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Tyrosyl-tRNA Synthetase Acts as an Asymmetric Dimer in Charging tRNA. A Rationale for Half-of-the-Sites Activity†

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ABSTRACT: Tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* is a classical example of an enzyme with half-of-the-sites activity. The enzyme crystallizes as a symmetrical dimer that is composed of identical subunits, each having a complete active site. In solution, however, tyrosyl-tRNA synthetase binds tightly, and activates rapidly, only 1 mol of Tyr/mol of dimer. It has recently been shown that the half-of-the-sites activity results from an inherent asymmetry of the enzyme. Only one subunit catalyzes formation of Tyr-AMP, and interchange of activity between subunits is not detectable over a long time scale. Paradoxically, however, the kinetics of tRNA charging are biphasic with respect to [Tyr], suggesting that both subunits of the dimer are catalytically active. This paradox has now been resolved by kinetic analysis of heterodimeric enzymes containing different mutations in each subunit. Biphasic kinetics with unchanged values of K_M for Tyr are maintained when one of the two tRNA-binding domains is removed and also when the affinity of the "inactive" site for Tyr is reduced by 2–58-fold. The biphasic kinetics do not result from catalysis at both active sites, but instead appear to result from two molecules of Tyr binding sequentially to the same site. A second molecule of Tyr perhaps aids the dissociation of Tyr-tRNA by displacing the tyrosyl moiety from its binding site. A monomer of the enzyme is probably too small to allow both recognition and aminoacylation of a tRNA molecule. This could explain the requirement for the enzyme to function as an asymmetric dimer.

Tyrosyl-tRNA synthetase (YTS/YTS)[1] from *Bacillus stearothermophilus* catalyzes aminoacylation of tRNA as a two-step reaction (eq 1 and 2). The enzyme comprises two

$$E + Tyr + ATP \rightleftarrows E{\cdot}Tyr\text{-}AMP + PP_i \quad (1)$$

$$E{\cdot}Tyr\text{-}AMP + tRNA \rightarrow E + Tyr\text{-}tRNA + AMP \quad (2)$$

subunits of identical composition and crystallizes as a symmetrical dimer (Blow & Brick, 1985). Each monomer has a complete active site, but YTS/YTS exhibits half-of-the-sites activity in that only 1 mol of Tyr is bound tightly, and 1 mol of Tyr-AMP formed rapidly per mol of dimer (Fersht, 1975; Fersht et al., 1975). Paradoxically, the Tyr dependence of tRNA charging kinetics has two phases, implying that 2 mol of Tyr bind during each turnover (Jakes & Fersht, 1975). Further, each enzyme dimer behaves asymmetrically as the same subunit is used for every turnover in the steady state (Ward & Fersht, 1988). The second subunit does not have sufficient catalytic activity in formation of E·Tyr-AMP to produce a second phase in tRNA charging kinetics. This raises two questions. First, does binding of 2 mol of tRNA cause both subunits to act catalytically? Second, if not, does the second mole of Tyr bind to the subunit that forms Tyr-AMP or to the "inactive" subunit?

We now answer these questions using simple and direct experiments that are based upon kinetic analysis of heterodimers that have been engineered by using the known domain structure of the enzyme (Carter et al., 1986; Ward & Fersht, 1988). Each subunit of YTS/YTS comprises two functional domains (Waye et al., 1983). The subunits interact through the N-terminal domains, which catalyze activation of Tyr (eq 1). The C-terminal domains are required for binding of tRNA but are not involved in contacts between the monomers. Deletion of the C-terminal domains produces ΔYTS/ΔYTS

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[1] Abbreviations: (for subunits of tyrosyl-tRNA synthetase) YTS, wild-type; ΔYTS, truncated wild-type [see Waye et al. (1983)]; YTS-(Asn-45), His → Asn-45 mutation; YTS(Ala-173), Gln → Ala-173 mutation; YTS(Gly-195), Gln → Gly-195 mutation.

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and has no significant effect on the kinetics of activation of Tyr (Waye et al., 1983).

Heterodimers of YTS/ΔYTS have been prepared by reversible denaturation of mixtures of parent homodimers (Cater et al., 1986; Bedouelle & Winter, 1986; Ward & Fersht, 1988). These enzymes are useful in investigation of half-of-the-sites activity since different mutations can be introduced into each subunit of the dimer. Each heterodimer is active at only one site, but the site used is randomly distributed between the subunits (Ward & Fersht, 1988). Each heterodimer thus consists of two populations, one activating Tyr at the full-length subunit and the other using the truncated subunit. There is no detectable interconversion between active and inactive sites when the enzymes turn over in the steady state (Ward & Fersht, 1988). tRNA must cross the subunit interface in order to be charged (Carter et al., 1986). Thus, enzyme that forms Tyr-AMP at the truncated subunit can use the intermediate to charge tRNA which is bound to the full-length subunit. Heterodimers that activate Tyr at the full-length subunit cannot charge tRNA.

