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Free Energy of Hydrolysis of Tyrosyl Adenylate and Its Binding to Wild-Type and Engineered Mutant Tyrosyl-tRNA Synthetases†

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Received April 4, 1986; Revised Manuscript Received June 2, 1986

ABSTRACT: The equilibrium constant for the formation of tyrosyl adenylate and pyrophosphate from ATP and tyrosine in solution has been measured by applying the Haldane relationship to wild-type and three mutant tyrosyl-tRNA synthetases from *Bacillus stearothermophilus*. The formation constant (=[Tyr-AMP][PP_i]/[ATP][Tyr]) at pH 7.78, 25 °C, and 10 mM $MgCl_2$ is $(3.5 \pm 0.5) \times 10^{-7}$. This corresponds to a free energy of hydrolysis of tyrosyl adenylate at pH 7.0 and 25 °C of −16.7 kcal mol^{-1}. All necessary rate constants had been determined previously for the calculations apart from the dissociation constant of tyrosyl adenylate from its enzyme-bound complex. This was measured by taking advantage of the 100-fold difference in hydrolysis rates of the tyrosyl adenylate when sequestered by the enzyme and when free in solution. These are technically difficult measurements because the dissociation constants are so low and the complexes unstable. The task was simplified by using mutants prepared by site-directed mutagenesis. These were designed to have different rate and equilibrium constants for dissociation of tyrosyl adenylate from the enzyme-bound complexes. The dissociation constants were in the range $(3.5–38) \times 10^{-12}$ M, with that for wild type at 13×10^{-12} M. The four enzymes all gave consistent data for the formation constant of tyrosyl adenylate in solution. This not only improves the reliability of the measurement but also provides confirmation of the reliability of the measured kinetic constants for the series of enzymes.

Aminoacyl adenylates are key intermediates in protein biosynthesis. Despite this importance, their free energies of formation and hydrolysis are unknown. The group transfer potentials of aminoacyl adenylates are expected to be high since they are mixed acid anhydrides (Jencks, 1957): the free energy of hydrolysis of luciferyl adenylate is −13.1 kcal mol^{-1} (Rhodes & McElroy, 1958), and the free energy of hydrolysis of acetyl adenylate is at least −13.3 kcal mol^{-1} (Jencks, 1957). It has become important for us in our studies of the tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* (Figure 1) to know the free energy of formation of tyrosyl adenylate from ATP and pyrophosphate. In these studies, we have measured for Figure 1 the dissociation constants of tyrosine (K_t), ATP (K'_a), and pyrophosphate (K_{pp}) and the rate constants k_3 and k_{-3} for the formation of enzyme-bound tyrosyl adenylate and its pyrophosphorolysis for wild-type and a series of mutant tyrosyl-tRNA synthetases (Wells & Fersht, 1986; Ho & Fersht, 1986). Clearly, if we can measure the dissociation constant of tyrosyl adenylate (K_{ta}) from the enzyme–tyrosyl adenylate complex then we can calculate the equilibrium constant (K_{eq}) for the formation of tyrosyl adenylate and pyrophosphate from tyrosine and ATP from a simple ther-

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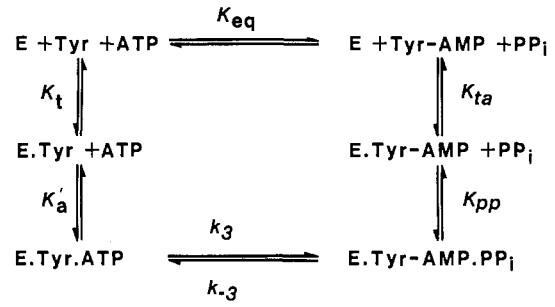
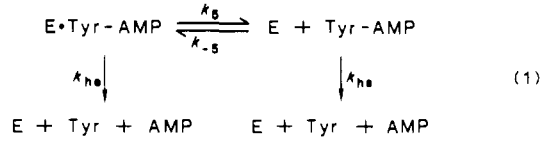


FIGURE 1: Thermodynamic relationships between the enzyme substrate complexes, showing how K_{eq} can be measured from a simple thermodynamic cycle.

modynamic cycle ($K_{eq} = k_3K_{pp}K_{ta}/k_{-3}K'_aK_t$).

