Tyr~AMP], to give the complex [E · Tyr~AMP,ATP,Tyr]. This complex probably slowly forms an [E · $(Tyr\sim AMP)_2$] complex which hydrolyses rapidly and does not accumulate. *Similarly, the monomeric valyl-enzyme is shown to have two active sites.* An [E · Val~AMP,ATP,Val] complex is formed which probably slowly gives an unstable [E · $Val\sim AMP)_2$] complex. In view of this and the recent demonstrations that several aminoacyl-tRNA synthetases are composed of repeating sequences it is suggested that all of these enzymes have at least two active sites. The second site is difficult to detect by normal steady-state kinetic measurements and binding assays as these enzymes exhibit negative cooperativity of substrate binding and half-of-the-sites reactivity. A mechanism based on interacting sites is proposed that could account for these observations: changes in binding energy at one site may be coupled with catalysis at the other to give large rate enhancements. However, this cannot account for the high specificity in the acylation of tRNA, a "verification" procedure seems essential. The proposed mechanism is quite general for catalysis and could be a reason why so many nonregulatory enzymes have subunits.

The tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* exhibits half-of-the-sites reactivity in apparently forming only one tyrosyl adenylate and binding only one tyrosine per enzyme dimer (Bosshard *et al.*, 1975; Fersht *et al.*, 1975). In this paper we investigate the role of the second site using as a probe the stimulation of the hydrolysis of the enzyme–aminoacyl adenylate complex by tyrosine and ATP. We shall show that the complex binds strongly and synergistically both ligands and that an evanescent enzyme · $(Tyr\sim AMP)_2$ complex is slowly formed and rapidly hydrolyzed. Also, once formed, both sites are approximately equally reactive toward hydrolysis.

Valyl-tRNA synthetase from *B. stearothermophilus* is a monomer of molecular weight 110,000, fractionally larger than the dimeric tyrosyl-enzyme of molecular weight 95,000. Koch and coworkers (Hartley, 1973; Koch *et al.*, 1974) have proved that this enzyme, along with certain others, including the leucyl-enzyme (MW 110,000 monomer), is composed of two repeating sequences which presumably arise from gene duplication and fusion. Subsequently it has been shown by other groups that the isoleucyl (MW 112,000, monomer), and the leucyl (MW 105,000, monomer) activating enzymes from *Escherichia coli* are also composed of two identical repeating sequences (Kula, 1973; Waterson and Konigsberg, 1974). In view of this we have also investigated the binding properties of the valyl-tRNA synthetase–valyl adenylate complex and we have found that this monomeric enzyme also has a second site which binds valine and ATP, and probably also forms an evanescent enzyme · $(Val\sim AMP)_2$ complex.

The following experiments are described in the Experimental Section: the enzyme-bound aminoacyl adenylates are prepared from ^{14}C-labeled amino acids and, after separating from free ATP and amino acid, their hydrolysis rates are determined; the binding of amino acid and/or ATP is measured by the stimulation of hydrolysis rate; the hydrolysis rates in the presence of added ATP and amino acid are also measured indirectly from the rate of consumption of ATP as the enzyme released on hydrolysis of the aminoacyl adenylate is free to recycle and form more aminoacyl adenylate; finally the binding of the amino acid and ATP to the enzyme-bound aminoacyl adenylate is measured directly by equilibrium dialysis and equilibrium gel filtration.

Experimental Section

Materials and apparatus and the activating enzymes (all from *B. stearothermophilus*) are described by Fersht *et al.* (1975a).

Methods and Kinetics Procedures. Unless otherwise stated all kinetic measurements were performed at 25 ± 0.1° in Tris-Cl buffers (pH 7.78, ionic strength 0.1, 0.144 M) and 10 mM $MgCl_2$. The aminoacyl-tRNA synthetases, which had been stored at −20° in 50% glycerol–water in Tris-Cl buffers, mercaptoethanol, and $PhCH_2SO_2F$[1] (phenylmethanesulfonyl fluoride) were prepared for kinetic purposes by desalting on a G-25 (fine) Sephadex column equilibrated with 0.144 M Tris-Cl (pH 7.78), 10 mM $MgCl_2$, 10 mM mercaptoethanol, and 10^{-4} M $PhCH_2SO_2F$.

Preparation and Hydrolysis of Enzyme-Bound Aminoacyl Adenylates. Stock solutions of enzyme–aminoacyl ade-

† From the MRC Laboratory of Molecular Biology, Cambridge CB2 2QH, England. *Received June 19, 1974.*

[1] Abbreviation used is: $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride.

nylate complexes were prepared daily and stored on ice. A solution (0.2 ml) of 7 μM tyrosyl-tRNA synthetase, 20 mM $MgCl_2$, 50 μM ATP, and 8.8 μM [^{14}C]tyrosine (141 Ci/mol) in 228 mM Tris-Cl (pH 7.78) containing 5 units of inorganic pyrophosphatase was desalted on either a 1 × 10 cm or 1 × 25 cm G-25 (fine) Sephadex column equilibrated with the same buffer and $MgCl_2$ and containing 10 mM mercaptoethanol and 10^{-4} M $PhCH_2SO_2F$ at 4° or room temperature.

Stock solutions of valyl-tRNA synthetase–valyl adenylate were prepared as above using 40 μM valine (73 Ci/mol) except that the complex, due to its instability, was always desalted at 4° using a 1 × 10 cm column.

The rates of hydrolysis of the complexes were monitored by adding 100 μl of complex to an equal volume of an aqueous solution of amino acid and/or ATP (adjusted to pH 7.8); 20 μl aliquots were periodically taken, added to a 2.5-cm Schleicher and Schüll BA 85 nitrocellulose filter washed with 3.0 ml of buffer, and dried, and the residual enzyme–adenylate complex was retained on the filter assayed in a toluene-based scintillant as described by Fersht *et al.* (1975a).

