Biochemistry

Volume 14, Number 1 January 14, 1975

Active Site Titration and Aminoacyl Adenylate Binding Stoichiometry of Aminoacyl-tRNA Synthetases†

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ABSTRACT: A simple, rapid, and economical procedure is described for the determination of the number of catalytically competent active sites on aminoacyl-tRNA synthetases based on the stoichiometry of aminoacyl adenylate formation. On mixing tRNA synthetase, cognate amino acid, [γ-^{32}P]ATP, and inorganic pyrophosphatase under suitable conditions there is an initial rapid stoichiometric "burst" (rate constant k_1) of depletion of ATP as enzyme bound aminoacyl adenylate is formed. There is then an initially linear decrease in ATP concentration as the complex hydrolyzes (with rate constant k_2) releasing enzyme to form further adenylate. Provided $k_2 \ll k_1$ the initial burst gives the stoichiometry of aminoacyl adenylate formation. Complexes which are too unstable to be isolated by the usual gel or nitrocellulose disk filtration procedure may be assayed in this way. This technique has been applied to five highly purified aminoacyl-tRNA synthetases. The tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* is shown to bind only one aminoacyl adenylate per dimer.

In the course of a systematic investigation into the mechanism of the charging of tRNA by aminoacyl-tRNA synthetases we require a simple routine procedure for the determination of the number of catalytically competent active sites on the protein, as a criterion of enzyme purity and ligand binding stoichiometry.

Catalytic competence may be measured either by tRNA charging or by reactions dependent on aminoacyl adenylate formation: in general, the latter have been the method of choice. Some enzyme–aminoacyl adenylate complexes are sufficiently stable to be isolated by gel filtration and this has been the basis of some successful assays and determination of binding stoichiometry (Norris and Berg, 1964). Yarus and Berg (1970) have presented a very efficient procedure isolating the complex on nitrocellulose filters. However, as Boeker and Cantoni (1973) have pointed out these procedures give only a *minimum* estimate of the stoichiometry, due to the dynamic nature of the equilibria involved.

We wish to present a more generally useful procedure which will detect labile as well as stable enzyme–aminoacyl adenylate complexes.

The basis of the method is the stoichiometric formation of 1 mol of pyrophosphate and depletion of 1 mol of ATP on the formation of 1 mol of adenylate. The reaction conditions are adjusted so that there is a rapid burst of enzyme–aminoacyl adenylate formation followed by a relatively slow turnover of substrate (eq 1). By using [γ-^{32}P]ATP the residual ATP is measured either by adsorption on charcoal, to which the radioactive products do not bind or in special cases by thin-layer chromatography.

The addition of inorganic pyrophosphatase ensures that the formation step k_1 is irreversible.

$$[^{32}\mathrm{P}]\mathrm{ADP} + \mathrm{AA} + \mathrm{E} \underset{k_1}{\xrightarrow{\text{fast}}} \mathrm{AA}\sim\mathrm{AMP}\cdot\mathrm{E} \xrightarrow[k_2]{\text{slow}} \begin{matrix}\text{products} \\ + \\ \mathrm{E}\end{matrix} + [^{32}\mathrm{P}]\mathrm{P} \quad (1)$$

Experimental Section

Apparatus

Emission from ^{32}P was monitored using either a Nuclear Chicago gas flow counter or a Unilux I scintillation counter. The double beam stopped-flow spectrofluorimeter, which will be described in detail elsewhere, consisted of a 150-W Xenon arc lamp and f/3.5 grating monochromator as light source, a six jet tangential mixing chamber and 0.173-mm^2 quartz observation cell, an EMI 9558 QB photomultiplier and cut off filter at right angles to the exciting

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light for monitoring emission, and an EMI 9781 A photomultiplier for monitoring the excitation beam. The fluorescent light was compared with the excitation using a Tektronix 564B storage oscilloscope and differential amplifier. The dead time (determined from the oxidation of dichloroindophenol by ascorbic acid) was 1.7 msec.

Materials

[γ-^{32}P]ATP (2000 Ci/mol) and other radiochemicals were obtained from the Radiochemical Centre, Amersham. Yeast inorganic pyrophosphatase was obtained from Worthington Biochemicals. Methionyl-tRNA synthetase was prepared from *Escherichia coli* EM 20031 as described by Atkinson *et al.* (1973). Aminoacyl-tRNA synthetases from *Bacillus stearothermophilus* were isolated as described by Koch *et al.* (1974), Koch (1974), and Jakes (1975). Other reagents were obtained commercially and recrystallized where necessary.