The dissociation constants of aminoacyl adenylates from aminoacyl-tRNA synthetases are generally considered to be immeasurably low. A measurement of 4.4 nM for phenylalanyl adenylate from yeast phenylalanyl-tRNA synthetase has been reported (Lin et al., 1983). Measurement of kinetic rate constants have been hampered by the instability of the aminoacyl adenylates in solution (Blanquet et al., 1972). The aminoacyl adenylates are sequestered by their enzymes, and so the enzyme-bound aminoacyl adenylate complexes are relatively stable and can be isolated. We have in this study utilized the difference in stability of tyrosyl adenylate when free in solution and when bound to the enzyme to measure the dissociation constant from the kinetics of hydrolysis (eq 1).



(1)

The rate of hydrolysis for tyrosyl adenylate when bound to wild-type enzyme is over 100-fold slower than the equivalent rate in free solution at pH 7.8 (Fersht, 1975; Mulvey & Fersht, 1977). The observed hydrolysis rate when the concentration of unligated enzyme (E) is varied can, therefore, be used to calculate the fraction of tyrosyl adenylate that is bound to enzyme. This allows the dissociation constant of tyrosyl adenylate from the enzyme to be calculated.

Both the reliability and ease of the measurements are increased by the existence of the series of mutant tyrosyl-tRNA synthetases that have been altered in their properties of binding tyrosyl adenylate (Fersht et al., 1985b). Since the free energy of formation of tyrosyl adenylate is independent of the enzyme used in the thermodynamic cycle, analysis of both wild-type and mutant tyrosyl-tRNA synthetases should produce the same overall free energy change for Figure 1. The kinetic constants of the many mutant enzymes vary considerably, and so mutants can be selected for which the relevant rate constants fall within an "observation window", where the relevant kinetic analysis is simplified.

Experimental Procedures

Materials

Reagents were obtained from Sigma and radiochemicals from Amersham International. Wild-type and mutant tyrosyl-tRNA synthetases were expressed in *Escherichia coli* JM101 hosts from M13mp9 templates constructed as described previously (Carter et al., 1984) and purified to electrophoretic homogeneity according to Wells and Fersht (1986). Nitrocellulose disks (BA 85) were obtained from Schleicher and Schuell.

Methods

Kinetic Analysis. Unless indicated otherwise, all experiments were carried out at 25 °C in a standard buffer of 144 mM Tris-HCl, pH 7.78, 10 mM $MgCl_2$, 0.1 mM phenylmethanesulfonyl fluoride, and 14 mM 2-mercaptoethanol. Kinetic constants for TyrTS(Cys → Gly51) were determined as before (Wells & Fersht, 1986) except that the dissociation constant of tyrosine was measured kinetically from the loss of label from [γ-^{32}P]ATP label rather than by equilibrium dialysis.

Formation of Enzyme–Tyrosyl Adenylate Complexes. A mixture containing 5–8 μM enzyme, 2 mM MgATP, 10 mM ^{14}C-labeled tyrosine (500 Ci/mol), and 1 unit/mL inorganic pyrophosphatase in the standard buffer was incubated at 4 °C for 5 min. Excess ligands were removed by gel filtration at 4 °C on a 1 × 25 cm Sephadex G-50 (superfine) column (Pharmacia) equilibrated in standard buffer. The eluent was monitored by absorbance at 280 nm, and the excluded peak was collected, usually at a concentration of 0.25–0.4 μM. Complexes were prepared daily and at this concentration were stable when stored on ice.

Dissociation of Tyrosyl Adenylate from the Enzyme-Bound Complexes. Vessels that were used to contain the dilute solutions of enzyme required for the kinetic experiments were treated as follows to eliminate the effects of adsorption of protein. Polypropylene bottles (150 mL) were filled with bovine serum albumin (0.1 mg/mL) and incubated at 25 °C overnight to saturate sites that could adsorb protein. The bottles were repeatedly washed with buffer before use in kinetic experiments. Further treatment with albumin was not found to be necessary in subsequent use. Other containers were tested but gave erratic losses of enzyme-bound tyrosyl adenylate, perhaps caused by either adsorption sites on the plastic and/or low levels of nucleophiles remaining on the plastic surface.

The enzyme-bound ^{14}C-labeled tyrosyl adenylate complex was diluted to 0.2 nM into standard buffer, containing various concentrations of unligated tyrosyl-tRNA synthetase, and incubated at 25 °C. (Stock solutions of unligated enzymes were pretreated by incubating at 25 °C with small quantities (<1 unit/mL) of inorganic pyrophosphatase. This removed any traces of pyrophosphate that remained in the enzyme preparation and thus eliminated artifacts arising from pyrophosphorolysis.) Aliquots (5 mL) were periodically filtered through presoaked nitrocellulose disks and washed with chilled standard buffer (5 mL). The fraction of enzyme-bound tyrosyl adenylate complex remaining in solution was determined by the retention of [^{14}C]tyrosine on the filters. The first-order decay was followed for 1–3 half-lives, each time course containing 10 or more time points.