Exchange of [^{3}H]Tyrosine into Tyrosyl-tRNA Synthetase-[^{14}C]Tyr~AMP. A solution (200 μl) of tyrosyl-tRNA synthetase-[^{14}C]Tyr~AMP (0.76 μM) prepared as above was added to an equal volume of 2.16 μM [^{3}H]tyrosine (80 Ci/mol) and 140 μM ATP; 20-μl aliquots were periodically taken and the rate of decrease of enzyme-bound [^{14}C]adenylate and concomitant increase in [^{3}H]adenylate assayed by nitrocellulose filtration as above.

Hydrolysis of ATP due to Aminoacyl Adenylate Formation. A stock solution of the enzyme (0.1 ml) prepared as above was added to an equal volume of [γ-^{32}P]ATP, cognate amino acid, with or without 1 unit of inorganic pyrophosphatase in the same buffer, and $MgCl_2$ concentration. Aliquots were periodically taken and the residual ATP concentration was assayed by retention on charcoal and monitoring with a gas-flow counter or by chromatography on PEI sheets as described by Fersht *et al.* (1975a). Possible formation of orthophosphate in the absence of pyrophosphatase was checked by chromatography on PEI sheets. Some experiments were performed using [^{14}C]ATP (6 Ci/mol) and separating the AMP from ADP and ATP by developing on PEI sheets with 1.0 N formic acid (Randerath and Randerath, 1964).

ATPase Activity of Tyrosyl-tRNA Synthetase in the Absence of Amino Acid Position of Cleavage of ATP. A solution (0.2 ml) of 1.3 μM tyrosyl-tRNA synthetase, 50 μM [γ-^{32}P]ATP (10 Ci/mol), and 10 mM $MgCl_2$ in pH 7.78 Tris-Cl (0.144 M) was incubated at 25°. Periodically parallel aliquots of 10 μl were taken. One was spotted directly onto the origin of a PEI strip and the [γ-^{32}P]orthophosphate separated from the [γ-^{32}P]ATP and pyrophosphate as described by Fersht *et al.* (1975a). The other was incubated for a few seconds with inorganic pyrophosphatase to hydrolyze any pyrophosphate formed and chromatographed as above.

The initial rates of hydrolysis of ATP at varying concentrations were similarly monitored under the above conditions but using [^{14}C]ATP to give [^{14}C]AMP or by measuring the residual [γ-^{32}P]ATP by adsorption on charcoal as described earlier. Similar experiments at a fixed concentration of ATP were performed with the valyl-enzyme.

Active Site Titration. The tyrosyl-enzyme was routinely assayed by adding 20 μl of enzyme (~2 μM) to 50 μl of 8 μM [^{14}C]tyrosine (140 Ci/mol), 50 μM ATP in 144 mM Tris-Cl (pH 7.8), and 10 mM $MgCl_2$. After incubating for 1 min 50 μl was filtered through a presoaked nitrocellulose filter washed with 3.0 ml of 10 mM $MgCl_2$/Tris-Cl, dried, and monitored on a scintillation counter as above. The specific activity of the tyrosine was determined under the same conditions. The observed stoichiometry of the binding of aminoacyl adenylate was multiplied by 1.1 to normalize to that observed by the ATP burst procedure (Fersht *et al.*, 1975a).

Binding of Amino Acids Determined from Equilibrium Dialysis. Ten cells, constructed to the design of Englund *et al.* (1969), were mounted in a thermostated housing at 25°. One chamber of each cell contained aminoacyl-tRNA synthetase and 1 unit/ml of yeast inorganic pyrophosphatase in pH 7.78 Tris, 10 mM $MgCl_2$, 10 mM mercaptoethanol, and 5×10^{-5} M $PhCH_2SO_2F$. The other chamber contained the same buffer and concentration of pyrophosphatase varying concentrations of cognate ^{14}C-labeled amino acid with or without ATP. The two chambers were separated by a Sartorius membrane (SM 11539). After equilibration for 2 hr triplicate aliquots of 5 μl were taken and the amino acid was assayed with the scintillation counter.

Binding of ATP Determined from Equilibrium Gel Filtration. A solution of enzyme (95 μl of either 8.6 μM tyrosyl- or 2.7 μM valyl-tRNA synthetase) incubated in 10 mM $MgCl_2$, 144 mM Tris (pH 7.8), 1 unit/ml of inorganic pyrophosphatase, [^{14}C]ATP (either 20 μM, 5 Ci/mol; or 51 μM, 12 Ci/mol); and either 120 μM tyrosine or 500 μM valine was added to 1 ml of G-25 (fine) Sephadex packed in a 1-ml tuberculin syringe and equilibrated with the same buffered solution. Individual drops of eluent were collected in scintillation vials and the ATP was assayed. In some experiments [^{3}H]H_2O was added to check the drop size (43.9 μl).

Results

In the absence of added ligands the aminoacyl adenylates hydrolyze to give free enzyme, amino acid, and AMP, but in the presence of added cognate amino acid and ATP (and inorganic pyrophosphatase) as the labeled complex hydrolyses fresh unlabeled complex is formed by the enzyme which had been released. This is illustrated in Figure 1 which shows the time course of ^{3}H label exchanging into tyrosyl-tRNA synthetase-[^{14}C]Tyr~AMP after the addition of excess [^{3}H]Tyr and ATP.

(1) Direct Determination of Hydrolysis Rates of Enzyme-Bound Aminoacyl Adenylates. (a) TYROSYL-TRNA SYNTHETASE. The complex of tyrosyl-tRNA synthetase-[^{14}C]tyrosyl adenylate collected from the G-25 column generally had 0.9–1.0 bound tyrosyl adenylates as judged by its specific radioactivity and the absorbance of the enzyme. On filtering through the nitrocellulose filters 97–100% of the counts were retained. In the absence of added ligands the complex slowly hydrolyzed at 25° with rate constant 5.7 ± 0.1 × 10^{-5} sec^{-1}. On the addition of excess tyrosine and ATP the hydrolysis rate constant increased to 45.4 ± 1.2 × 10^{-5} sec^{-1}. The rate constant followed saturation kinetics with respect to tyrosine, the K_M being 4.0 ± 0.3 μM. The K_M for ATP was very low, ~0.09 μM. Tyrosine by itself stimulated hydrolysis, K_S = 144 ± 14 μM, k_{max} = 17.5 ± 0.5 × 10^{-5} sec^{-1} at 0.4 μM enzyme. ATP by itself had little effect. In all cases excellent first-order decay curves were observed (see Figure 2).