Enzyme concentrations were calculated from the following values of molecular weight and A_{280} (A_{280} per mg per ml): tyrosyl, 2 × 47,500, 1.39; tryptophanyl, 2 × 35,000, 1.32; valyl, 1 × 110,000, 2.2; methionyl, 2 × 66,000, 1.55 (all from *B. stearothermophilus,* Koch *et al.* (1974)); methionyl (*E. coli*) 2 × 86,000, 1.39 (Blanquet *et al.*, 1973).

Methods

Active Site Titration. [γ-^{32}P]ATP, $MgCl_2$ (10 mM), Tris-Cl (pH 7.8, 144 mM), cognate amino acid (saturating concentration, usually 1 mM), and yeast inorganic pyrophosphatase (2 units/ml) were incubated at 25°. Triplicate aliquots (for zero time readings usually 10 or 20 μl, containing 5000 cpm) were separately mixed with 100 μl of 7% perchloric acid followed by 200 μl of a rapidly swirled suspension of 2% activated charcoal (Norit) in water. The requisite aminoacyl-tRNA synthetase was added to the reaction mixture and aliquots were periodically taken and quenched as above. The quenched samples were filtered through Whatman GF/C glass fiber filters, which were washed and dried and the [γ-^{32}P]ATP which was adsorbed to the charcoal was monitored on the gas flow counter. (A scintillation counter may also be used (Boeker and Cantoni, 1973).)

Alternatively, 25- or 50-μl aliquots of the quenched samples were spotted directly onto Polygram PEI sheets without the addition of charcoal. The [^{32}P]orthophosphate was separated from the [γ-^{32}P]ATP by developing for 0.5–1 hr with 0.5 M KH_2PO_4 (pH 3.5) (Randerath and Randerath, 1964; Cashel *et al.*, 1969). The PEI sheet was dried, cut into 1-cm strips, suspended in Bray's (1960) solution, and monitored in the scintillation counter. (The orthophosphate travels with the ion front.)

The stock solutions of [γ-^{32}P]ATP were periodically tested for hydrolysis by thin-layer chromatography on PEI sheets.

The hydrolysis of pyrophosphate by yeast inorganic pyrophosphatase was assayed by a similar procedure using PEI sheets.

Gel Filtration of the Tryptophanyl-tRNA Synthetase–Tryptophanyl Adenylate Complex. A solution (0.6 ml) of 4 μM tryptophanyl-tRNA synthetase, 10 mM $MgCl_2$, 1 mM ATP, and 150 μM [^{3}H]tryptophan (16 Ci/mol) in 144 mM Tris-Cl (pH 7.8) was incubated at 0° for 5 min. The enzyme-bound adenylate was isolated at 4° on a 1 × 10 cm G-25 Sephadex (fine) column equilibrated with 144 mM Tris-Cl and 10 mM $MgCl_2$. After correction for the absorbance of the adenylate a value of 1.6 mol of bound adenylate/mol of enzyme was calculated from the A_{280} and specific radioactivity. In the absence of ATP or enzyme no radioactivity was detected in the fraction used for the above calculation. This fraction could be rechromatographed with no loss of specific activity. A similar procedure was adopted for the valyl enzyme.

Gel and Membrane Filtration of the Tyrosyl-tRNA Synthetase–Tyrosyl Adenylate Complex. 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 288 mM Tris-Cl (pH 7.8) containing 7 units of inorganic pyrophosphatase was incubated at 25° for 5 min. The enzyme-bound adenylate was isolated at room temperature on a 1 × 25 cm G-25 Sephadex (fine) column equilibrated with the Tris and $MgCl_2$; 20 μl of the fraction containing the enzyme-bound adenylate (0.92 μM) was spotted onto a Schleicher and Schull BA 85 nitrocellulose filter and dried and the ^{14}C was monitored (4180 cpm) using a toluene-based scintillant (12.5 g of 2,5-diphenyloxazole and 0.75 g of 1,4-bis[2-(5-phenyloxazyl)]benzene in 2.5 l. of toluene); 20 μl of the same fraction was filtered through a nitrocellulose filter and washed with 3.0 ml of 10 mM $MgCl_2$ and 144 mM Tris (pH 7.8) to isolate the enzyme-bound adenylate, as described by Yarus and Berg (1970). After drying the ^{14}C was monitored as above (4070 cpm). A further 20 μl was incubated for 5 min at 25° with an additional 20 μl of 50 μM ATP and 8.8 μM [^{14}C]tyrosine in tris-Cl and $MgCl_2$. The 40 μl was then filtered as above and the ^{14}C retained on the filter was found to be 4500 cpm. A blank omitting enzyme gave 114 cpm.