We now show that tyrosyl-tRNA synthetase maintains half-of-the-sites activity when charging tRNA and that biphasic kinetics are most reasonably explained by two molecules of Tyr binding to the same site.

Experimental Procedures

Materials

Reagents were purchased from Sigma (London), Cambridge Biotechnology Ltd, BDH, and Amersham International.

Production of Mutant Enzymes. Enzymes were prepared as described by Fersht et al. (1985), Lowe et al. (1985), and Ward and Fersht (1988).

Transfer RNA. Isolation of $tRNA^{Tyr}$ from *B. stearothermophilus* is a difficult procedure that gives poor yields. tRNA from *Escherichia coli* has, therefore, often been used for characterization of charging kinetics [for example, see Carter et al. (1986) and Bedouelle and Winter (1986)]. This homologue varies from the authentic substrate both in base sequence and posttranscriptional modifications. These problems were decreased in the current work by using tRNA encoded by a synthetic gene (constructed by T. Borgford & T. E. Gray). The gene was expressed in *E. coli*, producing tRNA identical in sequence with that from *B. stearothermophilus* but with posttranscriptional modifications characteristic of its host. The kinetics of aminoacylation of the synthetic tRNA (Table I) by YTS/YTS are very similar to those for a preparation of authentic *B. stearothermophilus* tRNA in terms of K_M for Tyr and K_M for ATP, but the value of k_{cat} is slightly decreased. This decrease does not affect interpretation of results since values of k_{cat} sometimes differ between different preparations of authentic *B. stearothermophilus* tRNA. A single preparation of synthetic tRNA was used for the whole of the work reported in this paper.

Methods

Measurement of Enzyme Concentration. Active-site titration by filtration through nitrocellulose disks was used to determine the concentration of tyrosyl-tRNA synthetase (Wilkinson et al., 1983). The assay consists of measuring accumulation of the stable enzyme-bound Tyr-AMP complex in the presence of inorganic pyrophosphatase (see eq 1). The intermediate does not accumulate fully on the YTS(Asn-45), YTS(Ala-173), and YTS(Gly-195) mutants. The assay conditions were, therefore, modified so that these enzymes reach saturation (Lowe et al., 1987; Ward & Fersht, 1988). Each dimer forms 1 mol of Tyr-AMP/mol of dimer upon extrapolation to infinite concentration of Tyr. The intermediate does not accumulate above this level.

Table I: Comparison of Characteristics of Charging Kinetics Using Authentic or Synthetic tRNA[a]

	Tyr dependence				ATP dependence	
tRNA	K_{M1} (μM)	k_{cat1} (s^{-1})	K_{M2} (μM)	k_{cat2} (s^{-1})	K_M (mM)	k_{cat} (s^{-1})
authentic[b]	2.0	1.3	120	5.4	2.5	3.7
synthetic[c]	2.2	0.92	130	3.8	2.3	2.9

[a] Experimental conditions and methods of data analysis are given under Methods. Values of k_{cat} were obtained by extrapolation to infinite ATP and Tyr assuming Michalis–Menten kinetics. Kinetics were characterized by using 10–20 nM wild-type enzyme. Tyr dependence of tRNA charging was determined by using 10 mM ATP and 0.3–300 μM Tyr. ATP dependence was determined by using 100 μM Tyr and 0.2–12.5 mM ATP. [b] $tRNA^{Tyr}$ isolated from *B. stearothermophilus* was used as substrate. [c] tRNA encoded by the synthetic gene was employed as substrate.

Kinetic Procedures. All experiments were performed at 25 °C and in 144 mM Tris-HCl, pH 7.8, containing 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 0.1 mM phenylmethanesulfonyl fluoride. Rates of pyrophosphate exchange were measured as described by Calendar and Berg (1966). The kinetics of aminoacylation of tRNA were determined by the method of Wilkinson et al. (1983). Kinetic data were analyzed by using the Enzfitter program (Leatherbarrow, 1987).