In the absence of added ligands or the presence of saturating concentrations of tyrosine and ATP the rate con-

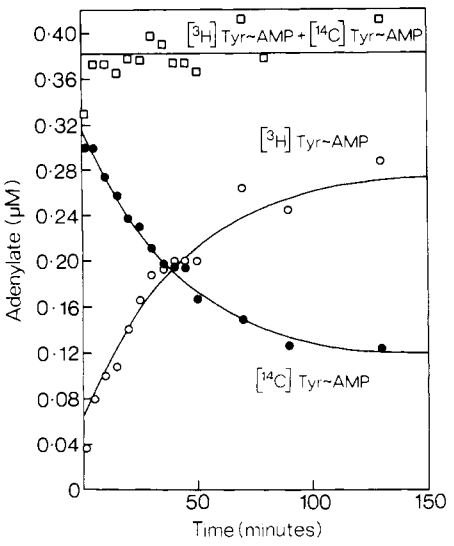


FIGURE 1: Exchange of [^{3}H]Tyr into tyrosyl-tRNA synthetase-[^{14}C]Tyr~AMP on mixing 0.76 μM enzyme (0.8 mol of [^{14}C]-Tyr~AMP/mol) with an equal volume of 2.16 μM [^{3}H]Tyr and 140 μM ATP. Under the reaction conditions the total concentration of adenylate did not decrease during the time course as sufficient ATP was available to regenerate the complex. The labels distributed statistically (solid curves are calculated from the data in Table I).

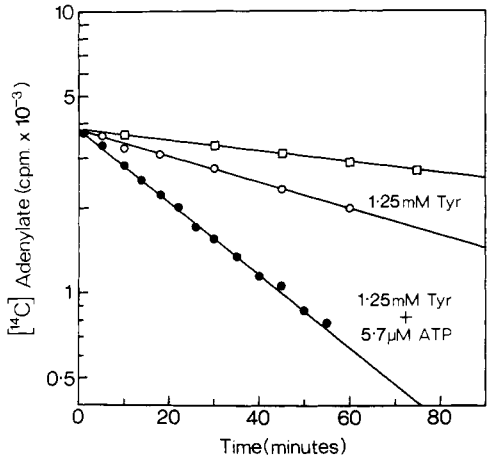


FIGURE 2: Loss of [^{14}C]Tyr from tyrosyl-tRNA synthetase-[^{14}C]-Tyr~AMP. Conditions as in text. (□) No added ligands; (○) added tyrosine (saturating); (●) added tyrosine and ATP (both saturating).

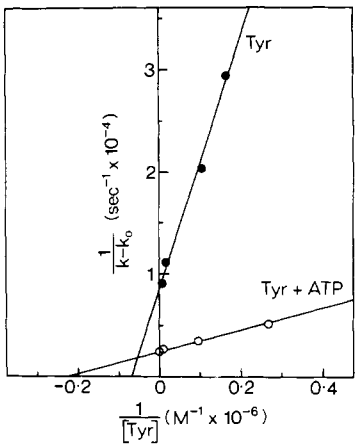


FIGURE 3: Binding of tyrosine to tyrosyl-tRNA synthetase-[^{14}C]-Tyr~AMP. Reciprocal plots for the binding of tyrosine (●) and tyrosine in the presence of added ATP (○) as reflected in increased hydrolysis rates. The results are corrected for the spontaneous hydrolysis (k_0 = 5.7 × 10^{-5} sec^{-1}). The concentration scale for tyrosine alone (●) is expanded by a factor of 10.

TABLE I: Rate and Dissociation Constants for the Hydrolysis of Tyrosyl-tRNA Synthetase-[^{14}C]Tyr~AMP Complexes.[a]

Complex	Dissociation Constants (μM) Tyrosine	ATP	$k_{hydrolysis}$ (sec^{-1} × 10^5)
[E·[^{14}C]Tyr~AMP][b]			5.7 ± 0.1
[E·[^{14}C]Tyr~AMP Tyr,ATP]	4.0 ± 0.3[b]	~0.09[c]	45.2 ± 1.2[b,c]
[E·[^{14}C]Tyr~AMP Tyr][b]	144 ± 14		17.5 ± 0.5
[E·[^{14}C]Tyr~AMP ATP]		~3.2[d]	5.7[b]

[a] 25°, pH 7.78 (144 mM Tris, μ = 0.1), 5 mM mercaptoethanol, 5 × 10^{-5} M $PhCH_2SO_2F$, 10 mM $MgCl_2$, and 5 units of inorganic pyrophosphatase/ml. Determined by retention of enzyme complex on nitrocellulose filters. [b] 0.4 μM complex. [c] 0.16 μM complex. [d] Calculated from the other three dissociation constants.

stants were independent of the concentration enzyme-aminoacyl adenylate. However, the stimulation by tyrosine increased at lower concentrations of the complex. Presumably tyrosine increases the dissociation constant of the complex.

The data and experimental conditions are illustrated and summarized in Figures 2 and 3 and Table I.

The important conclusion is that the complex binds additional tyrosine and ATP.

