Characterization of Isoleucyl-tRNA Synthetase from *Staphylococcus aureus*

I: KINETIC MECHANISM OF THE SUBSTRATE ACTIVATION REACTION STUDIED BY TRANSIENT AND STEADY-STATE TECHNIQUES

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Andrew J. Pope‡‡, Jacques Lapointe††, Lucy Mensah‡, Neil Benson‡, Murray J. B. Brown‡, and Keith J. Moore‡

From the ‡Department of Molecular Recognition, SmithKline Beecham Pharmaceuticals, New Frontiers Science Park North, Harlow, Essex, United Kingdom and the †Département de Biochimie, Faculté des Sciences et de Génie, Université Laval, Québec G1K 7P4, Canada

The kinetic mechanism for the amino acid activation reaction of *Staphylococcus aureus* isoleucyl-tRNA synthetase (IleRS; E) has been determined from stopped-flow measurements of the tryptophan fluorescence associated with the formation of the enzyme-bound aminoacyl adenylate (E-Ile-AMP; Scheme 1). Isoleucine (Ile) binds to the E-ATP complex ($K_4 = 1.7 \pm 0.9 \mu M$) ~35-fold more tightly than to E ($K_1 = 50–100 \mu M$), primarily due to a reduction in the Ile dissociation rate constant ($k_{d4} \sim 100–150 \text{ s}^{-1}$, cf. $k_{d4} = 3 \pm 1.5 \text{ s}^{-1}$). Similarly, ATP binds more tightly to E (Ile) ($K_5 = 70 \mu M$) than to E ($K_2 = 2.5 \text{ mM}$). The formation of the E-isoleucyl adenylate intermediate, E-Ile-AMP, resulted in a further increase in fluorescence allowing the catalytic step to be monitored ($k_{+5} = 60 \text{ s}^{-1}$) and the reverse rate constant ($k_{-2} = 150–200 \text{ s}^{-1}$) to be determined from pyrophosphorylation of a pre-formed E-Ile-AMP complex ($K_5 = 0.25 \text{ mM}$). Scheme 1 was able to globally predict all of the observed transient kinetic and steady-state PPi/ATP exchange properties of IleRS by simulation. A modification of Scheme 1 could also provide an adequate description of the kinetics of tRNA aminoacylation ($k_{cat, tr} = -0.35 \text{ s}^{-1}$) thus providing a framework for understanding the kinetic mechanism of aminoacylation in the presence of tRNA and of inhibitor binding to IleRS.

Activating the carboxyl group of amino acids with ATP to form the corresponding aminoacyl adenylate prior to transfer to tRNA is the first step in protein biosynthesis. The activation of each amino acid is catalyzed by a specific aminoacyl-tRNA synthetase (aaRS) which first catalyzes the activation (and stabilization) of the amino acid as a mixed anhydride adenylate, and the subsequent acyl transfer to the corresponding cognate tRNA (for a review, see Ref. 1). The high level of phylogenetic divergence between these enzymes in prokaryotes and eukaryotes (2, 5–6), together with their essential role in protein synthesis, makes aminoacyl-tRNA synthetases excellent targets for the development of selectively acting antibacterial agents.

The best known example of such a compound is pseudomonic acid A (PS-A) which specifically inhibits bacterial isoleucyl-tRNA synthetase (IleRS) around 10,000-fold more potently that the corresponding mammalian enzyme (7,8) and is a highly effective antibiotic. *Staphylococcus aureus* infections, particularly of the respiratory tract, are a major clinical problem and, because of the emergence of resistance to “classical” antibiotics (e.g. β-lactams), this organism is an important target for the development of new antibiotics. However, resistance to PS-A itself is emerging although not yet a clinically relevant problem (e.g. Refs. 9 and 10). Therefore, we have considerable interest in understanding the reaction cycle and inhibition of *S. aureus* IleRS by PS-A in order to aid the design of novel inhibitors and to understand the mechanisms of PS-A resistance. Following overexpression of *S. aureus* IleRS (11), reagent quantities of this enzyme are now available. In this paper, we describe the construction of a minimal reaction mechanism for amino acid activation by IleRS which adequately describes all of the steady-state and transient kinetic properties of this enzyme. Accompanying papers describe a detailed characterization of the kinetics and mechanism of inhibitor binding (12), and an analysis of the effects of ligand binding upon proteolysis of IleRS (13).

MATERIALS AND METHODS

The preparation of IleRS (12) and the methods used for steady-state ATP/PPi, exchange and tRNA aminoacylation reactions (15) were as described in the accompanying paper (12). Isoleucyl adenylate (Ile-ol-AMP; Fig. 3) was synthesized at SmithKline Beecham. Stoichiometric E-Ile-AMP complex was prepared by incubating IleRS with excess [MgATP] and [Ile] followed by gel filtration (Pharmacia Fast Desalt) to remove excess substrates. Adenylated IleRS was stable for several hours when stored on ice (data not shown). Stopped-flow studies, and the resulting data analysis (16–19), were performed with an Applied Photophysics SM17MV instrument at 22 °C in 50 mM Tris-HCl, pH 7.9, 10 mM MgCl₂ (buffer B) as described (12).

RESULTS

Steady-state Measurements of IleRS Tryptophan Fluorescence—Although IleRS (E) from *S. aureus* possesses 18 tryptophan residues distributed fairly uniformly throughout the sequence (11), examination of the equilibrium enzyme fluorescence of various complexes showed that, with the exception of E-ATP, these differed markedly in their fluorescence yields (Fig. 1). This provided the opportunity to determine the rates and equilibria for their inter-conversion directly by stopped-flow techniques.

The intrinsic protein fluorescence intensity is increased by
around 7% in the E-Ile binary complex and by around 17% upon the catalytic formation of E-Ile-AMP (Fig. 1). However, enzyme fluorescence was not altered by the presence of >5 mM Mg-ATP, MgPP₆, or MgApA (adenosine 5'-tetraphospho-5'-adenosine; data not shown). Interestingly, binding of Ile-ol-AMP (Fig. 10a) yielded an identical enzyme fluorescence to E-Ile-AMP formed during the reaction cycle, suggesting that the conformation of the enzyme-ligand complex is similar in both cases. The fluorescence change induced by Ile-ol-AMP was used to monitor those interactions that are spectroscopically silent (e.g. MgATP binding, see Fig. 4) using a kinetic competition approach (see below).