Kinetic Analysis of Enzymes That Can Bind 2 mol of Substrate and Form Only 1 mol of Product per Turnover. Consider the following general scheme.

$$E \underset{\pm S}{\overset{K_{M1}}{\rightleftharpoons}} E\cdot S \underset{\pm S}{\overset{K_{M2}}{\rightleftharpoons}} E\cdot S_2 \xrightarrow{k_{cat\,2}} E + P + S \qquad (3)$$

$$E\cdot S \xrightarrow{k_{cat\,1}} E + P$$

K_{M1} and K_{M2} are the apparent dissociation constants for E·S and E·S_2, respectively. The rate constants for formation of product are k_{cat1} and k_{cat2}. Let total concentration of enzyme be $[E]_0$ and assume $[S] \gg [E]_0$; then

$$\frac{v}{[E]_0} = \frac{\dfrac{k_{cat1}[S]}{K_{M1}} + \dfrac{k_{cat2}[S]^2}{K_{M1}K_{M2}}}{1 + \dfrac{[S]}{K_{M1}} + \dfrac{[S]^2}{K_{M1}K_{M2}}} \qquad (4)$$

Fitting of Data for Biphasic Kinetics. It is uncertain whether the biphasic dependence upon [Tyr] for aminoacylation by TyrTS results from (1) binding of 2 mol of Tyr/turnover or (2) enzyme existing as two populations with different kinetic characteristics (see Discussion). The second model would give

$$v = \frac{k_{cat}' f [E]_0 [S]}{K_{M1}' + [S]} + \frac{k_{cat2}' (1 - f)[E]_0}{K_{M2}' + [S]} \qquad (5)$$

where f represents the fraction of $[E]_0$ with a value of $k_{cat} = k_{cat1}'$ and $K_M = K_{M1}'$. The remaining enzyme has a value of $k_{cat} = k_{cat2}'$ and $K_M = K_{M2}'$.

Let $k_{cat1}'f = k_1''$ and $k_{cat2}'(1 - f) = k_2''$; then eq 5 rearranges to

$$\frac{v}{[E]_0} = \frac{\dfrac{k_1''[S]}{K_{M1}'} + \dfrac{k_2''[S]}{K_{M2}'} + \dfrac{(k_1'' + k_2'')[S]^2}{K_{M1}'K_{M2}'}}{1 + \dfrac{[S]}{K_{M1}'} + \dfrac{[S]}{K_{M2}'} + \dfrac{[S]^2}{K_{M1}'K_{M2}'}} \qquad (6)$$

Equations 4 and 6 are clearly very similar and observed rates

Table II: Kinetic Properties for the Activation of Tyr by the Full-Length Subunits in Different Heterodimers[a]

enzyme	K_M for Tyr (μM)	K_M for ATP (mM)	k_{cat} (s^{-1})
YTS/YTS[d]	2.1	1.0	8.2
YTS/ΔYTS[d]	2.3	1.3	7.7
YTS(Asn-45)/YTS(Asn-45)[b]	7.0	1.5	0.0037
YTS(Asn-45)/ΔYTS[b]	7.6	1.6	0.0039
YTS(Gly-195)/YTS(Gly-195)[c]	50	1.0	0.060
YTS(Gly-195)/ΔYTS[c]	62	1.1	0.070
YTS(Ala-173)/YTS(Ala-173)[c]	220	1.9	1.6
YTS(Ala-173)/ΔYTS[c]	260	1.1	1.3

[a] The assay consisted of following the time course of active-site titration at 25 °C. Concentration of enzyme was 0.6–2.0 μM, and $MgCl_2$ was maintained at 10 mM greater than ATP. Tyr dependence was measured at 50 μM ATP, and ATP dependence was determined at 10 μM Tyr except for *b*, where Tyr dependence was measured at 30 mM ATP and ATP dependence was determined at 30 μM Tyr. Values of K_M for Tyr, therefore, approximate to K_t (*c*) or K_t' (*b*), and values of K_M for ATP approximate to K_a (*c*) or K_a' (*b*) (see Figure 1). Values of k_{cat} were obtained by extrapolation to infinite concentration of both Tyr and ATP assuming Michaelis–Mentin kinetics with $K_t = K_t'$ and $K_a = K_a'$. Values were determined at high substrate concentrations (1.5–80 μM Tyr for Tyr dependence or 0.1–30 mM ATP for ATP dependence so that formation of Tyr-AMP by wild-type active sites of heterodimers reaches completion by the first time point (Ward & Fersht, 1988), allowing subsequent monitoring of slow formation of the intermediate at the mutant active site. Values given for heterodimers, therefore, apply to the mutant active site. [b] Data from Ward and Fersht (1988). [d] Values from kinetics of pyrophosphate exchange (Ward & Fersht, 1988).

in [Tyr] dependence of tRNA charging fit equally to each model. At low [S], both models predict Michaelis–Menten kinetics with $k_{cat1} = k_1''$ and $K_{M1} = K_{M1}'$. At high [S], agreement between the two models is no longer precise, but the following relationships hold to within ±10%: $k_{cat2} = (k_1'' + k_2'')$ and $K_{M2} = K_{M2}'$. The values quoted in Table III were calculated by fitting to model 1 since this is the most likely to be correct and because the values obtained are very similar regardless of which model was used.