Stopped-Flow Fluorescence Studies. The formation of aminoacyl adenylate may sometimes be monitored by the change in the tryptophan fluorescence of the enzyme (Blanquet *et al.*, 1972). One syringe of the stopped-flow spectrofluorimeter contained 2 mM L-methionine (or 100 μM L-tyrosine), 0.5 μM methionyl (or tyrosyl)-tRNA synthetase (from *B. stearothermophilus*) in 10 mM $MgCl_2$, and 144 mM Tris-Cl (pH 7.8) incubated at 25°. The other syringe contained various concentrations of ATP in 10 mM $MgCl_2$ and Tris at 25°. The decrease in fluorescence (excitation at 290 nm, emission at >330 nm) due to aminoacyl adenylate formation on mixing was monitored.

Results

In eq 2, where E = aminoacyl-tRNA synthetase, AA =

$$E \xrightarrow[ATP,AA]{k_1} E\cdot(AA\sim AMP)_n \xrightarrow{k_2} E + \text{products} \quad (2)$$

cognate amino acid, E · AA ~ AMP is the enzyme bound adenylate, and k_1 and k_2 are the pseudo-first-order rate constants for the formation and hydrolysis of the aminoacyl adenylate complex at the given concentrations of AA and ATP, it may be shown that

$$[ATP]_t = [ATP]_0 - n[E]_0\left(\frac{k_1}{k_1 + k_2}\right)\left(\frac{k_1}{k_1 + k_2} - \frac{k_1 e^{-(k_1 + k_2)t}}{k_1 + k_2} + k_2 t\right) \quad (3)$$

where $[ATP]_0$ is the initial concentration of ATP, $[ATP]_t$, that at time t, and $[E]_0$ the total enzyme concentration ($[ATP]_0 \gg [E]_0$).

If $k_2 \ll k_1$, then at $t \gg (k_1 + k_2)^{-1}$

$$[ATP]_t = [ATP]_0 - n[E]_0 - n[E]_0 k_2 t \quad (4)$$

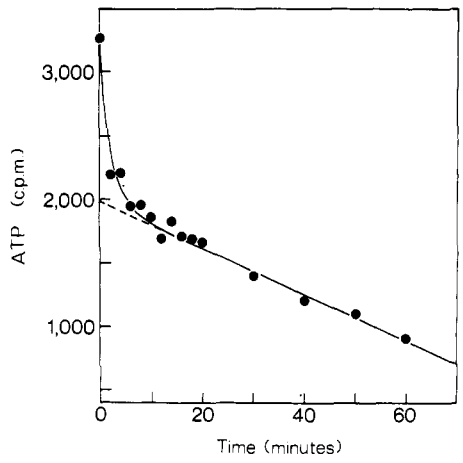

FIGURE 1: Formation of valyl adenylate from valine (1 mM), ATP (5.6 μM), and valyl-tRNA synthetase in the presence of pyrophosphatase at 25°, 10 mM $MgCl_2$, and pH 7.8.

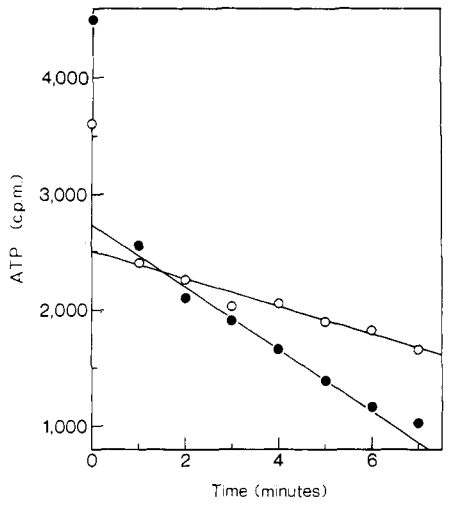

FIGURE 2: Formation of tyrosyl adenylate (○) and methionyl adenylate (●) from tyrosyl-tRNA synthetase (8.8 μM), ATP (31 μM), and tyrosine (0.24 mM), and from methionyl-tRNA synthetase (1 μM from *E. coli*), ATP (5.6 μM), and methionine (1 mM) in the presence of pyrophosphatase as in Figure 1.