**Transient Kinetics of Substrate and Inhibitor Binding to S. aureus IleRS**—We have characterized the elementary rate and/or equilibrium constants involved in substrate binding, activation, and Ile-ol-AMP binding using stopped-flow according to the minimal mechanism shown in Scheme 1. The rate and equilibrium constants derived (Table I, Scheme 1) provide an adequate description of all of the experimental steady-state and transient kinetic data as described below.

**Binding of Ile-ol-AMP (k₁₊ and k₋₁)—**See below² for explanation of the notation used throughout and accompanying (12, 13) papers. The binding of excess Ile-ol-AMP to IleRS was monitored directly by rapid mixing of the enzyme and inhibitor in a stopped-flow apparatus (Fig. 2A). The observed rate constant, \( k_{	ext{obs}} \), of the exponential increase in protein fluorescence (amplitude ~17%) varied linearly with Ile-ol-AMP concentration (Fig. 2B), consistent with a simple bimolecular reaction (Scheme 1; \( k_{	ext{obs}} = k_{\text{on}}[\text{Ile-ol-AMP}] + k_{\text{off}} \)). Although \( k_{\text{on}} = 2.4 \times 10^{5} \text{M}^{-1} \text{s}^{-1} \), the intercept defining \( k_{\text{on}} \) was too small to be determined accurately (\(< 0.5 \text{ s}^{-1} \)) from Fig. 2B. However, direct measurement of \( k_{\text{on}} \) for Ile-ol-AMP via displacement experiments yielded a value of ~0.07 s⁻¹ (12), deriving an overall \( K_{\text{d}} \sim 30 \text{ nM} \).

**Binding of Isoleucine (k₁₊ and k₋₁)—**Similar experiments to those described above but with the substrate i-isoleucine (Ile), also resulted in a single exponential increase in protein fluorescence (Fig. 3A). The experiment shown in Fig. 3 provided an estimate of \( k_{\text{on}} = 3.1 \pm 0.3 \times 10^{5} \text{M}^{-1} \text{s}^{-1}, \ k_{\text{off}} = 142 \pm 14 \text{ s}^{-1} \) (Fig 3B; \( K_{\text{d}} = 46 \pm 6 \mu \text{M} \)). Six independent repeat experiments yielded mean (±S.D.) values for each parameter of \( k_{\text{on}} = 2.2 \pm 0.5 \times 10^{5} \text{M}^{-1} \text{s}^{-1}, \ k_{\text{off}} = 131 \pm 50 \text{ s}^{-1} \), and hence \( K_{\text{d}} = 60 \pm 27 \mu \text{M} \). This large error in \( K_{\text{d}} \) limits the reliability of other equilibrium constants in the mechanism, particularly \( K_{\text{d}} \), which have been estimated, in part, from a thermodynamic linkage argument involving the estimate of \( K_{\text{d}} \) (see below). Nevertheless, these rate and equilibrium constants are consistent with the [Ile] dependence of the fluorescence amplitudes (Fig. 3C; \( K_{\text{d}} = 56 \pm 10 \mu \text{M} \)) and provide unambiguous evidence that IleRS can bind Ile (\( K_{\text{d}} \sim 50–100 \mu \text{M} \)) in the absence of Mg-ATP. The value for \( k_{\text{on}} \) is lower than expected for a diffusion limited binding process (typically \( >10^{8} \text{ M}^{-1} \text{s}^{-1} \)), although several mechanisms can give rise to such behavior (14, 20, 36) and for the purposes of Scheme 1, Ile binding can be considered an elementary step.

**Binding of MgATP (k₁₊ and k₋₁)—**The steady-state protein fluorescence intensity of IleRS was not detectably changed upon addition of 5 mM Mg-ATP (Fig. 1), a concentration approx-

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² Equilibrium (dissociation) or steady-state constants for step n are termed \( K_n \) and \( K_n' \) for reactions performed in the absence and presence of tRNA, respectively. The rate and equilibrium constants are defined explicitly in Scheme 1 (Table I). The internal equilibrium constant, \( K_n \), and the external equilibrium constant, \( K_n' \), are written in the forward direction. \( k_{\text{on}} (= k_{\text{on}}') \) is the equilibrium dissociation constant for inhibitor binding to \( E \). Where elementary rate and equilibrium constants cannot be directly extracted from derivative plots of the observed rate constants, \( k_{\text{on}} \) and \( k_{\text{on}}' \) against concentration, the limiting rate constants and apparent equilibrium constants have the suffix \( \text{app} \) (e.g. \( k_{\text{on}} \text{app} \) or \( K_{\text{d}} \text{app} \)). The \( k_{\text{on}} \) values for the aminocacylation and ATPPP₆ exchange reactions are \( k_{\text{cat,tr}} \) and \( k_{\text{cat,ex}} \), respectively.

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**FIG. 1.** Equilibrium tryptophan fluorescence of IleRS complexes relative to that of free IleRS (=100%); E, free enzyme; E-Ile, IleRS-Ile complex; E-ATP-Mg, IleRS-ATP-Mg complex; E-Ile-AMP, IleRS-isoleucine-adenylate (catalytic) complex; E-Ile-AMP, IleRS-isoleucinol adenylate (inhibitor) complex.