Experiments to measure the rate of breakdown of enzyme-bound tyrosyl adenylate at relatively high concentrations of added unligated enzyme (100–200 nM) were performed in 1.5 mL Eppendorf tubes, using 12–25 nM enzyme [^{14}C]tyrosyl adenylate complex and taking 50-μL aliquots for nitrocellulose disk filtration. Acid-precipitable background was determined by adding an aliquot (50 μL) to 5% trichloroacetic acid (1 mL) and collecting the precipitate on a glass fiber filter as before.

Chase Reactions with Excess Cold Tyrosine and ATP. The rate of dissociation of [^{14}C]tyrosyl adenylate from the enzyme-bound complex was also measured by "chasing off" with high concentrations of unlabeled tyrosine and ATP. A solution containing [^{14}C]tyrosyl adenylate–enzyme complex (200 nM) and 1 unit/mL inorganic pyrophosphatase in standard buffer was incubated at 25 °C. To initiate the chase reaction,

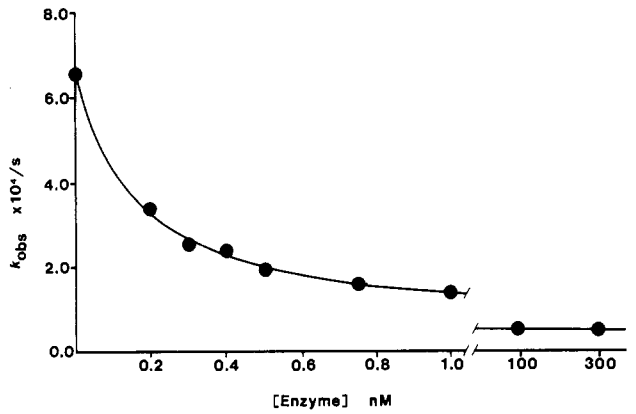


FIGURE 2: Dependence of observed decay rate of tyrosyl adenylate (k_{obsd}) on free enzyme concentration for the wild-type enzyme. k_5 is calculated from the [E] = 0 intercept, k_{he} from k_{obsd} at infinite free enzyme and k_{-5} from the curvature. Data were fitted by using a nonlinear least-squares fit program (R. J. Leatherbarrow, unpublished results).

MgATP and tyrosine were added to final concentrations of 10 mM and 200 μM, respectively. Aliquots (10 μL) were spotted onto nitrocellulose disks and washed with 5 mL of chilled buffer as above.

Stability of Enzyme at High Dilution. A solution of enzyme (1 nM) in the standard buffer was incubated at 25 °C under standard conditions. Aliquots (1 mL) were added periodically to a solution (100 μL) of 2 μM [^{14}C]tyrosine, 20 mM MgATP, and 1 unit/mL inorganic pyrophosphatase for active-site titration. Triplicate aliquots were filtered onto nitrocellulose disks. All enzymes studied retain full activity for the time studied (at least 2 h) under these conditions.

Covalent Modification. In experiments conducted for more than 1 h, significant deviation from first-order decay was seen in the total counts measured by filtration through nitrocellulose disks. This was found to be caused by the accumulation of a fraction containing [^{14}C]tyrosine which was acid-precipitable, implying covalent modification. Such modification has been noted frequently in this laboratory and has been the subject of two recent papers on other aminoacyl-tRNA synthetases. Kern et al. (1985) report covalent aspartylation of Asp-tRNA synthetase, and Rapaport et al. (1985) report covalent modification of Phe-tRNA synthetase. The covalent modification is slow, with a maximum observed rate of 2×10^{-5} s^{-1}, and does not affect the kinetics of this study. The radioactivity just increases the background counts and was subtracted accordingly as follows. In experiments parallel with those where samples were filtered through nitrocellulose disks, an aliquot (5 mL) of reaction mixture was added to 200 μL of 75% trichloroacetic acid, which was then filtered through a Whatman GF/C glass fiber filter disk, which retained the precipitated protein.