(b) VALYL-TRNA SYNTHETASE. The complex isolated by gel filtration had 0.8-0.9 bound valyl adenylates as judged by the specific radioactivity. Filtration of a solution at room temperature through the nitrocellulose filter led to only a 55-60% retention of counts. Prior chilling of the complex and washing with cold buffer gave increased retentions of 90% or greater. The lower accuracy of the kinetic determinations and only a smaller stimulation of hydrolysis rate by added ligands rendered this system less suitable for detailed study than the tyrosyl-enzyme. The following was observed; over a 20-fold range of enzyme-valyl adenylate concentration (0.2-4 μM) the isolated complex hydrolyzed with rate constant 1.23 × 10^{-4} sec^{-1}; on addition of valine (500 μM) the rate constant doubled to 2.4 × 10^{-4} sec^{-1}; ATP by itself had little effect but the addition of both 80 μM ATP and 833 μM valine increased the rate constant to 6 × 10^{-4} sec^{-1}. Again the stimulation by amino acid increased at low concentrations of the enzyme-aminoacyl adenylate.

(2) Indirect Determination of Hydrolysis Rates of Enzyme-Bound Aminoacyl Adenylates. (a) BLANK REACTION. The enzyme preparations had weak ATPase activities due to the presence of trace amounts of impurities (Fersht *et al.*, 1975b). The preparation of tyrosyl-tRNA synthetase hydrolyzed ATP to ADP and P_i (without any production of pyrophosphate) with a K_M of 2.2 ± 0.4 μM and k_{cat} of 4 ± 1 × 10^{-4} sec^{-1}, based on the total enzyme concentration. Similar activity was observed with the valyl ligase. As we wished to measure accurately the rate of hydrolysis of ATP by the aminoacyl-tRNA synthetase due to

TABLE II: Hydrolysis of Valyl-tRNA Synthetase-[^{14}C]Val~AMP Isolated by Gel Filtration.[a]

Enzyme (μM)	Valine (μM)	ATP (μM)	k (sec^{-1} $\times$ 10^4)
4.14	0	0	1.23
0.3	0	0	1.26
0.2	0	208	1.2
0.2	500	0	2.4
0.78	1000	47	5.0
0.78	1000	47	5.4
0.3	833	80	6.0

[a] Conditions as in Table I.

aminoacyl adenylate formation control experiments were performed to determine the contribution of the ATPase activity. (1) The enzyme preparation was incubated with ATP and amino acid in the absence of pyrophosphatase and the rate of orthophosphate release measured. (2) The enzyme was incubated with ATP and pyrophosphatase in the absence of amino acid, and either the [^{14}C]AMP from [^{14}C]ATP or the ^{32}P produced from [γ-^{32}P]ATP was measured. The results are summarized in Table III.

(b) ATP HYDROLYSIS DUE TO AMINOACYL ADENYLATE FORMATION. In the presence of inorganic pyrophosphatase and tyrosine (1.25 mM) the tyrosyl activating enzyme hydrolyzed ATP to AMP *via* aminoacyl adenylate formation with a turnover number of 9.5 $\pm$ 1.2 $\times$ 10^{-4} sec^{-1}. This was found by dividing the initial rate of hydrolysis of ATP by the enzyme concentration, allowing 1.1 active sites/mol (Fersht *et al.*, 1975a). The initial rate measurements were made using the three different techniques: adsorption of [γ-^{32}P]ATP on charcoal; separation of either [^{32}P]orthophosphate from [γ-^{32}P]ATP or [^{14}C]AMP from [^{14}C]ATP by thin-layer chromatography. The different kinds of "blank" hydrolytic reactions were measured under the same conditions, omitting either amino acid and/or inorganic pyrophosphatase, and subtracted from the overall hydrolytic rates (see Table III). There is reasonable agreement among the different methods showing clearly that the overall turnover number for the hydrolysis of ATP *via* aminoacyl adenylate formation is in the range 7–13 $\times$ 10^{-4} sec^{-1} rather than the value of 4.52 $\pm$ 0.12 $\times$ 10^{-4} sec^{-1} observed for the loss of [^{14}C]tyrosyl adenylate from the enzyme complex which had been isolated by gel filtration and hydrolyzed under the same conditions. Similarly, it is seen in Tables II and III that whereas the rate constant for the loss of [^{14}C]valyl adenylate from valyl-tRNA synthetase-[^{14}C]valyl adenylate is 5.2 $\pm$ 0.2 $\times$ 10^{-4} sec^{-1}, the turnover number for ATP hydrolysis *via* valyl adenylate hydrolysis under the same conditions (47 μM ATP, 1 mM Val, 0.78 μM enzyme) is 9.5 $\pm$ 0.4 $\times$ 10^{-4} sec^{-1} M^{-1}.

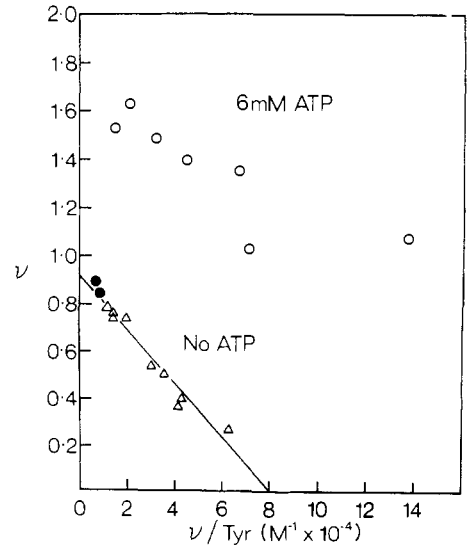


FIGURE 4: Binding of tyrosine to tyrosyl-tRNA synthetase-Tyr~AMP studied by equilibrium dialysis. 13 μM enzyme, 8–130 μM [^{14}C]tyrosine, 144 mM Tris (pH 7.78), 10 mM $MgCl_2$, and 1 unit of inorganic pyrophosphate/ml. (○) 6 mM ATP added; (●) no added ATP, (△) results from Fersht *et al.* (1975b); ν, number of moles of [^{14}C]tyrosine bound/mole of enzyme.

Direct Determination by Equilibrium Dialysis of the Binding of Amino Acid to the Aminoacyl Adenylate Complex. Enzyme bound aminoacyl adenylate was formed *in situ* by dialyzing cognate amino acid and ATP into the chamber containing aminoacyl-tRNA synthetase and inorganic pyrophosphatase (*cf.* Berthelot and Yaniv, 1970).