(Equation 4 may be obtained intuitively by just assuming $k_1 \gg k_2$ in the reaction scheme.)

The concentration of ATP should initially decrease in a rapid exponential burst which is followed by a linear decrease with time. The linear portion extrapolates back to a value of $[ATP]_0 - n[E]_0$, *i.e.*, a "burst" of $n[E]_0$. If $[ATP]_0$ is only two or three times greater than $[E]_0$ then the burst is not a simple exponential as the aminoacyl adenylation step is not pseudo first order in ATP but the linear portion will still extrapolate back to $[ATP]_0 - n[E]_0$ provided $k_1 \gg k_2$.

In the presence of inorganic pyrophosphatase the reaction of the aminoacyl-tRNA synthetases conformed to the above theory (see Figures 1 and 2). The initial burst of ATP depletion for the reaction of the tyrosyl, tryptophanyl, and two methionyl enzymes occurred before the first measurement (1 min). However, the burst for the valyl enzyme was slow ($t_{1/2} \sim 1.3$ min). The rate constants for the formation of aminoacyl adenylate for the tyrosyl and methionyl enzymes from *B. stearothermophilus* were measured by stopped-flow fluorescence. The value of k_1 for the methionyl enzyme from *E. coli* was calculated approximately from the steady-state data of Lawrence *et al.* (1973).

TABLE I: Stoichiometry, Rate Constants, and Experimental Conditions for the Formation of Aminoacyl Adenylate Complexes at 25° and pH 7.8.

Enzyme	Enzyme (μM)	ATP (μM)	n[a]	k_1[b] ($sec^{-1} \times 10^3$)	k_2[c] ($sec^{-1} \times 10^4$)
Valine[d]	1.4	5.6	0.96	9[e]	2.3
	2.8	5.6	0.93	9	2.3
Tyrosine[d]	2.8	11	1.18	40[f]	8
	5.6	11	1.10	40[f]	11
	8.8	31	1.13	140[f]	19[g]
Tryptophan[d]	1.0	5.5	1.24		
	1.4	5.1	1.68		1.7
	1.4	5.1	1.42		2.3
	2.1	7	1.44		2.2
Methionine[d]	0.9	9.8	2.15	4400[f,h]	26
				60	
Methionine[i]	1.0	5.6	2.22	800[j]	28
	1.6	5.5	2.19	800[j]	22
	1.6[k]	5.5	2.10		13

[a] Number of moles of aminoacyl adenylate/mole of enzyme. [b] Formation rate constant. [c] Decomposition rate constant (eq 1). [d] From *B. stearothermophilus*. [e] Determined from the time course of ATP depletion. [f] From stopped-flow fluorescence. [g] pH 8.0. [h] Biphasic, nonequivalent sites. [i] From *E. coli*. [j] Estimated from the data of Lawrence *et al.* (1973). [k] pH 7.0.

The apparent rate constant for the decomposition of the enzyme adenylate complex, k_2, was determined from the linear portions of ATP against time (eq 4). The experimental conditions and rate constants are summarized in Table I. It is seen that k_1 is at least 40 times greater than k_2 in all cases. The criterion that $k_1 \gg k_2$ is satisfied and the initial burst is equal to $n[E]_0$.

n was calculated from the number of counts retained on charcoal from the aliquots taken before the addition of enzyme, after suitable correction for the dilution on addition of enzyme (X, cpm), the value extrapolated back to zero time from the linear portion (Y, cpm), the pyrophosphate retained at total completion of reaction (X_∞), and the initial ATP and enzyme concentrations ($[ATP]_0$, $[E]_0$)

$$n = \frac{X - Y}{X - X_\infty} \frac{[ATP]_0}{[E]_0}$$

Alternatively as we found that (X_∞/X) is reproducibly 0.065, the formula

$$n = 1.07 \frac{X - Y}{X} \frac{[ATP]_0}{[E]_0}$$

was used.