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**TABLE 1**

| Rate and equilibrium constants for substrate binding, activation and inhibitor binding to S. aureus IleRS | \( E = \text{IleRS} \) |
|---|---|
| **Rate/equilibrium constants** | \( E = \text{IleRS} \) |
| Ile + E \( \leftrightarrow \) Ile \( E \) | \( k_{\text{on}} (= k_{\text{on}}') = 60 \pm 27 \mu \text{M} \) |
| Ile + E \( \leftrightarrow \) Ile \( E \) | \( k_{\text{on}} (= k_{\text{on}}') = 60 \pm 27 \mu \text{M} \) |
| ATP + E \( \leftrightarrow \) ATP | \( k_{\text{on}} (= k_{\text{on}}') = 60 \pm 27 \mu \text{M} \) |
| E \( \leftrightarrow \) Ile-AMP | \( k_{\text{on}} (= k_{\text{on}}') = 60 \pm 27 \mu \text{M} \) |
| E \( \leftrightarrow \) Ile-AMP | \( k_{\text{on}} (= k_{\text{on}}') = 60 \pm 27 \mu \text{M} \) |
| E-Ile-AMP + PP₆ \( \leftrightarrow \) E-Ile-AMP + PP₆ | \( k_{\text{on}} (= k_{\text{on}}') = 60 \pm 27 \mu \text{M} \) |
| Ile-ol-AMP + E \( \leftrightarrow \) Ile-ol-AMP | \( k_{\text{on}} (= k_{\text{on}}') = 60 \pm 27 \mu \text{M} \) |
When changes in IleRS protein fluorescence, when $E \,(0.1 \,\mu M)$ was rapidly mixed with varying [Ile-ol-AMP] (1.6–50 $\mu M$) in the stopped-flow apparatus. Solid lines are the best fits to a single exponential defining an observed rate constant, $k_{o-b}$, dependence of $k_{o-b}$ from three independent experiments such as those shown in A on (Ile-ol-AMP) (mean ± S.E.) defining $k_{o-b} = 2.4 \pm 0.2 \times 10^9 \,\text{M}^{-1} \,\text{s}^{-1}$ and $k_{o-b} < 0.5 \,\text{s}^{-1}$.

**Fig. 2.** Binding of Ile-ol-AMP to IleRS measured using stopped-flow. A, changes in IleRS protein fluorescence, $E \,(0.1 \,\mu M)$ was rapidly mixed with varying [Ile-ol-AMP] (1.6–50 $\mu M$) in the stopped-flow apparatus. Solid lines are the best fits to a single exponential defining an observed rate constant, $k_{o-b}$, dependence of $k_{o-b}$ from three independent experiments such as those shown in A on (Ile-ol-AMP) (mean ± S.E.) defining $k_{o-b} = 2.4 \pm 0.2 \times 10^9 \,\text{M}^{-1} \,\text{s}^{-1}$ and $k_{o-b} < 0.5 \,\text{s}^{-1}$.

The solid line is the best fit to a hyperbola ($K_1 = 56 \pm 10 \,\mu M$).  

$$E \cdot \text{Ile-ol-AMP} \quad k_{-i} \quad \frac{k_{o-b} \text{[ATP]}}{E} \quad k_{-2} \quad E \cdot \text{ATP}$$

**Scheme 2**

approximately equal to $2 \times K_2$ and $\sim$10-fold higher than the $K_m^{\text{ATP}}$ measured in the steady state tRNA aminoacylation reaction (see below, Fig. 9), suggesting that ATP binding was spectroscopically silent. We therefore employed a transient kinetic competition approach (21) to characterize the binding of Mg-ATP to $E$. In these experiments, ATP is pre-mixed in the syringe of the stopped-flow apparatus with a ligand (e.g. Ile-ol-AMP) which is both competitive with ATP (Fig. 10) (12) and which induces a fluorescence change upon binding to $E$ (Figs. 1 and 2). This mixture is then rapidly mixed with $E$ such that the two ligands bind to $E$ simultaneously and in parallel according to Scheme 2 and the transient kinetics of $E$-Ile-ol-AMP formation are monitored spectroscopically. The kinetics and/or thermodynamics of ATP binding are inferred from the effect of ATP on the kinetics of Ile-ol-AMP binding (21). A series of kinetic competition experiments were performed by mixing $0.1 \,\mu M$ $E$ with a sample of $0.5 \,\mu M$ Ile-ol-AMP plus varying concentrations of Mg-ATP. Mixing $E$ in Ile-ol-AMP alone yielded, as expected (Fig. 2), a single exponential increase in protein fluorescence ($k_{o-b} \sim 2.5 \,\text{s}^{-1}$, amplitude $\sim$16%, Fig. 4A). When Mg-ATP and Ile-ol-AMP were mixed with $E$, $k_{o-b}$ for the formation of $E$-Ile-ol-AMP decreased with increasing [ATP] (Fig. 4), as expected for the competition between the two ligands where the binding of the spectroscopically silent ligand (ATP) is rapidly reversible relative to the rate of Ile-ol-AMP binding (21). Analysis of the data according to Equation 1 yields an estimate for $K_2$ of $\sim 2.5 \pm 0.2 \,\mu M$ (mean ± S.D., $n = 3$) and an estimate of $k_{o-b}$ ($\sim 0.1 \,\text{s}^{-1}$) consistent with that determined directly (Fig. 2), as shown,

$$k_{o-b} \sim \frac{k_{o-b} \text{[Ile-ol-AMP]} \, k_{-i} \, (1 + [\text{ATP}])}{K_1} \quad (\text{Eq. 1})$$

As predicted, the estimate of $K_2$ was independent of the concentration of Ile-ol-AMP used (defining $k_{o-b} > 10 \,\text{s}^{-1}$; see also Fig. 9), and was similar to that obtained from the activation of the aminoacylation reaction by ATP (see below, 2.7 mm; Table III). These data therefore provide clear evidence that ATP can bind to $E$ in the absence of the co-substrate, Ile (and vice versa, see Fig. 3), such that a minimal mechanism must include random order of addition of both substrates. Simulations of the experiment shown in Fig. 4A by numerical integration techniques (using Scheme 1, Table I) provide an adequate description of the experimental data (compare Fig. 4, A and C).

**Fig. 3.** Binding of Ile to *S. aureus* IleRS measured using stopped-flow protein fluorescence. A, fluorescence transients obtained when $0.1 \,\mu M$ $E$ was mixed with 20–100 $\mu M$ Ile; B, dependence of $k_{o-b}$ from panel A on [Ile], defining $k_{o-b} = 3.1 \pm 0.3 \times 10^6 \,\text{M}^{-1} \,\text{s}^{-1}$, $k_{-i} = 142 \pm 14 \,\text{s}^{-1}$, $K_i = 46 \pm 6 \,\mu M$; C, dependence of the fluorescence amplitude (in arbitrary units) on [Ile]. The solid line is the best fit to a hyperbola ($K_i = 56 \pm 10 \,\mu M$).