Results

Characteristics of Mutant Enzymes. The mutants YTS(His → Asn-45)/YTS(His → Asn-45) and YTS(Gln → Ala-173)/YTS(Gln → Ala-173) have been characterized in previous studies (Leatherbarrow & Fersht, 1987; Lowe et al., 1987; see Tables III and IV in the present work). His-45 stabilizes the transition state for activation of Tyr but interacts only weakly with substrates in the ground state. Mutation of His → Asn-45 removes this interaction with the transition state, drastically decreasing catalytic rate, but having little affect on affinity for free substrates (Leatherbarrow & Fersht, 1987), Gln-173 forms part of the binding site for Tyr by hydrogen bonding to the α-ammonium group (Blow & Brick, 1985). Mutation of this residue to Ala accordingly weakens binding of Tyr. Affinity for ATP is also reduced, and the energy of the transition state for formation of Tyr-AMP is raised (Lowe et al., 1987).

The effect of mutating Gln → Gly-195 has been studied only in the steady state (Fersht et al., 1985). The crystal structure of enzyme-bound Tyr-AMP (Blow & Brick, 1985) suggests that this change would decrease affinity for Tyr since Gln-195 appears to form a hydrogen bond with the α-carboxyl group of the substrate. Detailed pre-steady-state analysis in the present work (Tables III and IV) shows that mutation of Gln → Gly-195 results in a 6.5-fold decrease in affinity for Tyr and a slightly increased affinity for ATP. The rate constant for activation of Tyr appears to be decreased by a factor of about 350.

Kinetic Properties of Each Subunit in Heterodimers Are Similar to Those of the Parental Homodimers. The following heterodimers were produced: YTS/ΔYTS, YTS(Asn-45)/ΔYTS, YTS(Ala-173)/ΔYTS, YTS(Gly-195)/ΔYTS. Each enzyme forms 0.95–1.07 mol of Tyr-AMP/mol of dimer, strongly suggesting that each has half-of-the-sites activity [see Ward and Fersht (1988)]. These measurements also imply that at least 95% of each preparation is active enzyme. The enzymes were shown to be pure by FPLC gel filtration (Ward et al., 1986).

Previous studies of YTS/ΔYTS and YTS(Asn-45)/ΔYTS indicate that half of each enzyme forms Tyr-AMP on the full-length subunit during the first turnover. The remainder forms the intermediate on the truncated subunit (Ward & Fersht, 1988). The kinetic properties for the activation of Tyr by the full-length subunit in YTS/ΔYTS cannot be isolated from those of the truncated subunit since each monomer has a wild-type active site. However, the kinetics of pyrophosphate exchange by this enzyme average the activity of the two subunits. The observed values of K_M for Tyr, K_M for ATP, and k_{cat} are not significantly different from those of the wild-type (Table II), suggesting that the full-length subunit of the heterodimer functions normally.

Activation of Tyr at a mutant active site may be much slower than that at a wild-type subunit. Appropriate heterodimers may thus display biphasic kinetics of active-site titration (Ward & Fersht, 1988). Under suitable conditions, formation of Tyr-AMP at the wild-type subunit reaches 0.5 mol/mol of enzyme dimer by the first time point, and then a further 0.5 mol of intermediate/mol of dimer accumulates slowly at the mutant site with the same rate constant as that of the mutant dimer. This behavior was exploited in order to characterize the kinetics of the full-length subunit in heterodimers (see Figure 1 and Table II). The values of K_M for Tyr, K_M for ATP, and k_{cat} were measured, and in each case, the full-length subunit in the heterodimers has properties

Table III: Charging Kinetics of Heterodimers When Affinity of Full-Length Subunit for Tyr Is Varied[a]

binding of Tyr		Tyr dependence of charging kinetics				
enzyme	K_t (μM)	enzyme	K_{M1} (μM)	k_{cat1} (s^{-1})	K_{M2} (μM)	k_{cat2} (s^{-1})
$[YTS]_2$	12[b]	YTS/YTS	2.2	0.92	130	3.8
$[YTS]_2$	12[b]	YTS/ΔYTS	1.7	0.52	96	1.7
$[Asn\text{-}45]_2$	23[c]	Asn-45/ΔYTS	2.1	0.45	120	1.5
$[Gly\text{-}195]_2$	78[d]	Gly-195/ΔYTS	2.1	0.42	160	1.5
$[Ala\text{-}173]_2$	700[e]	Ala-173/ΔYTS	2.1	0.39	150	1.1