RESULTS

Dissociation Constants of Tyrosyl Adenylate Complexes. The dissociation and subsequent hydrolysis of tyrosyl adenylate from both wild-type and selected mutant enzymes was slow enough for the reactions to be monitored manually without recourse to rapid reaction equipment. For all enzymes studied, the decay of the noncovalently enzyme-bound complex followed simple first-order kinetics. The observed rate constant for the decay decreases with increasing concentrations of added free enzyme and approaches a limiting value at concentrations of about 200 nM (Figure 2). This observation is consistent with the hypothesis that the overall hydrolysis rate of tyrosyl adenylate is decreased from that measured in free solution

Table I: Rate and Equilibrium Constants for Steps in the Overall Hydrolysis of Enzyme–Tyrosyl Adenylate Complexes[a]

enzyme	k_5 (s^{-1})	k_{-5} (s^{-1} M^{-1})	k_{he} (s^{-1})	K_{ta} (M)
wt	6.72×10^{-4}	5.09×10^{7}	5.14×10^{-5}	1.32×10^{-11}
Δwt[b]	5.18×10^{-4}	5.60×10^{7}	5.24×10^{-5}	9.25×10^{-12}
Thr → Gly51	2.65×10^{-3}	6.86×10^{7}	4.89×10^{-5}	3.86×10^{-11}
Thr → Ala51	2.78×10^{-4}	8.01×10^{7}	6.05×10^{-5}	3.47×10^{-12}

[a] K_{ta} is the dissociation constant of tyrosyl adenylate from the enzyme, calculated by $K_{ta} = k_5/k_{-5}$. Kinetic constants are as defined in eq 1 and were measured at pH 7.78 and 25 °C. [b] Experiment performed on truncated mutant, lacking the tRNA binding domain (Waye et al., 1983). Standard errors are typically less than ±5% for k_5 and ±10% for k_{-5}.

(Fersht, 1975; Mulvey & Fersht, 1977) by being sequestered by the tyrosyl-tRNA synthetase that had been added to the solution, as described by eq 1. This scheme can be solved analytically, subject to certain conditions (see Appendix) to give

$$k_{obsd} = \frac{k_{he}k_{hs} + k_{he}k_{-5}[E] + k_5k_{hs}}{k_{he} + k_5 + k_{-5}[E] + k_{hs}} \quad (2)$$

k_{hs} has been measured previously ($k_{hs} = 7.6 \times 10^{-3}$ s^{-1}; Mulvey & Fersht, 1977). Thus, k_{he}, k_{-5}, and k_5 can be determined by curve fitting the dependence of the observed decay rate k_{obsd} on the free enzyme concentration (Figure 2).

The individual values of the rate and equilibrium constants for each mutant (Table I) fall into a consistent pattern. Δwt, a truncated form of the enzyme that lacks the tRNA-binding domain (Waye et al., 1983) has a value of the dissociation constant K_{ta} which is very similar to that of the wild type, about 10 pM. The mutation Thr→Gly-51, which is known to lower the binding energy of tyrosyl adenylate in the transition state by 0.28 kcal mol^{-1} (Fersht et al, 1985a), leads to a 3-fold increase in dissociation constant. Conversely, the mutation Thr → Ala-51, which is known to increase the binding energy of tyrosyl adenylate by 0.96 kcal mol^{-1}, decreases the dissociation constant by a factor of 4. The second-order rate constants for the association of tyrosyl adenylate with the enzymes are all very similar; the discrimination in binding is determined mainly by the dissociation rate constant. The rate constant for the hydrolysis of the enzyme-bound tyrosyl adenylate is also very similar for each enzyme. The rate constants for dissociation (k_5) vary from about only 5 to 50 times higher than the rate constant for hydrolysis on the enzyme (k_{he}). It could well be for other certain other aminoacyl-tRNA synthetases that k_{he} is greater than k_5. Values of k_5 measured by curve fitting are consistent with values measured by a "chase" experiment on the addition of excess unlabeled tyrosine and ATP to the preformed tyrosyl adenylate complex.

Calculation of the Equilibrium Constant for the Formation of Tyrosyl Adenylate in Solution. The equilibrium constant K_{eq} for the reaction in solution (eq 3) is independent of the

$$E + Tyr + ATP \rightleftharpoons E + Tyr\text{–}AMP + PP_i \quad (3)$$

enzyme used, allowing both confirmation of the result and selection of a mutant enzyme with kinetic parameters within an optimal "observation window" (Appendix). It is seen from Figure 1 that

$$K_{eq} = k_3K_{pp}K_{ta}/K'_aK_tk_{-3} \quad (4)$$

with constants as defined in Figure 1. With experimental data from this study for TyrTS(Thr → Gly-51) and Δwt (Table II) and from Ho and Fersht (1986), and Wells and Fersht (1986) for wild type and TyrTS(Thr → Ala-51), values for

Table II: Kinetic and Thermodynamic Data for the Formation of Tyrosyl Adenylate by Δwt and Thr → Gly51[a]

enzyme	K_t (μM)	K'_a (mM)	k_3 (s^{-1})	k_3 (s^{-1})	K_{pp} (mM)
Δwt[b]	12	5.2	34	16.0	0.85
Thr → Gly51	12	2.07	13.1	16.3	0.36

[a] Kinetic constants as defined in Figure 1. [b] Truncated mutant, lacking the tRNA binding domain (Waye et al., 1983). Forward reaction for Δwt was taken from Wells and Fersht (1985). Standard errors are typically ±5% for rate processes and ±10% for equilibrium constants.