**Reaction Cycle of *S. aureus* IleRS**

**Fig. 4.** A and C. Fluorescence amplitude of tRNA aminoacylation reaction resulted in a 16–17% increase in enzyme fluorescence (cf. Fig. 1). Although the time course of the change in species concentration predicted by Scheme 1 is complex (see below), due to the particular combination of fluorescence yields associated with each of the intermediates, the observed fluorescence transients could, under most conditions, be described by the sum of two exponentials (e.g. Fig. 5A). The fast phase of the transient (observed rate constant $k_{o-b}$) had an [Ile] dependence consistent with that observed previously (Fig. 3) and an equivalent transient was observed in the absence of ATP. Furthermore, this rate constant was invariant when IleRS was mixed into varying ATP concentrations in the presence of a fixed concen-
Fig. 4. Kinetic competition of Ile-ol-AMP and Mg-ATP binding to IleRS. A, stopped-flow traces obtained from mixing 0.1 \text{mM} E with 0.5 \text{mM Ile-ol-AMP} plus 0, 2, and 10 \text{mM ATP-Mg}. B, dependence of \( k_{\text{obs}} \) on [ATP] (0.02–15 \text{mM}) from experiments such as those shown in A. The solid line is the best fit of the data to Equation 1 yielding \( K_2 = 2.5 \text{mM} \). C, kinetic simulation of the predicted fluorescence transients (arbitrary units) for the experiments shown in A using Scheme 1 and the data in Table I.

The slower transient observed in these mixing experiments, \( k_{\text{obs},2} \) (at 7%), showed a hyperbolic dependence in either [ATP] (Fig. 5C) or [Ile] (data not shown, Table II), most likely due to the first-order catalytic formation of the E-Ile-AMP intermediate since the fluorescence yield of E-Ile-AMP is about 7% greater than that of E-Ile (or E-ATP, E-Ile-ATP) alone (Fig. 1). The [ATP] dependence of \( k_{\text{obs},2} \) was analyzed according to Equation 2 (\([L] = [\text{Mg-ATP}]\)) to obtain estimates of the maximal observed rate constant, \( k_{\text{max}} = k_{5,\text{app}} + k_{5,\text{app}} \), the apparent \( K_d \) of the binding step, \( K_3,\text{app} \) and the apparent reverse rate constant for the first order step, \( k_{5,\text{app}} \). We emphasize the apparent nature of these terms as opposed to the elementary nature of the rate and equilibrium constants reported in Table I and derived as described,

\[
k_{\text{obs}} = \frac{k_{5,\text{app}} [L]}{K_3,\text{app} + [L]} + k_{-5,\text{app}} \quad \text{(Eq. 2)}
\]

The value of \( k_{5,\text{app}} \) was too low to be determined reliably in these experiments, but had a value of \(<2 \text{s}^{-1} \) such that \( k_{\text{max}} = k_{5,\text{app}} + k_{5,\text{app}} \) approximates to \( k_{5,\text{app}} \). According to Scheme 1, the apparent irreversibility of the chemical cleavage step
(k_{5,app} < 2 \text{ s}^{-1}) is a consequence of the weak binding of PP_i, the rapid and thermodynamically favorable release of which from the E-Ile-AMP-PP_i complex makes the observed reverse rate negligible.

To determine if the maximal apparent rate constant observed at high [ATP] in Fig. 5C (57 s^{-1}) was the elementary rate constant for E-Ile-AMP-PP_i formation, we conducted a series of experiments in which the concentration dependence of k_{obs,2} in either Ile or ATP was measured in the presence of fixed concentrations of the other substrate (Table II). The maximal observed rate constant, k_{max}, was dependent upon [Ile], reaching a limiting value near 55 s^{-1} at high concentrations of both ATP and Ile (Table II). This rate constant likely reflects the true elementary rate constant for E-Ile-AMP-PP_i formation which was confirmed in a number of other experiments conducted at saturating concentrations of Ile and ATP (not shown). Kinetic simulations of these experiments (Scheme 1, Table I) yielded fluorescence transients similar to those observed experimentally (compare Fig. 5, A and D) and furthermore, predicted accurately the apparent maximal rate constants for k_{obs,2} at different [Ile] (Table II).

The apparent equilibrium constants for ATP and Ile (K_{3,app} = 75 \text{ mM} and K_{4,app} = 15 \text{ mM}, respectively; Table II) only approximate to the true equilibrium constants, K_3 and K_4, when the pseudo first-order association and first-order dissociation rate constants are much larger than the rate constant for the subsequent (signal generating) chemical step. For ATP binding to E-Ile, kinetic simulations were able to predict the observed fluorescence transients using a value of K_3 (70 \text{ mM}) approximately equal to the K_{3,app} value from Table II (75 \text{ mM}). However, similar kinetic simulations indicate that the K_{4,app} value for Ile binding to E-ATP (15 \text{ mM}) is an overestimate of the true K_4 (1.7 ± 0.9 \text{ mM}, Table I). Indeed, experiments described below suggest that the dissociation of Ile from E-Ile-ATP (k_{-4} ~ 3 ± 1.5 \text{ s}^{-1}) occurs more slowly than the rate of the chemical cleavage step (~60 \text{ s}^{-1}). As such, the non-equivalence of the best estimate of K_3 and the K_{4,app} for Ile activation of the chemistry step (Table II) is to be expected. Finally, based on reasonably reliable estimates for three of the four equilibrium constants in Scheme 1 defining the formation of the E-ATP-Ile complex, thermodynamic linkage with associated error propagation (primarily in K_i, see above), suggests K_4 = 1.7 ± 0.9 \text{ mM}.

Despite this slight uncertainty, the major conclusion is that the binding of either substrate to E reduces the K_4 for the formation of the ternary E-ATP-Ile complex by >10-fold compared with the K_4 obtained for either substrate binding to free E (Figs. 3 and 4).