[a] Experimental conditions and methods of data analysis are given under Methods. K_t is the absolute dissociation constant for the E·Tyr complex. Values of k_{cat} were obtained by extrapolation to infinite ATP and Tyr assuming Michaelis–Menten kinetics. tRNA encoded by the synthetic gene was used as substrate. The following abbreviations are used: $[YTS]_2$, wild-type enzyme; Asn-45, full-length subunit with His→Asn-45 mutation; Gly-195, full-length subunit with Gln→Gly-195 mutation; Ala-173, full-length subunit with Gln→Ala-173 mutation. Tyr dependence of tRNA charging was determined by using 10 mM ATP, 0.3–300 μM Tyr, and 10–40 nM enzyme. [b] Data from Wells and Fersht (1985). [c] Data from Leatherbarrow and Fersht (1987). [d] Value determined from kinetics of pyrophosphate exchange by following loss of label from [γ-^{32}P]ATP at various [Tyr] (Wells & Fersht, 1986). [e] Data from Lowe et al. (1987).

Table IV: Charging Kinetics of Heterodimers When ATP-Binding Affinity and Catalytic Activity of Full-Length Subunit Are Varied[a]

absolute constants for binding of ATP and catalysis			ATP dependence of charging		
enzyme	K_a' (mM)	k_3 (s^{-1})	enzyme	K_M for ATP (mM)	k_{cat} (s^{-1})
$[YTS]_2$[b]	4.7	38	YTS/YTS	2.3	2.9
$[YTS]_2$[b]	4.7	38	YTS/ΔYTS	1.8	1.4
$[Asn\text{-}45]_2$[c]	1.6	0.0032	Asn-45/ΔYTS	2.1	1.4
$[Gly\text{-}195]_2$[d]	1.2	0.11	Gly-195/ΔYTS	2.5	1.3
$[Ala\text{-}173]_2$[e]	36–180	8–40	Ala-173/ΔYTS	2.6	1.2

[a] Experimental procedures are given under Methods. Absolute rate and binding constants (see Figure 1) were determined by monitoring formation of E·Tyr-AMP in the pre-steady-state. ATP dependence of charging was studied in the steady state. Values of k_{cat} were obtained by extrapolation to infinite ATP and Tyr assuming Michalis–Menten kinetics. tRNA encoded by the synthetic gene was used as substrate. Abbreviations as for Table III. ATP dependence of tRNA charging was determined by using 100 μM Tyr, 0.2–12.5 mM ATP, and 10–40 nM enzyme. [b] Data from Wells and Fersht (1985). [c] Data from Leatherbarrow and Fersht (1987). [d] Value of K_a' could not be measured, and so the value quoted is K_a determined from kinetics of formation of enzyme-bound Tyr-AMP using 2 μM enzyme, 10 μM Tyr, and 0.1–30 mM ATP. [K_a for wild-type enzyme is 3.5 mM (J. Knill-Jones and A. R. Fersht, unpublished results).] The value of k_3 was calculated by extrapolation to infinite ATP and Tyr assuming Michaelis–Menten kinetics and $K_a = K_a'$. [e] Data from Lowe et al. (1987).

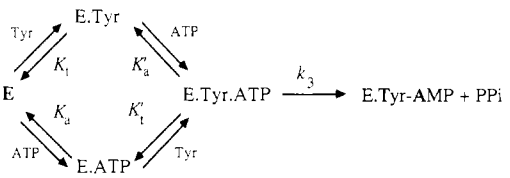

FIGURE 1: Kinetic scheme for formation of enzyme-bound Tyr-AMP.

similar to those of the same subunit in parental full-length homodimers (Table II).

The kinetics of activation of Tyr depend on the structure of only the N-terminal domains (Waye et al., 1983). Any kinetic properties of heterodimers are unlikely to be due to changes in structure of the C-terminal domains since the functioning of these domains also appear to be unaffected, either by any of the mutations used or by location in heterodimers rather than full-length homodimers. Thus, each heterodimer has similar kinetics for charging of tRNA, and the only change from the wild-type is that the value of k_{cat} is halved (Tables III and IV). This is because only 50% of the enzyme in solution turns over at the truncated subunit (which has the wild-type active site in each case), and only Tyr activated at this subunit can be used to aminoacylate tRNA (Ward & Fersht, 1988; Carter et al., 1986). Kinetic analysis thus indicates that the functioning of each domain in the full-length subunit of heterodimers is not changed relative to the parental homodimers.