Our initial experiments were conducted in the absence of inorganic pyrophosphatase and totally artefactual results were obtained due to the reverse of the first reaction, k_1, by mass action.

Comparison with Gel and Nitrocellulose Disk Filtration and Other Techniques. An extensive and rigorous study by Bosshard *et al.* (1975) isolating the adenylate complexes by gel filtration showed that the tyrosyl-tRNA synthetase

formed 0.75–1.1 tyrosyl adenylates/dimer and the tryptophanyl enzyme, 1.8–2.0. Our experiments using this technique gave values of 0.86 and 1.6, respectively, and 0.94 for the valyl enzyme, which may be compared with 1.1, 1.4, and 0.94 from the "burst" assays. The nitrocellulose filter assay gave a value of 1.0 for the tyrosyl enzyme.

The binding stoichiometry of the methionyl enzyme from *E. coli* has been studied by Blanquet *et al.* (1972) using the aminoalkyl adenylate and equilibrium dialysis, and also fluorescence quenching on the *in situ* formation of aminoacyl adenylate on the addition of methionine and ATP. Their results are in excellent agreement with ours.

Discussion

The proposed method of determining the stoichiometry of aminoacyl adenylate formation and binding from the depletion of ATP during reaction is very satisfactory, being simple, rapid, and reproducible. The required condition for a "burst" assay, that $k_1 \gg k_2$ in eq 3, is satisfied at low concentrations of ATP. Typically 5–10 μM concentrations of ATP were used in this study, but in most cases lower concentrations could be used judging from the values of k_1 in Table I. The simplest method of assaying the concentration of ATP, the adsorption on charcoal, requires an enzyme active site concentration of about 20–50% of that of the ATP in order to measure an appreciable *difference*. Using small volumes of about 100 μl, only 1–50 μg of enzyme are required for an assay depending on k_1 and k_2. This may be reduced even further by separating the [γ-^{32}P]ATP from the [^{32}P]orthophosphate by thin-layer chromatography on PEI sheets when the *ratio* of the two may be measured.

Only one radioactive compound is required for all the synthetases. The disadvantage of the short half-life of ^{32}P is not too serious. The [γ-^{32}P]ATP obtainable from Amersham Radiochemical Centre has typical specific activities of 1–2000 Ci/mol so that the effective shelf life is about 2 months as specific activities of only about 50 Ci/mol are required.

Comparison with Other Methods. The burst assay will detect any intermediate where $k_1 > k_2$. Gel and nitrocellulose disk filtration will isolate aminoacyl adenylates given the above condition and also k_2 is slow compared with the isolation time. The filtration procedures should always be calibrated against a burst method or the rigorous steady-state gel filtration technique used by Boeker and Cantoni (1973) in order not to overlook labile sites. As far as time and low consumption of protein are concerned the burst method is superior to steady-state gel filtration. It is also more economical in protein than the routine gel filtration procedure. The most efficient assay for routine use is the nitrocellulose filtration when it is applicable and has been standardized as suggested. For example, we find that it is suitable for the tyrosyl but not for the valyl-tRNA synthetase unless the solutions are chilled.

The results of applying the technique to the five aminoacyl-tRNA synthetases which had been purified to at least 90% homogeneity as judged by the usual criteria are listed in Table I. The monomeric valyl-tRNA synthetase forms one aminoacyl adenylate/molecule, the dimeric methionyl enzymes 2/molecule, the dimeric tryptophanyl enzyme 1–2/molecule, but the dimeric tyrosyl enzyme appears to form only 1 aminoacyl adenylate/dimer. This latter result has also been obtained by Bosshard *et al.* (1975) from gel filtration experiments. They argue that the enzyme exhibits "half-of-the-sites" reactivity. The tyrosyl-tRNA synthetase from *E. coli* is also a dimer of similar molecular weight to the *B. stearothermophilus* enzyme. It has been deduced from nitrocellulose filtration that this also forms only 1 aminoacyl adenylate/dimer (Krajewska-Grynkiewicz *et al.*, 1973). However, gel filtration experiments show that two are formed (Chousterman and Chapeville, 1973).

In the following paper (Fersht, 1975) we shall show how the overall rate of hydrolysis of ATP determined from the linear portion of the burst assay also gives important information on the number of catalytically competent sites.

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