Binding of Isoleucine to the E-ATP Binary Complex (k_{+4} and k_{-4})—Although Table II predicts that Ile binds more tightly to E-ATP than to the free enzyme, there is no information concerning the rate constants k_{-4} and k_{+4}. Initial experiments to monitor the association kinetics of Ile with E-ATP (prior to the chemical step) proved difficult to interpret since, at low [Ile], k_{obs,1} (defining the formation of the ternary E-ATP-Ile complex) had a similar rate constant and amplitude to k_{obs,2} (defining the catalytic formation of E-ATP-PP_i). In an attempt to kinetically resolve the bimolecular Ile binding step from the first order catalytic step, we conducted experiments at high [Ile] (up to 300 \text{ mM}) although this necessarily led to rapid transients and associated uncertainty (Fig. 6A).

The apparent observed rate constant increased approximately linearly with [Ile] (Fig. 6B), yielding an intercept of 22 ± 13 s^{-1} and a slope of 1.6 × 10^6 \text{ M}^{-1} \text{ s}^{-1}. These values only represent k_{+4} and k_{+4} when the bimolecular formation of E-ATP-Ile occurs much more rapidly than the decay of the ternary complex (at ~60 \text{ s}^{-1}). However, the predicted species distribution (based on Scheme 1, Table I) suggests that both processes are likely to be kinetically linked below 150 \text{ mM} Ile (Fig. 6D). Only the data between 150 and 250 \text{ mM} Ile (which yielded k_{obs} ~ 450 s^{-1}; close to the upper limit for reliable measurements on our apparatus) could be used to estimate the elementary rate constants (k_{-4} ~ 1.6 × 10^6 \text{ M}^{-1} \text{ s}^{-1}) and, therefore, we can estimate only k_{+4} ~ 10 \text{ s}^{-1}. This value is, as we noted above, significantly less than the estimate of the rate of the chemistry step (about 60 \text{ s}^{-1}) such that rapid equilibrium assumptions associated with Ile binding to E-ATP are invalid. Despite the difficulty in obtaining a reliable estimate for k_{+4}, and hence K_4, from either Table I or Fig. 6 or by thermodynamic linkage with other well defined equilibrium constants we obtain an estimate of K_4 = 1.7 ± 0.9 \text{ mM} and hence k_{-4} ~ 3 ± 1.5 \text{ s}^{-1} from an estimate of k_{+4}. Kinetic simulation of the experiments (Fig. 6A, Scheme I, Table I, k_{+4} ~ 3 ± 1.5 \text{ s}^{-1}) provided an adequate description of the experimental transients (Fig. 6C) and of the apparent rate constant for the chemistry step at different [Ile] (Table II). We believe the combined data justifies the estimate of k_{+4} and hence K_4 with appropriate caveats regarding the uncertainty associated with these value.

Effect of Order of Mixing of Substrates—Binding of ATP to free E was sufficiently weak (K_4 = 2.5 \text{ mM}) to be expected to be a rapid equilibrium on the stopped-flow time scale, an assumption we have incorporated into simulations of Scheme 1 (k_{-2} = 10^7 \text{ M}^{-1} \text{ s}^{-1}, k_{+2} = 2.5 × 10^4 \text{ s}^{-1}, e.g. Fig. 6D). As such, we would expect no difference in the kinetics of E-AMP-Ile formation when the E-ATP complex was mixed with Ile and when both substrates were mixed with E simultaneously. To test this hypothesis (Fig. 7), a fixed concentration of the pre-bound substrate was maintained in both stopped-flow syringes, so that any pre-established equilibria were not perturbed upon subsequent mixing with the second substrate. As expected, the stopped-flow time course obtained was identical whether or not ATP was pre-mixed with E (Fig. 7A, note that the traces have been offset for clarity). In contrast, the fluorescence amplitude observed following pre-binding of Ile to E reflects the difference

### Table II

| Fixed substrate | Variable substrate | K_{4,app} (\text{M}) | K_{5,app} (\text{M}) | Observed k_{5,app} (s^{-1}) | Simulated k_{5,app} (s^{-1}) |
|-----------------|--------------------|---------------------|---------------------|-----------------------------|-----------------------------|
| ATP · Mg | Isoleucine | 16 ± 7 | 53 ± 6 | 57 | 58 |
| 1 mM | 15 | 15 | 54 ± 5 | 58 |
| 2 mM | 72 | 42 ± 1.8 | 41 |
| 30 mM | 71 | 57 ± 2.6 | 58 |
| 60 mM | 71 | 57 ± 2.6 | 58 |
in fluorescence intensity between $E$-Ile and $E$-Ile-AMP (Fig. 1). Kinetic simulation of the experiments in Fig. 7A (Scheme 1, Table I) provided a reasonable description of the experimental data (Fig. 7B) with exponential rate constants (46 s$^{-1}$) comparable to those observed experimentally (40–44 s$^{-1}$).

**Pyrophosphorolysis of E-Ile-AMP**—Since the $E$-Ile-AMP complex is relatively stable (rate constant for decay of $E$-Ile-AMP $< 3 \times 10^{-4}$ s$^{-1}$; data not shown), it could be isolated using rapid gel-filtration allowing measurement of the reverse rate constant for the catalytic step ($k_{-\beta}$ in Scheme 1) via pyrophosphorolysis of $E$-Ile-AMP. Rapid mixing of freshly isolated $E$-Ile-AMP with Mg-PP$_i$ resulted in a decrease in enzyme fluorescence (Fig. 8A) corresponding to the reaction $E$-Ile-AMP-PP$_i$ $\rightarrow$ $E$-Ile-AMP (Δ$F$$\approx$8%). The observed rate constant for this process showed a hyperbolic dependence on [PP$_i$] reaching a limiting observed rate constant of 100 s$^{-1}$ and a yielding a value for $K_{m}$ of 250 μM. When the binding of PP$_i$ is followed by the one-step irreversible formation of $E$-Ile-AMP, the limiting rate constant of 100 s$^{-1}$ would correspond to $k_{-\beta}$. However, simulations of the experiments in Fig. 8A with $k_{-\beta}$ = 100 s$^{-1}$ yield simulated transients significantly slower than observed experimentally. We have used a value of $k_{-\beta}$ = 150–200 s$^{-1}$ to obtain transients consistent to those observed experimentally (data not shown). Although the mechanistic basis for this albeit minor difference (1.5–2-fold) is not readily evident, the complex, bisubstrate reversible nature of the pyrophosphorolysis reaction will necessarily lead to a net flux back toward $E$-Ile-AMP-PP$_i$, to a certain extent and hence reduce the macroscopic apparent maximal rate of pyrophosphorolysis ($k_{\text{max,app}}$ = 100 s$^{-1}$) relative to the true rate constant ($k_{-\beta}$ = 150–200 s$^{-1}$). Regardless of the absolute estimate of $k_{-\beta}$ (100–200 s$^{-1}$), the major conclusions are that PP$_i$ binding is weak ($K_{m}$ = 250 μM), the chemistry step is readily reversible and it has an internal equilibrium constant ($=k_{+g}/k_{-g}$) significantly less than unity (0.3–0.4).