Activation of Tyr and transfer to tRNA occur at the truncated subunit of heterodimers (Carter et al., 1986). Apart from the halving of the value of k_{cat}, the kinetics of charging of tRNA by heterodimers (Tables III and IV) are very similar to the kinetics of the wild-type (Table I), indicating that the functioning of the truncated subunit is not altered by inclusion in heterodimers.

Biphasic Kinetics Do Not Result from Charging of 2 mol of tRNA per Turnover. Deletion of a tRNA-binding domain from YTS/YTS generates YTS/ΔYTS, which charges tRNA with values of K_M for Tyr and K_M for ATP that are very similar to those of the wild-type (Tables III and IV). The deletion does not, therefore, lead to any detectable delocalized structural reorganization. The heterodimer has only 1 binding site for tRNA but retains biphasic kinetics (Figure 2), indicating that this kinetic behavior cannot result from charging 2 mol of tRNA per turnover.

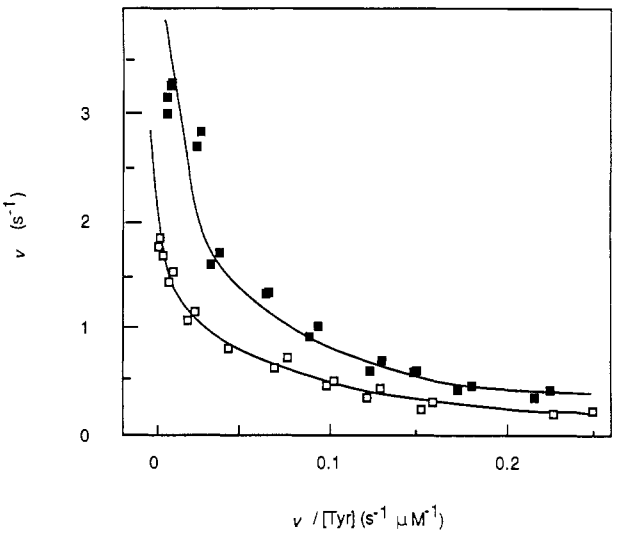

FIGURE 2: Tyr dependence for charging of synthetic tRNA by YTS/YTS (filled symbols) and YTS/ΔYTS (open symbols). Experimental details given in legends to Tables I and III.

Effect of Varying the Full-Length Subunit on the Kinetics of Charging by Heterodimers. The full-length subunit was changed in order to investigate its function in aminoacylation of tRNA. None of the following changes has a significant effect on charging kinetics (Tables III and IV). (a) Variation of affinity for Tyr: The value of K_t for the full-length subunit was varied over a range of 58-fold. (b) Variation of affinity for ATP: The magnitude of K_a' was altered over a range of at least 23-fold. (c) Variation of catalytic rate constant: The value of k_3 was varied over a range of 12 000-fold.

Discussion

The Paradox: Tyrosyl-tRNA Synthetase Shows Half-of-the-Sites Activity in Formation of Tyr-AMP but Has Biphasic Kinetics for Charging of tRNA. Tyrosyl-tRNA synthetase catalyzes aminoacylation of tRNA as a two-step reaction: First, Tyr is activated by formation of enzyme-bound Tyr-AMP, and then it is used to charge tRNA (Jakes & Fersht, 1975). The enzyme exhibits half-of-the-sites activity in formation of Tyr-AMP (Fersht, 1975; Fersht et al., 1975), but dependence upon [Tyr] for the kinetics of tRNA charging has two phases, suggesting paradoxically that 2 mol of Tyr can bind during each turnover (Jakes & Fersht, 1975). Data presented in the current work show that the enzyme maintains half-of-the-sites activity when charging tRNA. Two molecules of Tyr appear to bind sequentially to the same site and so produce the biphasic kinetics.

Biphasic Kinetics Do Not Result from Charging of 2 mol of tRNA per Dimer. There are several possible explanations for the biphasic kinetics of tRNA charging. The first hypothesis to be tested was that catalysis occurs at both active sites of the dimeric enzyme only in the presence of tRNA and high [Tyr], which would lead to charging of 2 mol of tRNA during each turnover and produce biphasic kinetics. This proposal is disproved in the current work by deleting one of the two tRNA-binding domains. The resultant heterodimer retains biphasic Tyr dependence of charging (Figure 2), despite being able to bind only 1 mol of tRNA/dimer.

Biphasic Kinetics Do Not Result from Binding of Tyr to Both Subunits of the Dimer. We have constructed a family of heterodimers in order to investigate the role of the subunit

of tyrosyl-tRNA synthetase that does not have catalytic activity. The full-length subunit of heterodimers cannot catalyze aminoacylation of tRNA (Carter et al., 1986), and so when this subunit is varied (with the truncated subunit retaining the wild-type active site), the role of the noncatalytic subunit can be tested directly.