TABLE III: Overall Rate of Aminoacyl Adenylate Hydrolysis Determined from Depletion of ATP.[a]

ATP (μM)	$k_{overall}$[b] (sec^{-1} $\times$ 10^4)	Method[c]	k_{blank}[d] (sec^{-1} $\times$ 10^4)	Method[c]	$k_{overall-blank}$ (sec^{-1} $\times$ 10^4)
		(a) Tyrosyl-tRNA Synthetase[e]			
12	13.3	A	4.1	A(1)	9.2
12	11.4	B	4.1	B(1)	7.3
48	13.4	B	4.9	B(2)	8.5
48	15.4	C	2.4	C(1)	13
		(b) Valyl-tRNA Synthetase[f]			
47	11.6	B	1.6	B(1)	10.0
47	10.7	C	1.8	C(2)	8.9

[a] Conditions as in Table I, all determinations in duplicate. [b] Overall turnover number for the depletion of ATP in the presence of enzyme, cognate, amino acid, ATP, and inorganic pyrophosphatase. The turnover number is defined as equal to the initial reaction rate divided by the concentration of enzyme and number of active sites/mole (1.1 for tyrosyl, 1.0 for valyl). [c] Determined by: A = adsorption of [γ-^{32}P]ATP on charcoal; B = separation of [^{32}P]orthophosphate from [γ-^{32}P]ATP on PEI sheets; C = separation of [^{14}C]AMP from [^{14}C]ATP on PEI sheets. [d] Hydrolysis of ATP in either absence of pyrophosphatase (1) or absence of amino acid (2) using methods A, B, and C. [e] 1.2–1.4 μM. [f] 0.78 μM.

TABLE IV: Binding of Valine to the Valyl-tRNA Synthetase in the Presence and Absence of ATP.[a]

[Valine] (μM)	moles of Valine Bound/mole of Enzyme	
	No ATP	6 mM ATP
820	(0.52 ± 0.50)	1.52 ± 0.36
400	0.81 ± 0.10	2.23 ± 0.18
200	0.85 ± 0.08	1.37 ± 0.19
115	0.54 ± 0.10	1.47 ± 0.10
50	0.57 ± 0.02	1.42 ± 0.04

[a] 25°, pH 7.78, 144 mM Tris, 10 mM $MgCl_2$, 8 μM enzyme, and 1 unit of inorganic pyrophosphatase/ml.

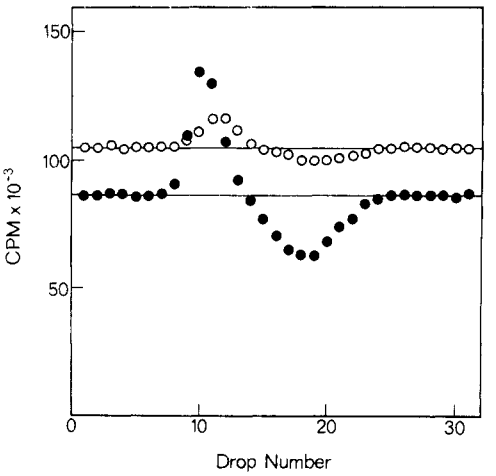


FIGURE 5: Binding of ATP to aminoacyl-tRNA synthetase aminoacyl adenylates studied by equilibrium gel filtration. 8.6 μM tyrosyl-tRNA synthetase (●) or 2.7 μM valyl-tRNA synthetase (○) was incubated with 20 μM [^{14}C]ATP, cognate amino acid, 10 mM $MgCl_2$, 144 mM Tris, and inorganic pyrophosphatase and gel filtered in the same buffer.

Under these conditions there is additional binding of the amino acid to the complex. Whereas, in the absence of ATP only 0.9–0.95 mol of tyrosine bind to 1 mol of the tyrosyl-tRNA synthetase dimer about 1.6 mol bind in the presence of ATP (see Figure 4). Similarly it is seen in Table IV that whereas under saturating conditions of valine in the absence of ATP ($K_S \sim 10$ μM; Bosshard *et al.*, 1975) only 0.6–0.8 mol of valine bind/mol of enzyme; 1.4–2 bind in the presence of ATP. As under the reaction conditions both enzymes form about 1 mol of enzyme-bound aminoacyl adenylate/mol there must be unreacted amino acid bound at a second site.

Some of the measurements on the valyl-tRNA synthetase entailed only small differences in ligand concentration between either side of the dialysis membrane due to the relatively low enzyme concentration used (Table IV). These measurements are considered reliable since (a) the reproducibility of sampling is high (standard error is ±0.2%), (b) the control experiments omitting ATP were performed in parallel and the trend in ratio between the two sets of experiments is clear, and (c) these experiments have now been performed using the valyl-tRNA synthetase from *E. coli* at ten times the enzyme concentration of this study with similar results (unpublished data).

Under the reaction conditions less than 3% of the ATP should have hydrolyzed during the course of the experiments.

Direct Determination by Equilibrium Gel Filtration of the Binding of ATP to the Aminoacyl Adenylate Complex. In order to avoid complications arising from ATP hydrolysis during the course of equilibrium dialysis, the method of Hummel and Dreyer (1962) was used to measure the binding of ATP to the aminoacyl adenylate complexes. The complexes were formed *in situ* as in the equilibrium dialysis experiments. The amounts of [^{14}C]ATP carried by the protein through the Sephadex column and the subsequent "trough" were determined by assaying the individual drops of eluent (see Figure 5). (In the absence of protein the drop size was remarkably constant, the standard deviation being less than 0.5%. However, the presence of protein diminished the drop size; 2.7 μM valyl-tRNA synthetase caused a decrease of 1% or less and 8.6 μM tyrosyl-tRNA synthetase about 3%.) In the presence of 20 μM unbound ATP, the tyrosyl-tRNA synthetase carried 1.9 mol of ATP/mol of enzyme (area under peak = 1.93 mol of ATP/mol; area under trough = 1.86 mol of ATP/mol) while the valyl-tRNA synthetase carried 1.1 (peak = 1.2; trough = 1.0) (see Figure 5). An experiment using 51 μM ATP and 2.5 μM valyl-tRNA synthetase, with [^{3}H]H_2O added to the buffer as an internal marker to calibrate the drop size, gave a value of about 1.5 for the stoichiometry (peak = 1.56, trough 1.47). This shows directly that ATP binds to the tyrosyl-tRNA synthetase tyrosyl adenylate complex with a dissociation constant of much less than 20 μM, and also binds to the valyl complex with a dissociation constant of around 50 μM.