**Steady-state Kinetics of tRNA Aminoacylation and PP$_i$/ATP Exchange**—The results above allowed the construction of a kinetic mechanism for substrate activation in the absence of tRNA (Scheme 1) which could adequately describe all of the transient kinetic data. It was of interest, however, to determine whether the same mechanism could also describe the steady-state kinetics of PP$_i$/ATP exchange and, to a lesser extent, of the full tRNA aminoacylation reaction. The steady-state parameters describing both the exchange and aminoacylation reactions were determined at saturating concentrations of one substrate while varying the concentration of the other substrate (Table III). Unfractionated *Escherichia coli* MRE600 tRNA (~4 μM functionally active tRNA$^{15}$) was used as the acceptor species in aminoacylation measurements. Analysis of progress curves at a range of tRNA concentrations using an integrated form of the Michaelis-Menten equation confirmed that the $K_{m,\text{tRNA}}$ was ≤0.1 μM (data not shown) consistent with similar values reported for *E. coli* IleRS (~0.1 μM) (22). In addition, the steady-state kinetic parameters (Table III) are broadly comparable to those reported previously for *E. coli* IleRS (22). Therefore, *S. aureus* IleRS is able to utilize tRNA-Ile from *E. coli* effectively as a substrate.

The $k_{\text{cat}}$ for PP/ATP exchange performed in the absence of tRNA ($k_{\text{cat,ex}} = 18$ s$^{-1}$ at 22°C, Table III) was ~50-fold higher than for tRNA aminoacylation ($k_{\text{cat,ex}} = 0.35$ s$^{-1}$, Table III). Scheme 1 was able to provide an adequate description of the steady-state kinetics of ATP/PP, exchange (e.g., simulated $k_{\text{cat,ex}}$ = 22 s$^{-1}$, data not shown, observed $k_{\text{cat,ex}} = 18 \pm 2$ s$^{-1}$, Table III). Since both the internal equilibrium constant for the chem-
Fig. 7. Effect of the mixing order on IleRS tryptophan fluorescence transients observed upon mixing with Ile (30 μM) and MgATP (4 mM). A, lower trace (E + ATP, Ile), E was rapidly mixed into Ile and ATP. This trace has been offset (by +0.01 units) from the other traces for clarity (k_{obs} = 44.0 ± 1.2 s^{-1}, amp = 15%). EATP + Ile; E was preincubated with ATP at the final concentration (4 mM) and then rapidly mixed into a mixture of 30 μM Ile plus 4 mM ATP (k_{obs} = 44.0 ± 1.2 s^{-1}, amplitude = 14.5%). E-Ile + ATP, as for E-ATP + Ile except that E was preincubated with 30 μM Ile (k_{obs} = 34.7 ± 1.6 s^{-1}, amp = 7.5%). All fluorescence transients were best described by a single exponential fit (as shown). B, kinetic simulation of the predicted fluorescence transients (arbitrary units) for the experiments shown in A using Scheme 1 and the data in Table I.

Fig. 8. Pyrophosphorolysis of the E-Ile-AMP complex by MgPP_i, measured using stopped-flow fluorescence. A, stopped-flow fluorescence traces following rapid mixing of E-Ile-AMP with MgPP_i, as indicated (data shown over 50 ms for clarity); B, concentration dependence of k_{obs} in MgPP_i, defining k_{max} = k_{app} + k_{atp} = 110 s^{-1} and k_{app} = k_{app}/k_{a} = 250 μM.

Table III

| Kinetic parameter | tRNA aminoacylation | PP/ATP exchange |
|-------------------|---------------------|------------------|
| K_m,Ile (μM)      | 5 ± 3               | 10 ± 2           |
| K_m,ATP (μM)      | 240 ± 18            | ND*              |
| K_m,tRNA (μM)     | <0.1                | NA               |
| k_{cat} (s^{-1})  | 0.35 ± 0.05         | 18.0 ± 2         |
| k_{cat}/k_{m,Ile} (M^{-1}s^{-1}) | 1.8 x 10^5          | 1.8 x 10^5       |

* ND, not done.
\* NA, not applicable.

The rate constant k_{cat} define the k_{cat,ex} for the enzyme (where chemistry is rate-limiting), the data in Table I suggest a k_{cat,ex} of 18–24 s^{-1}, consistent with that observed experimentally assuming 100% active enzyme (16–20 s^{-1}). Furthermore, since the rate constant for Ile dissociation from E-Ile-AMP (k_{d} = 3 ± 1.5 s^{-1}) is less than the rate constant for the catalytic step (k_{cat} = 60 s^{-1}), the k_{cat,ex}/K_m,Ile determined using saturating concentrations of ATP (1.8 x 10^6 M^{-1}s^{-1}, Table III) should approximate to the true association rate constant, k_{a} measured by stopped-flow (1.7 x 10^6 M^{-1}s^{-1}, Fig. 6B).