The biphasic kinetics for tRNA charging could result from binding of 2 mol of Tyr during aminoacylation of 1 mol of tRNA. One molecule of Tyr could be expected to bind to each subunit of the dimer. This could correlate with the observation that the E·Tyr-AMP complex, unlike E·Tyr complex, can bind a second mole of Tyr (Fersht, 1975). The hypothesis was tested by construction of heterodimers where the Tyr-binding affinity of the full-length subunit is changed. The value of K_t was varied by up to 58-fold with no detectable effect on the kinetics of charging (Table III). This shows that binding of Tyr to the full-length subunit does not produce biphasic dependence upon the substrate, disproving the idea that Tyr-binding energy is transferred from the noncatalytic subunit, across the subunit interface, to the catalytic subunit. Half-of-the-sites activity is, therefore, displayed by tyrosyl-tRNA synthetase when charging tRNA.

Two Moles of Tyr Appear To Bind Sequentially to the Same Subunit of Tyrosyl-tRNA Synthetase during Charging of 1 mol of tRNA. This idea is supported by the following observations. First, the experimental data (Table III) suggest that the biphasic kinetics are caused by 2 mol of Tyr binding to the catalytically active truncated subunit during aminoacylation of 1 mol of tRNA. Second, the tyrosyl-tRNA synthetase binds 2 mol of radiolabel in the presence of [^{14}C]Tyr-tRNA and [^{14}C]Tyr (Jakes & Fersht, 1975). Third, repeated binding to the same subunit during a single turnover would be consistent with the long-lasting functional asymmetry of the dimer (Ward & Fersht, 1988).

The first mole of Tyr presumably dissociates from the binding site on the truncated subunit to allow access of the second mole of Tyr. A plausible scheme is

$$\text{E·Tyr-AMP} + \text{tRNA} \rightarrow \text{E·Tyr-tRNA} \xrightarrow{\text{I}} \text{E·Tyr·Tyr-tRNA} \rightarrow \text{E} + \text{Tyr-tRNA} + \text{Tyr} \quad (7)$$

The step where AMP dissociates from the enzyme is unclear, but inspection of the crystal structure of E·Tyr-AMP (Blow & Brick, 1985) suggests that enzyme-bound AMP sterically hinders entrance to, or exit from, the Tyr-binding site. AMP, therefore, probably dissociates at the step shown. The Tyr moiety of Tyr-tRNA could leave its binding site at step I, and the product could remain associated with the enzyme via the tRNA. This would leave the Tyr-binding site accessible to a second mole of the substrate.

Binding of Additional Substrate May Be a Common Method To Increase Rate in Enzyme Catalysis. Binding of a second mole of Tyr thus appears to increase catalytic rate via an unknown mechanism. A number of aminoacyl-tRNA synthetases display half-of-the-sites activity and biphasic kinetics with respect to the cognate amino acid (Schimmell & Soll, 1979). For some of these enzymes, it has been shown that binding of a second mole of amino acid increases flux through the rate-limiting step in charging, which is dissociation of charged tRNA (Yarus & Berg, 1969; Helene et al., 1971; Schimmel & Soll, 1979). Tyrosyl-tRNA synthetase may utilize additional substrate binding in this way, but this cannot be shown directly since the rate of dissociation of Tyr-tRNA from the enzyme is too fast to measure manually (Ward & Fersht, unpublished results).

Limitations in Studying Mechanism Using Steady-State Kinetics. Steady-state kinetics detect only substrate and product; intermediates are not seen directly. Proof of the mechanism proposed above requires development of assays allowing detailed analysis of charging in the pre-steady-state since biphasic kinetics could simply result from division of the enzyme into two populations that have different kinetic properties. This possibility is very unlikely since neither activation of Tyr nor ATP dependence of charging is biphasic (Tables I and II). The two populations would thus have to differ only in dependence upon [Tyr] in the charging reaction. Further, four different heterodimers have very similar biphasic kinetics with values of K_M close to those of the wild-type and values of k_{cat} that are halved (Tables III andIV). This argues against the existence of two distinct populations since mutation could change the distribution between these populations and so alter the kinetics. However, this possibility cannot be formally disproved without detailed pre-steady-state analysis of tRNA charging.