Discussion

(*a*) *Both the Monomeric Valyl-tRNA Synthetase and Dimeric Tyrosyl-tRNA Synthetase have Two Sets of Binding Sites.* Although the tyrosyl-tRNA synthetase from *B. stearothermophilus* binds only 1 mol of tyrosine and only one tyrosyl adenylate per mol of enzyme there is a second set of binding sites for ATP and tyrosine which becomes accessible when the first is occupied with tyrosyl adenylate. The evidence for this is that the binding of these ligands to the tyrosyl adenylate complex may be detected either by the stimulation of the hydrolysis rate of the tyrosyl adenylate or by equilibrium dialysis and gel filtration procedures. Tyrosine increases the hydrolysis rate of the complex, either by direct activation or increasing the dissociation rate constant, binding with a K_s of 144 μM. This dissociation constant decreased on the addition of ATP to 4 μM. The apparent dissociation constant of ATP from the [E · Tyr~MP, Tyr] complex is about 0.09 μM, compared with a K_M of 3.9 mM for the formation of tyrosyl adenylate (Fersht *et al.*, 1975b). The much tighter binding of ATP shows that this stimulation is not an artefact associated with the reaction of tyrosine and ATP with the free enzyme. Under conditions where the active site titration procedure shows that only one tyrosyl adenylate is bound per mole, equilibrium dialysis studies of the binding of tyrosine in the presence of ATP indicate two tyrosine moieties are bound. Also, equilibrium gel filtration shows that an additional mole of ATP is bound as well (Figures 4 and 5). Similar kinetic and binding measurements demonstrate that the monomeric valyl-tRNA synthetase binds simultaneously 1 mol each of valyl adenylate, ATP, and valine.

This study in conjunction with the structural evidence that the valyl-tRNA synthetase is composed of two fused repeating sequences (Koch *et al.*, 1974) suggests that the

two activating enzymes are similar in many ways. The diversity of this class of enzymes may not be as great as the subunit characteristics would indicate.

The following evidence suggests that the two binding sites on each enzyme are architecturally similar. However, it is based on a discrepancy of a factor of 2 in reaction rates and some caution must be borne in mind.

(*b*) *The Second Sites Appear to have Weak Catalytic Activity.* This is inferred from an apparent contradiction that arises when the rate constants for the hydrolysis of the enzyme-bound aminoacyl adenylates are determined by two different methods. On adding enzyme bound ^{14}C-labeled adenylate (E · [^{14}C]AA~AMP) to a solution of amino acid and ATP there is a loss of enzyme-bound radioactivity as the complex hydrolyzes. There is a simultaneous loss of ATP as the newly released enzyme forms (unlabeled) aminoacyl adenylate from the ATP and amino acid.

If there were only one active site, eq 1 should hold (where PP = pyrophosphate, which is hydrolyzed by pyrophosphatase to orthophosphate).

$$\mathrm{ATP} + \mathrm{AA} \xrightarrow[\mathrm{E}]{k_1} \mathrm{E{\cdot}AA{\sim}AMP} \xrightarrow{k_2} \mathrm{E} + \mathrm{AA} + \mathrm{AMP} \quad (1)$$

$$+ \mathrm{PP} \downarrow (2\mathrm{P_i})$$

In eq 1, where $k_1 > k_2$, it is easily shown that

$$\mathrm{d[ATP]}/\mathrm{d}t = k_2[\mathrm{E{\cdot}AA{\sim}AMP}] \quad (2)$$

and also

$$\mathrm{d[E{\cdot}AA{\sim}AMP]}/\mathrm{d}t = k_2[\mathrm{E{\cdot}AA{\sim}AMP}]$$

Under conditions of saturating binding of tyrosine and ATP to the tyrosyl-tRNA synthetase-[^{14}C]tyrosyl adenylate complex, k_2 is directly determined from the loss of [^{14}C]adenylate to be $4.54 \pm 0.12 \times 10^{-4}$ sec^{-1}. But when determined from the rate of hydrolysis of ATP and eq 2 a value of $9.5 \pm 1.2 \times 10^{-4}$ sec^{-1} is obtained, approximately twice the expected value. Similarly when k_2 for the hydrolysis of the valyl complex is determined directly a value of $5.2 \pm 0.2 \times 10^{-4}$ sec^{-1} is found, but application of eq 2 gives $9.5 \pm 0.4 \times 10^{-4}$ sec^{-1} under the same conditions.

In order to overcome this apparent contradiction, both sites on the enzymes must be catalytically active in order to give a factor of 2. An intermediate E(AA~AMP)$_2$ must be formed where there is a 50% probability of a particular, *e.g.*, ^{14}C-labeled, bound aminoacyl adenylate hydrolyzing quickly. Equation 1 must be enlarged to include two sites. For example, eq 3 fits the experimental observations.

In eq 3 k_2, k_2', k_2'', and k_2''' are the apparent rate constants for the hydrolysis of the complexes as indicated and $k_1' < k_2'', k_2'''$ as only one aminoacyl adenylate accumulates.