Table III: Equilibrium Constants for Formation of Tyrosyl Adenylate in Solution

enzyme	K_1[a] (M^{-1})	K_{ta} (M)	K_{eq} for [Tyr–AMP][PP_i]/[Tyr][ATP][b]
wt	2.48×10^4	1.32×10^{-11}	3.27×10^{-7}
Δwt	2.70×10^4	9.25×10^{-12}	2.49×10^{-7}
Thr → Gly51	1.15×10^4	3.85×10^{-11}	4.44×10^{-7}
Thr → Ala51	1.12×10^5	3.47×10^{-12}	3.89×10^{-7}

[a] $K_1 = (k_3/K'_aK_t)/(k_{-3}/K_{pp})$; $K_{eq} = K_1K_{ta}$. [b] Mg^{2+} salts of PP_i and ATP.

K_{eq} have been calculated and are listed in Table III. The narrow spread of values is quite satisfactory, considering the number of individual rate and equilibrium constants involved in the calculations. The average value for K_{eq} is $(3.5 \pm 0.5) \times 10^{-7}$, which corresponds to a free energy change of 8.8 kcal mol^{-1}.

Phosphate Group Transfer Potential. The phosphate group transfer potential is defined as the free energy released on hydrolysis of a phosphate ester or anhydride. For tyrosyl adenylate this reaction is

$$\text{Tyr–AMP} + H_2O \rightleftharpoons \text{Tyr} + \text{AMP} \quad (5)$$

and can be represented as the sum of two equations

$$\text{Tyr–AMP} + MgPP_i \rightleftharpoons \text{Tyr} + MgATP \quad (6)$$

$$H_2O + MgATP \rightleftharpoons MgPP_i + \text{AMP} \quad (7)$$

The free energy change under standard conditions (pH 7) for eq 7 has been measured as −7.7 kcal mol^{-1} (Jencks & Gilchrist, 1964). In order to add eq 6 and 7 and calculate the phosphate group transfer potential for tyrosyl adenylate, the free energy change measured for reaction 6 at pH 7.78 must be corrected to pH 7.0. The only relevant ionization in this range is that of magnesium pyrophosphate, which has a pK_a of 6.6. This leads to an additional 0.2 kcal for hydrolysis at pH 7.0 compared with pH 7.78. The biochemical standard free energy for tyrosyl adenylate production is thus 9.0 kcal mol^{-1}. From this it follows that the phosphate group transfer potential is 16.7 kcal mol^{-1}.

Discussion

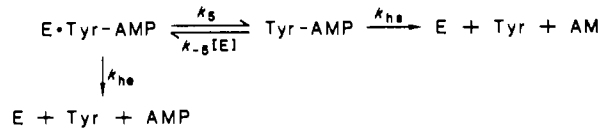
We have measured the dissociation constant of tyrosyl adenylate from wild type tyrosyl-tRNA synthetase to be 13 pM. From this, the formation constant, K_{eq}, for tyrosyl adenylate from ATP and tyrosine is calculated to be $(3.5 \pm 0.5) \times 10^{-7}$ at pH 7.78 and 25 °C. According to the Haldane equation, this value should be independent of the enzyme used. Similar studies on three mutant enzymes produced values for K_{eq} that are in satisfactory agreement. The internal consistency shows that the kinetic scheme proposed for the formation of tyrosyl adenylate describes the observed kinetic behavior for all four enzymes and also that our estimates of errors for the measurements of the many rate and equilibrium constants used in the calculations (Wells & Fersht, 1986) are satisfactory.