Functions of the Catalytically Inactive Subunit of Tyrosyl-tRNA Synthetase. Variation of K_t, K_a', or k_{cat} in the full-length subunit of heterodimers has no significant effect on the kinetics of charging at the truncated subunit (Tables III and IV). These data suggest that the functions of the full-length subunit are to bind tRNA (Waye et al., 1983) and possibly to stabilize the active conformation of the dimer [since monomeric enzyme does not activate Tyr (Jones et al., 1985; Ward et al., 1986, 1987)]. tRNA binds to one subunit and is then charged by using Tyr activated at the other subunit of the dimer (Carter et al., 1986). The model of synthetase/tRNA interaction (Bedouelle & Winter, 1986) suggests a biological rationale for half-of-the-sites activity in tyrosyl-tRNA synthetase. tRNA is a long molecule, and a single subunit of the enyzme is too small to be large enough both to recognize and charge tRNA. The enzyme is a dimer for reasons of recognition of tRNA and so needs only one active site per dimer.

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Differential Reactivity in the Processing of [*p*-(Halomethyl)benzoyl]formates by Benzoylformate Decarboxylase, a Thiamin Pyrophosphate Dependent Enzyme†

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ABSTRACT: A series of [*p*-(halomethyl)benzoyl]formates have been investigated as substrates for benzoylformate decarboxylase. These analogues vary from acting as normal substrates to acting as potent competitive inhibitors. The fluoro analogue is a substrate with K_m (190 μM) and turnover number (20 s^{-1}) similar to those of benzoylformate (K_m = 340 μM; 81 s^{-1}). The bromo analogue is a competitive inhibitor (K_i = 0.3 μM) and exhibits processing to eliminate bromide and form (*p*-methylbenzoyl)thiamin pyrophosphate. This modified cofactor hydrolyzes to form the *p*-methylbenzoate in quantitative yield. The chloro analogue [K_m(app) = 21 μM] partitions between these two pathways such that 0.6% of the analogue ultimately forms *p*-methylbenzoate. These data are consistent with the interpretation that the leaving group potential of the halogen determines the enzymic fate of the analogue and that the potent inhibition observed for the bromo analogue is due to covalent modification of the cofactor.

Benzoylformate decarboxylase (EC 4.1.1.7; benzoylformate carboxy-lyase; BFD[1]) from *Pseudomonas putida* catalyzes the formation of benzaldehyde from benzoylformate, an α-keto acid. Aside from its characterization as part of the mandelate pathway group (Hegeman, 1966a,b,c), this enzyme has been little studied. Until recently (Weiss et al., 1988), virtually nothing had been reported about the details of the enzymatic mechanism except for its strict requirement for thiamin pyrophosphate as a cofactor.

The proposed mechanism for the reaction catalyzed by BFD (Figure 1) is analogous to that for the formation of acetaldehyde from pyruvate catalyzed by pyruvate decarboxylase. The mechanism involves the formation of a covalent substrate–cofactor intermediate capable of stabilizing the carbanion generated by decarboxylation.

The K_m for benzoylformate has been measured as 1 mM and 0.08 mM in separate studies at pH 6.2 (Hegman, 1970) and pH 6.1 (Weiss et al., 1988), respectively. The K_m for TPP has been reported as 1 μM (Hegman, 1970). Benzoylformate decarboxylase has a higher substrate specificity than pyruvate decarboxylase. Only benzoylformate and para-substituted benzoylformates have been shown to be substrates (Weiss et al., 1988; Hegman, 1970). Pyruvate, α-ketobutyrate, and α-ketoglutarate are not substrates.

Previously, we have reported the inhibition of BFD by [*p*-(bromomethyl)benzoyl]formate (BrMeBF) (Dirmaier et al., 1986). We established that inhibition was due to an unusual enzymatic processing of BrMeBF resulting in decarboxylation, bromide ion elimination, and tautomerization to form a covalently modified cofactor (*p*-methylbenzoyl-TPP). In this paper we present further studies on a series of [*p*-(halomethyl)benzoyl]formates. We demonstrate that these analogues vary from acting as substrates for the enzyme to acting as potent competitive inhibitors. We postulate a common intermediate for these analogues and propose that halogen leaving group potential determines the partitioning between inhibition and normal substrate turnover.

Materials and Methods

Horse liver alcohol dehydrogenase (HLADH), thiamin pyrophosphate chloride (TPP), NADH, Hepes, and Tris were

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[1] Abbreviations: BFD, benzoylformate decarboxylase; BF, benzoylformate; MeBF, (*p*-methylbenzoyl)formate; BrMeBF, [*p*-(bromomethyl)benzoyl]formate; ClMeBF, [*p*-(chloromethyl)benzoyl]formate; FMeBF, [*p*-(fluoromethyl)benzoyl]formate; HOMeBF, [*p*-(hydroxymethyl)benzoyl]formate; TPP, thiamin pyrophosphate; HLADH, horse liver alcohol dehydrogenase; NADH, nicotinamide adenine dinucleotide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate; Tris, tris(hydroxymethyl)aminomethane.

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