The initial rate of hydrolysis of ATP in the steady state, under saturating concentrations of ATP and AA, is given by

$$\mathrm{d[ATP]}/\mathrm{d}t = \left[\mathrm{E}^{[^{14}\mathrm{C}]\mathrm{AA{\sim}AMP}}_{\mathrm{AA,ATP}}\right](k_2' + k_1') \quad (4)$$

and

$$\frac{\mathrm{d}}{\mathrm{d}t}\left[\mathrm{E}^{[^{14}\mathrm{C}]\mathrm{AA{\sim}AMP}}_{\mathrm{AA,ATP}}\right] = \left[\mathrm{E}^{[^{14}\mathrm{C}]\mathrm{AA\,AMP}}_{\mathrm{AA,ATP}}\right]\left(k_2' + k_1'\left\{\frac{k_2''}{k_2''' + k_2''}\right\}\right) \quad (5)$$

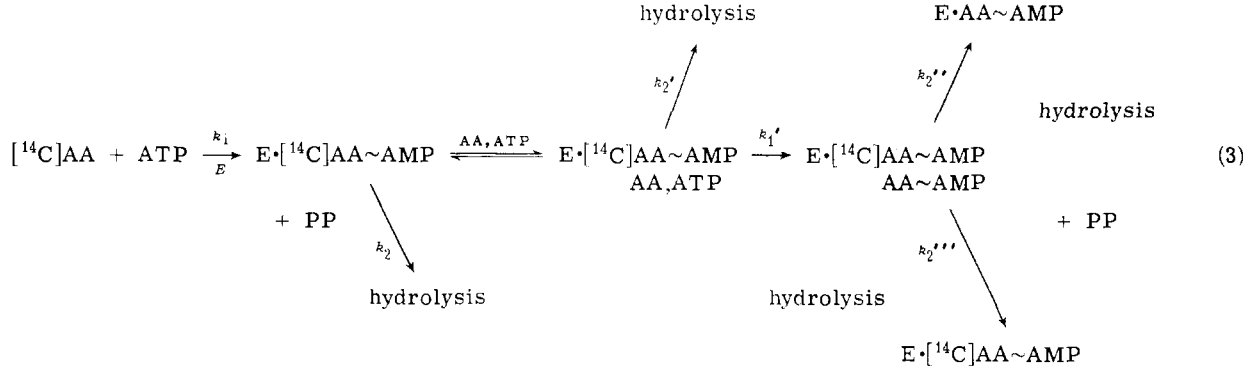
where the last bracketed term in (5) represents the probability of the complex hydrolyzing to lose ^{14}C.

If $k_2'' \sim k_2'''$ and $k_1' \sim k_2'$, then to a first approximation d[ATP]/dt = k_1'[complex] and d[complex]/dt = $\frac{1}{2}k_1'$[complex]. k_1' is then 9.5×10^{-4} sec^{-1} for both ligases. As $k_2'' \sim k_2'''$ it is likely that the E(Tyr~AMP)$_2$ complex is symmetrical and the valyl complex involves an approximate symmetry.

It is not suggested that both sites are catalytically active during tRNA charging but rather that the second site is an "effector" site with some residual activity. However, this weak activity does provide some evidence that the two sets of sites on the valyl-enzyme are structurally similar.

(*c*) *General Implications.* Irrespective of whether or not the rate and equilibrium measurements on the enzyme-bound aminoacyl adenylates have any physiological relevance in themselves, the conclusion derived from them, that a monomeric enzyme has two active sites, is of mechanistic importance. The valyl-tRNA synthetase seems typical of the monomeric activating enzymes in terms of molecular weight (~110,000; see Loftfield, 1972 for a review), and, as mentioned in the introduction, repeating sequences. It is therefore likely that all of the activating enzymes have at least two active sites. This is contrary to the currently accepted view that the monomeric enzymes of this class have only one active site.

The isoleucyl, valyl, and leucyl enzymes from *E. coli*, like the valyl-enzyme from *B. stearothermophilus*, appear to bind only one amino acid and one aminoacyl adenylate (Norris and Berg, 1964; Berthelot and Yaniv, 1970; Yaniv and Gros, 1969; Bosshard *et al.*, 1975; Fersht *et al.*, 1975a). The existence of the second site is hidden by "half-of-the-sites" reactivity in the last case and presumably for the other three. The current ideas on the specificity and reaction mechanism must be reexamined in the light of this.



(3)

It has been difficult to account for the high specificity of the aminoacyl-tRNA synthetases for the amino acid in the esterification of tRNA (Loftfield and Eigner, 1966). Whereas an amino acid larger than the natural substrate clearly may be excluded by steric hinderance from binding productively at the active site, a smaller amino acid will always bind to some extent although making fewer interactions with the enzyme. In this case either the binding of the smaller unnatural substrate will be weaker due to the missing interactions or the catalysis will be weaker if these interactions are important in causing strain, induced fit, or overcoming nonproductive binding. Pauling (1958) has calculated that a methylene group can contribute at the most a factor of 20 (1.8 kcal) to the binding energy. Also there is a considerable body of experimental data showing that a methylene group contributes 0.64 kcal (factor of 5) to hydrophobic bonding (see, for example, Gitler and Ochoa-Solano, 1968; Oakenfull and Fenwick, 1973). But the isoleucyl-enzyme from *E. coli* will not transfer valine to the tRNA, despite the valine being shorter than isoleucine by only one methylene group (Bergmann *et al.*, 1961; Loftfield and Eigner, 1966). Loftfield (1963) has shown that the fidelity of charging in general is better than 1 error in 3000, which was the limit of detection of his technique. Various "verification" mechanisms have been demonstrated and suggested. Baldwin and Berg (966) showed that the isoleucyl-tRNA synthetase valyl adenylate will not transfer the valine to isoleucyl-tRNA but is preferentially hydrolyzed. There are reports that misacylated tRNAs are deacylated by the synthetases (Yarus, 1972, 1973; Yarus and Mertes, 1973; Eldred and Schimmel, 1972; Ebel *et al.*, 1973).

We have attempted to find a mechanism that could increase specificity through interacting active sites. The following accounts for the observations of half-of-the-sites reactivity and negative cooperativity and could explain *apparent* high specificities in the pyrophosphate exchange reaction.