Aminoacyl adenylates have long been regarded as high-energy intermediates on the pathway of aminoacylation of tRNA. The high free energy of hydrolysis of these phosphate ester compounds arises mainly from two sources. First, the large entropy changes that accompany the hydrolysis of nucleoside phosphates and the decrease in the magnitude of enthalpy of hydrolysis with increasing charge (George et al., 1970) imply that solvent effects rather than electrostatic repulsion are the major factor in producing the large free energy of hydrolysis. This idea is supported by both molecular orbital calculations (Hayes et al., 1978) and experiments in nonpolar solvents (Wolfenden & Williams, 1985). Second, the carbonyl group in acyl adenylates destabilizes the phosphate ester by electron withdrawal. Thus, phosphate group transfer potentials higher than that for ATP have already been recorded for acetyl adenylate (−13.3 kcal mol^{-1}; Jencks, 1957) and luciferyl adenylate (−13.1 kcal mol^{-1}; Rhodes & McElroy, 1958). The free energy of hydrolysis of tyrosyl adenylate is calculated in this study to be 16.7 kcal mol^{-1}. The additional 3.4 kcal mol^{-1} of stabilization for this compound compared with that for acetyl adenylate is most likely caused by the presence of a charged amino group in the tyrosyl adenylate. This stabilizes the charge on the carboxylate ion of tyrosine produced on hydrolysis, thus increasing the free energy of hydrolysis. An identical additional stabilization of 3.4 kcal mol^{-1} is found when the free energies of hydrolysis of glycine ethyl ester and acetyl ethyl ester are compared (Jencks et al., 1960). This demonstrates that the additional stabilization is indeed due to the charged amino group of tyrosine rather than the phenolic side chain. The effects of such stabilization can also be seen in the differences in pK_a's for the carboxyl groups, 2.2 for tyrosine and 4.8 for acetate.

The tyrosyl-tRNA synthetase catalyzes the production of bound tyrosyl adenylate by changing the equilibrium constant for its formation from 3.5×10^{-7} in solution to 2.3 between enzyme-bound species (Wells & Fersht, 1986). This corresponds to an additional stabilization of tyrosyl adenylate provided by the enzyme of 9.3 kcal mol^{-1} compared with that for tyrosine and ATP. Site-directed mutagenesis has been applied to remove hydrogen bonding groups from the tyrosyl adenylate binding region of the enzyme active site, to determine the source of this stabilization. It has been shown that the residues Cys-35, Tyr-34, and His-48 are responsible for some 3 kcal mol^{-1} of stabilization energy. The remaining 6 kcal mol^{-1} may arise from two sources. First, there are other groups within the active site whose differential binding energy is currently being examined (D. M. Lowe and A. R. Fersht, unpublished results). Second, the free energies of hydrolysis of phosphoric and carboxylic acid anhydrides have been shown to be diminished by a lowering of the polarity of the reaction medium (Wolfenden & Williams, 1985), and therefore may be lower for the enzyme-bound equilibria.

This study has also demonstrated the importance of the preferential binding of tyrosyl adenylate compared with the binding of the transition state (Wells & Fersht, 1986). Tyrosyl adenylate in free solution is a highly reactive tyrosylating reagent. Unless it is bound tightly as an intermediate, tyrosyl adenylate will act as a relatively nonspecific tyrosylation agent with free nucleophiles, sometimes with detrimental consequences in vivo. Tight binding of tyrosyl adenylate also keeps the molecule within the active site cleft, where (comparing k_{he} with k_{hs}) the susceptibility to hydrolysis is lowered by 2 orders of magnitude. As protein synthesis is often a major consumer

Scheme I



of metabolic energy, it is clearly important that the servicing reactions, such as those catalyzed by tyrosyl-tRNA synthetase are as efficient as possible, and there will be strong selective pressure on the enzyme to ensure this. Enzyme catalysis is not just the process of enhancing the reaction rate of the catalysed reaction, but also involves the lowering of the rate of side reactions, in this case, hydrolysis. The enzyme tyrosyl-tRNA synthetase has evolved to generate tyrosyl-tRNA^{Tyr} both in a rapid manner and at high yield.

Appendix

Dissociation Constant Measurement for Tyrosyl Adenylate. The Observation Window. The breakdown of the enzyme-bound tyrosyl adenylate complex, by a combination of hydrolysis in free solution and within the active site can be represented by Scheme I. k_{he} represents the overall rate of hydrolysis at infinitely high concentrations of free enzyme. Under such conditions, any tyrosyl adenylate dissociating from the enzyme will be sequestered by the excess free enzyme faster than hydrolysis occurs.

Scheme I cannot be analyzed in general by using the steady-state approximation, because the concentration of free Tyr–AMP may become appreciable. The steady state applies when either k_{hs} or/and $k_{-5}[E] \gg k_5$. It is therefore necessary to treat the system as one which never attains a true steady state and solve the differential equations which describe the system accordingly.