The K_m and V_max values shown in Table III, however, provide no information concerning the kinetically preferred order of substrate addition. We therefore investigated the kinetics of aminoacylation at varying concentrations of ATP and Ile and at a fixed, saturating, concentration of tRNA (Fig. 9). The dependences of the initial rate on [MgATP] and [Ile] (Fig. 9, A and D) were globally fit to Equation 3 which defines the steady-state equation for an equilibrium-ordered mechanism in which the kinetically preferred path involves the initial rapid equilibrium binding of ATP with a dissociation constant K_{ATP} (= 2.7 ± 0.4 mM) followed by the binding of Ile with a Michaelis constant K_{Ile} = 3 ± 0.3 μM to form the quaternary E-tRNA-ATP-Ile complex (23, 24).

\[ v = \frac{V_{max} [MgATP] [Ile]}{K_{ATP} [MgATP] + K_I [Ile] + K_{ATP,Ile} + [MgATP] [Ile]} \] (Eq. 3)

Other models for bistructate addition (including random order of addition such as in Scheme 1) did not yield an adequate fit to the data. Although there appears to be a preferred order of substrate binding in the presence of tRNA, the K_m measured for ATP binding to E-tRNA in steady-state measurements (Scheme 4) is similar to K_{a} determined from transient kinetics in the absence of tRNA (2.5 versus 2.7 mM; Figs. 3 and 9). Initial attempts to simulate the tRNA aminoacylation reaction used a modification of Scheme 1 in which a first order step (at 0.35 s^{-1}, Table III) corresponding to the rate-limiting chemical transfer reaction was added following PP_i release (i.e. E-Ile-AMP + tRNA → E + Ile-tRNA). Due to the highly favorable release of PP_i, from the E-Ile-AMP-PP_i, intermediate which precedes the rate-limiting step for the reaction, the predicted K_m values for both Ile and ATP were >100-fold lower than observed experimentally (Table III). However, in a mechanism in which the rate-limiting transfer step occurs prior to PP_i release (E-Ile-AMP-PP_i + tRNA → E + Ile-tRNA + PP_i), the remaining rate constants in Scheme 1 yielded very similar steady-state kinet-
ics to those observed experimentally (compare Fig. 9, A versus C, for ATP and D versus F for Ile). Although a solution to the elementary tRNA aminoacylation mechanism was not the main aim of this study and notwithstanding the somewhat arbitrary modification of Scheme 1 to include a tRNA transfer prior to PPi release, the similarity of the experimental data (Fig. 9) and that predicted from the modified Scheme 1 is rather striking.

Catalysis of Ap4A Formation by IleRS—Scheme 1 provides an adequate description of all the data obtained in the absence of tRNA. However, all aaRSs studied thus far that can catalyze amino acid activation in the absence of tRNA are also able to catalyze the reaction of ATP with the enzyme bound adenylate to form Ap4A (e.g. Ref. 34). We therefore investigated whether this reaction was catalyzed by S. aureus IleRS and if so, whether the reaction would impact significantly on the analysis of the stopped-flow data according to Scheme 1. Rapid mixing of E plus saturating [Ile] with Ap4A led to a slow exponential increase in enzyme fluorescence, consistent with the formation of the E-Ile-AMP-ATP complex although the maximal observed rate constant, $k_{-8}$, was only 0.04 s$^{-1}$ at high concentrations of Ap4A (data not shown; $K_m = 200 \mu$M; Scheme 3). For those synthetases that have been studied in detail, the affinity of ATP for the E-Ile-AMP-ATP complex appears to be very low (e.g. $K_m = 11$ and 50 mM for E. coli LysRS and PheRS, respectively) (35). As such, $k_{-8}$ cannot be determined directly from the experiments we have performed. However, by analogy with other synthetases (e.g. E. coli PheRS) in which synthesis of Ap4A is slow ($0.25$ s$^{-1}$) and where the internal equilibrium favors ATP rather than Ap4A ($K_8 = 1$) (35), it is probable that for IleRS, $k_{-8} < k_{-8} = 0.04$ s$^{-1}$. As such, the catalysis of Ap4A formation by S. aureus IleRS is unlikely to contribute significantly to the experimental data and has therefore been disregarded in the construction of the minimal mechanism.

Steady-state Kinetics of Inhibition of by Isoleucinyl Adenylate (Ile-ol-AMP)—Isoleucinyl adenylate (Fig. 10 A) is the reduced, and therefore a non-hydrolyzable analogue of the normal activated amino acid intermediate (Ile-AMP) formed during the IleRS reaction cycle. Such amino-alkyl adenylates have been known for some time to be effective inhibitors of aaRSs (25). Ile-ol-AMP, was found to inhibit both the steady-state tRNA aminoacylation (Fig. 10) and PPi/ATP exchange reactions (data not shown) competitively with respect to both ATP and Ile.

Analysis of the data in Fig. 10 (in the presence of 5 mM ATP) using Equation 4 yielded an estimate for the apparent $K_i$ ($K_i$ (app) — 0.25 $\mu$M, where $S = $ Ile.

$$ V_{\text{max}} = \frac{K_m \text{Ile}}{[\text{Ile}]} \left(1 + \frac{[I]}{K_{\text{app}}}ight) $$

Since Ile-ol-AMP is competitive with both Ile (Fig. 10) and ATP (Fig. 4), $K_i$ (app) from Equation 4 is an overestimate of the true $K_i$ for Ile-ol-AMP binding. Extensive kinetic simulations, in which inhibition curves were generated for different values of $K_i$ and
the initial rates subsequently fit to Equation 4 allowed the empirical relationship between \( K_i \) and \( K_{\text{app}} \) in this system to be defined. The results of these simulations indicated that the \( K_{\text{app}} \) overestimated the true \( K_i \) by 3.2-fold yielding an estimate of \( K_i \approx 70 \, \text{nm} \). From the direct measurement of \( k_{\text{cat},\text{ex}} \) and \( k_i \), for Ile-ol-AMP (Fig. 2 (12)), we obtain \( K_i \approx 30 \, \text{nm} \) indicating that our kinetic mechanism is able to quantitatively account, within reasonable margins (45 ± 25 m\( \text{M} \)), for both the transient and steady-state kinetics of IleRS and for its inhibition by Ile-ol-AMP.