General Mechanism for Enhancement of Specificity and Catalysis through Interacting Sites. The basis of the mechanism is that the enzyme has (at least) two sets of binding sites. It is proposed that the substrates bind to the enzyme with negative cooperativity; that is, there is a steric interference to binding at the second site once the first site is occupied. These unfavorable steric interactions are relieved when either one or both sites form products. The relief of strain may then increase the reaction rate by lowering the activation energy. In this way the binding energy of both sites is used for catalysis. Three variations of the mechanism are given.

(a) CATALYSIS. In Scheme I, a "half-of-the-sites" case,

SCHEME I: Half-of-the-Sites Mechanism.

$$\begin{array}{ccccc} E & \overset{S}{\underset{K_S}{\rightleftharpoons}} & ES & \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} & EP \\ & & S\Big\Updownarrow K_S' & & S\Big\Updownarrow K_S'' \\ & & ES_2 & \overset{k_1'}{\underset{k_{-1}'}{\rightleftharpoons}} & EPS \end{array}$$

$$K_S = \frac{E \cdot S}{ES}, K_S' = \frac{ES \cdot S}{ES_2}, K_S'' = \frac{EP \cdot S}{EPS}$$

the unfavorable interactions are relieved when one site reacts. The substrate binds strongly to the enzyme when one site is occupied by products. The dissociation constant K_S' is much greater than K_S and K_S''. The maximum contribution to catalysis of the increased binding energy of the substrate as the reaction progresses is given by K_S'/K_S''.

In Scheme II, the coupled case, both sites react simulta-

SCHEME II: Coupled Mechanism.

$$\begin{array}{ccccc} E & \overset{S}{\underset{K_S}{\rightleftharpoons}} & ES & \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} & EP \\ & & S\Big\Updownarrow K_S' & & P\Big\Updownarrow K_P \\ & & ES_2 & \overset{k_1'}{\underset{k_{-1}'}{\rightleftharpoons}} & EP_2 \end{array}$$

$$K_P = \frac{EP \cdot P}{EP_2}$$

neously. The unfavorable interactions are relieved on product formation. If the difference between K_S and K_S' is due to the unfavorable interactions in ES_2 then the maximum contribution to catalysis caused by the relief of the unfavorable interactions is given by K_S'/K_S.

Scheme III involves an energetically unfavorable inter-

SCHEME III: Half-of-the-Sites Sequential Mechanism.

$$\begin{array}{ccccccc} E & \overset{S}{\underset{K_S}{\rightleftharpoons}} & ES & \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} & EP & \xrightarrow{k_2} & EP' \\ & & S\Big\Updownarrow K_S' & & K_S''\Big\Updownarrow S & & \\ & & ES_2 & & EPS & \xrightarrow{k_2'} & EP'S \end{array}$$

$$k_2 < k_{-1}$$

mediate, EP. This would normally be present at a low concentration so that the rate of production of EP′ is low (turnover number = k_2k_1/k_{-1}). However, if a second molecule of S binds strongly to EP and very weakly to ES the equilibrium will be displaced toward EP, increasing the reaction rate (to a maximum turnover number of k_1). The maximum contribution to catalysis caused by the displacement of the equilibrium is a factor of k_1/k_2.

(b) SPECIFICITY. Schemes I and II could account for such an observation that the isoleucyl-tRNA synthetase catalyzed pyrophosphate exchange reaction with isoleucine involves a 160-fold lower K_M than with valine (Loftfield and Eigner, 1966).[2] The requirement of two molecules of substrate in the transition state gives a factor of $(5\text{–}20)^2$ since two additional methylene groups are involved. However, in a *mixture* of valine and isoleucine the enzyme can discriminate against valine by a factor of only 5–20. This is because of the importance of the mixed complex enzyme–valine–isoleucine. Indeed, any model we construct falls down on this since the combination of the equilibrium constant between [E · Ile-Val] and [E · Ile_2] and the relative rate constants for the reaction to give Val~AMP and Ile~AMP can never be greater than the binding energy of one methylene group. Until a satisfactory mechanism to the contrary has been demonstrated it must be concluded that a "verification" mechanism is required to account for the fidelity of protein synthesis. However, the mechanism presented is important for catalysis.

Catalytic Advantages of Subunits. There are distinct catalytic advantages in the facility of using the binding en-

[2] It is probably not necessary to invoke any special mechanism to account for the factor of 160 in the above case; W. P. Jencks (personal communication) suggests that the factor of 5–20 per methylene group is an underestimate.

ergy of the substrate more than once during the reaction. A single site enzyme may catalyze a reaction involving an unfavorable equilibrium by tight binding of the unstable intermediate. Tight binding requires that the enzyme has a structure complementary to that of the intermediate. For maximum catalysis the structure of the enzyme should be complementary to that of the substrate in the transition state (Fersht, 1974). The transition states for the formation and subsequent reaction of the intermediate may be incompatible with tight binding. Similarly the tight binding of a high-energy product could lead to a very slow diffusion rate of the product from the enzyme. These difficulties are lessened with oligomeric enzymes and Schemes I, II, and III of the mechanism. The binding energy of additional substrate molecules rather than the product is used for catalysis.

Monod *et al.* (1965) have discussed the difficulties in explaining why so many nonregulatory enzymes are oligomeric. The catalytic advantages we have suggested may be an important reason for the abundance of these enzymes.

Lazdunski (Lazdunski *et al.*, 1971; Lazdunski, 1972) has proposed a different mechanism to account for half-of-the-sites enzymes. In this, a chemical step at one site is linked with a different chemical step at the other; the sites alternate in activity and function, asymmetry always being maintained. This mechanism does not appear to hold for the reactions described here since a symmetrical [E · $(AA\sim AMP)_2$] complex is formed in which both sites are equivalent.

The proposed mechanism may be tested experimentally by binding and kinetic studies. In the following paper (Fersht *et al.*, 1975b) we present evidence that tyrosyl adenylate is formed by the tyrosyl-tRNA synthetase from *B. stearothermophilus* by this mechanism.

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