In all experiments in this study, an excess of free enzyme [E] was added to the enzyme tyrosyl adenylate complex to ensure that, within the observed time range, the concentration of free enzyme remained constant. Under such conditions the differential equations describing eq 1 are linear.

$$\begin{aligned} d/dt[E\cdot TA] &= -(k_{he} + k_5)[E\cdot TA] + k_{-5}[E][TA] \\ d/dt[TA] &= k_5[E\cdot TA] - (k_{-5}[E] + k_{hs})[TA] \end{aligned} \quad \text{(A-1)}$$

where TA and E·TA represent the free and enzyme-bound tyrosyl adenylate species, respectively. Applying the Laplace transform

$$f(t) = \int_{-\infty}^{\infty} F(t) \exp(-st)\, dt = L\{F(t)\}$$

(see Stephenson, 1973) and writing $e\tilde{t}a = L\{E\cdot TA\}$ and $\tilde{t}a = \tilde{L}\{TA\}$ converts eq 1 into the matrix equation

$$\begin{bmatrix} s + k_{he} + k_5 & -k_{-5}[E] \\ -k_5 & k_{hs} + k_{-5}[E] + s \end{bmatrix} \begin{bmatrix} e\cdot\tilde{t}a \\ \tilde{t}a \end{bmatrix} = \begin{bmatrix} E\cdot TA_0 \\ 0 \end{bmatrix} \quad \text{(A-2)}$$

subject to the boundary conditions $[E\cdot TA] = [E\cdot TA]_0$ and $[TA] = 0$ at $t = 0$. This equation can be solved, and the inverse transform calculated to give an equation defining [E·TA] as a function of time. However, solving the equation

$$\det \begin{vmatrix} s + k_{he} + k_5 & -k_{-5}[E] \\ -k_5 & k_{hs} + k_{-5}[E] + s \end{vmatrix} = 0 \quad \text{(A-3)}$$

for s produces two roots, which are the two exponential factors in the equation $[ETA] = A \exp(-\lambda_1 t) + B \exp(-\lambda_2 t)$, which describes mathematically the biphasic decay of tyrosyl adenylate mathematically. From eq A-3

$$s^2 + (k_{he} + k_5 + k_{-5}[E] + k_{hs})s + k_{he}k_{hs} + k_5k_{hs} + k_{he}k_{-5}[E] = 0 \quad \text{(A-4)}$$

The two phases are clearly distinguishable, subject to the condition $\lambda_1 \gg \lambda_2$; i.e.

$$(k_{he} + k_5 + k_{-5}[E] + k_{hs})^2 \gg 4(k_{he}k_{hs} + k_5k_{hs} + k_{he}k_{-5}[E]) \quad \text{(A-5)}$$

In this study, all the enzymes were studied at concentrations sufficiently high to ensure that the $k_{-5}[E]$ term was the dominant one, and thus the inequality holds. Since $\lambda_1 \gg \lambda_2$, it follows directly from eq A-4 and quadratic theory that

$$\lambda_2 = k_{obsd} = \frac{k_{he}k_{hs} + k_{he}k_{-5}[E] + k_5k_{hs}}{k_{he} + k_5 + k_{-5}[E] + k_{hs}} \quad \text{(A-6)}$$

Under conditions where $k_{hs} \gg k_5$ (the dissociation rate is slow), this expression can be reduced to the steady-state approximation

$$k_{obsd} = k_{he} + \frac{k_5k_{hs}}{k_{-5}[E] + k_{hs}} \quad \text{(A-7)}$$

The limiting case where the free enzyme concentration approaches zero is difficult to study. At $[E] \simeq 0$ the condition $(\lambda_1 \gg \lambda_2)$ is not necessarily obeyed for systems where k_5 approaches k_{hs}. In addition, it becomes harder experimentally to maintain the condition $d[E]/dt = 0$. However, by the addition of large amounts of ATP and unlabeled tyrosine, any available active sites can be filled with cold tyrosyl adenylate complex, reducing the free enzyme concentration to zero. Under these conditions, $k_{obs} = k_5 + k_{he}$, enabling k_5 to be calculated. This value can be used to reduce the parameter redundancy involved in measuring $k_5[E]$. k_{he}, k_5, and k_{-5} were then determined by fitting the data to eq A-6 with a nonlinear least-squares fit program (R. J. Leatherbarrow, unpublished work) based on the Marquardt algorithm (Marquardt, 1963). The nature of eq A-6 and the assumptions made in the derivation mean that accurate independent estimation of these constants is only possible for enzymes within a narrow range of k_5 values.

Lower Limit for k_5. If k_5 is only slightly faster than k_{he}, then a large proportion of the hydrolysis will occur on the enzyme. Only small changes in observed decay rate will be observed as the free enzyme concentration is decreased. There will be a very low "signal" (in solution hydrolysis) to "noise" (on-enzyme hydrolysis) ratio. The signal to noise problem is made worse by the difficulties of studying the enzyme at concentrations lower than 0.2 nM, the region where most information is obtained about the $k_5/k_{-5}[E]$ ratio. In practice, the limit of resolution is approximately $k_5 = 2.5 \times 10^{-4}$ s^{-1}, about the dissociation rate constant for TyrTS(Ala-51).

Upper Limit. Above this threshold, any increase in k_5 will enhance the "signal:noise" ratio, enabling more accurate estimations of the kinetic constants. The upper limit of accurate data collection is determined by two factors. First, as k_5 becomes faster, the condition $\lambda_1 \gg \lambda_2$ no longer applies at low enzyme concentrations and the decay curve starts to appear biphasic. Furthermore, at such low free enzyme concentrations it is necessary to take 5-mL samples at each time point, and when $k_5 > 0.01$ s^{-1}, it becomes technically difficult to time these accurately.

Registry No. PP_i, 14000-31-8; ATP, 56-65-5; Tyr–AMP, 50466-77-8; L-Tyr, 60-18-4; tyrosyl-tRNA synthetase, 9023-45-4.

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Diphtheria Toxin Can Simultaneously Bind to Its Receptor and Adenylyl-(3′,5′)-uridine 3′-Monophosphate†

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Received May 12, 1986; Revised Manuscript Received July 9, 1986

ABSTRACT: Diphtheria toxin that was bound to receptors on BS-C-1 cells was able to bind approximately 1 molar equiv of adenylyl-(3′,5′)-uridine 3′-monophosphate (ApUp). In contrast, receptor-bound CRM197, a mutant form of toxin with greatly diminished affinity for dinucleotides, did not bind ApUp. Affinity of the dinucleotide for receptor-bound toxin differed from that for free toxin by less than an order of magnitude. These results indicate that the receptor site and the ApUp site on the toxin do not significantly overlap.

There is evidence suggesting that the receptor site and the dinucleotide site on diphtheria toxin (DT)[1]—which perform functionally and temporally distinct events in the intoxication process and lie on opposite ends of the primary structure—may overlap in the native toxin. Compounds containing multiple phosphate residues, such as nucleoside triphosphates or inositol hexaphosphate, inhibit both binding of the toxin to cells (Middlebrook & Dorland, 1979) and binding of NAD or the endogenous dinucleotide, adenylyl-(3′,5′)-uridine 3′-monophosphate (ApUp), to the toxin (Lory et al., 1980; Collins & Collier, 1984). A variety of evidence supports a model in which the dinucleotide binding site on the A moiety is in close proximity to a cationic, phosphate-binding region (P-site) on the B moiety (Lory et al., 1980; Proia et al., 1981). It has therefore been suggested that the P-site might actually correspond to the receptor site. If this were true, one would expect that association of the toxin with its receptor would prevent binding of dinucleotides.

To test this possibility we took advantage of the high affinity of ApUp for the dinucleotide site on DT. ApUp (whose source and function are uncertain) is tightly bound to a fraction of the toxin molecules in many preparations (Barbieri et al., 1981). Its binding is competitive with respect to NAD and various phosphorylated compounds (Collins & Collier, 1984). At 5 °C in 50 mM Tris-HCl buffer, pH 7.1, the K_d of ApUp for the toxin in solution is 9 pM and the half-life of the toxin-ApUp complex is about 60 min (Collins et al., 1984). Thus if receptor-bound toxin were able to bind ApUp with a similar affinity, the complex should be sufficiently stable to survive brief washing and allow accurate quantification. We therefore performed experiments to determine if toxin bound to receptors

† This work was supported by Grant AI-22021 from the National Institute of Allergy and Infectious Diseases and Grant CA-39217 from the National Cancer Institute. Partial support was received from the Shipley Institute of Medicine. J.T.B. was recipient of Postdoctoral Fellowship AI-06607 from the National Institute of Allergy and Infectious Diseases. C.M.C. was a predoctoral trainee of Grant AI-07116 from the National Institute of Allergy and Infectious Diseases.

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[1] Abbreviations: DT, diphtheria toxin; ApUp, adenylyl-(3′,5′)-uridine 3′-monophosphate; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/86/0425-6608$01.50/0