**DISCUSSION**

There is considerable interest in the potential therapeutic uses of selective inhibitors of aminoacyl-tRNA synthetases such as PS-A (12, 13) as anti-infective agents (e.g. Ref. 26). We have a long standing interest in the interactions of PS-A and of other inhibitors with IleRS, particularly in relation to the development of compounds with improved anti-bacterial or clinical properties (e.g. Refs. 27–30). The recently solved crystal structure of the *Thermus thermophilus* IleRS-PS-A complex (31) has added extra impetus to this endeavor. As a first step to taking a more rational approach to the design of IleRS inhibitors, we have investigated the reaction cycle of this enzyme from *S. aureus*, one of the key target organisms for PS-A (11).

**Minimal Kinetic Mechanism for Amino Acid Activation**—Significant changes in enzyme fluorescence (up to 17%) were observed during the reaction cycle or following binding of the reaction intermediate analogue, Ile-ol-AMP, allowing us to use stopped-flow techniques to follow the binding and activation of substrates. Fluorescence resonance energy transfer experiments using chromophoric inhibitors of IleRS that quench the intrinsic protein fluorescence of IleRS suggest that a significant proportion of the observed IleRS tryptophan fluorescence originates from a few residues close to the active site (12).

The transient kinetic experiments described here allowed the solution of a complete minimal reaction scheme for the binding and activation of substrates. With the possible exception of the dissociation rate constant for Ile from the E-ATP-Ile ternary complex (\( k_{\text{dis}} \), Scheme 1), we have been able to make estimates of most of the elementary rate constants or equilibrium constants that describe the IleRS amino acid activation reaction with reasonable accuracy (Scheme 1, Table I). Based on this minimal mechanism, kinetic simulations were performed which adequately describe the transient kinetics (including both the observed exponential rate constants and fluorescence amplitudes) and the steady-state kinetics of ATP/PP\_i exchange.

A key feature of the mechanism is the random order of addition of substrates to form the E-Ile-ATP ternary complex with thermodynamic linkage between the binding of the first and second substrate (e.g. ATP binds more tightly to E-Ile than to free E and vice versa). This confirms previous qualitative evidence for such linkage from steady-state experiments with *E. coli* IleRS (22).

The product of dissociation constants \( K_i \cdot K_4 = 4 \pm 2 \times 10^{-9} \, \text{m}^{-2} \) approximates to the product \( K_i \cdot K_4 \sim 2–6 \times 10^{-9} \, \text{m}^{-2} \) (depending on the value used for \( K_i \)) and which defines the equilibrium constant for the (path-independent) reaction \( E + \text{ATP} + \text{Ile} \leftrightarrow E-\text{ATP-Ile} \). By analogy with tyrosyl-tRNA synthetase (33), the ~35-fold difference in equilibrium constants observed in the presence of the co-substrate corresponds to about 2 kcal/mol, and may therefore result from the formation of only two or three additional uncharged hydrogen bonds. A second feature of Scheme 1 is the inherent reversibility of the chemistry step (\( k_{\text{cat},\text{ex}}/k_i \approx 0.3–0.4 \) which accounts for the known reversibility of overall ATP/PP\_i exchange and which adequately predicts the experimentally observed \( k_{\text{cat},\text{ex}} \) for this reaction (60 s\(^{-1}\) ~ 0.3–0.4 = 18–24 s\(^{-1}\)). However, the very weak affinity of PP\_i for the E-Ile-AMP-PP\_i complex (\( K_6 = 0.25 \, \text{mM} \)) drives the reaction toward stoichiometric formation of E-Ile-AMP when IleRS is mixed with Ile and ATP at submicromolar concentrations of enzyme.

The dissociation of Ile from the E-Ile-ATP complex (\( k_4 = 3 \pm 1.5 \, \text{s}^{-1} \)) is slower than the \( k_{\text{cat},\text{ex}} \) in the reverse direction (PP\_i \rightarrow ATP, 18 s\(^{-1}\)). Therefore, if this estimate is accepted with the associated caveats, the kinetically preferred pathway must then involve release of ATP from E-Ile-ATP at a rate governed by \( k_3 \) rather than via the sequential release of Ile (at 3 ± 1.5 s\(^{-1}\)) and then of ATP. A second consequence of a low value for \( k_4 \) is that the binding of Ile to E-ATP has a large commitment to catalysis (since \( k_4 > k_4 \)) and hence the \( k_{\text{cat},\text{ex}}/K_{\text{Ile}} \) (1.8 × 10\(^6\) M\(^{-1}\) s\(^{-1}\), Table III) is predicted to approximate to the true bi-molecular association rate constant for Ile binding to E-ATP (1.6 × 10\(^6\) M\(^{-1}\) s\(^{-1}\), Fig. 6B). Of course, Scheme 1 suggests that assumptions involving rapid equilibrium binding of Ile prior to catalysis are invalid.

**Comparison of Scheme 1 with the kinetics of tRNA Aminoacylation**—Our main focus has been to define the elementary rate constants that describe the amino acid activation reaction.
However, we have also examined the steady-state kinetics of tRNA aminoacylation, primarily to determine how closely Scheme 1 could predict the full tRNA aminoacylation reaction. The parameters determined in these experiments were broadly similar to those previously reported for the E. coli IleRS (22), although our data (Fig. 10) suggest a rapid-equilibrium ordered mechanism in which ATP binds first (Fig. 9) (24). However, analysis of the crystal structures of enzyme-substrate complexes of other aaRSs (e.g. Bacillus stearothermophilus TyrRS) (33), has led to questioning of the validity of an ordered substrate addition mechanism that arose from a steady-state kinetic analysis of TyrRS (33).

When considering the relationship between Scheme 1 and the complete tRNA aminoacylation reaction, it is clear that tRNA could bind to and dissociate from any of the enzyme intermediates. We have only two pieces of indirect evidence concerning their tRNA ligation states. First, proteolysis protec-
intermediates. We have only two pieces of indirect evidence that tRNA could bind to and dissociate from any of the enzyme intermediates. It is intuitive that any kinetic analysis of TyrRS (33), has led to questioning of the validity of an ordered sub-
plexes of other aaRSs (e.g. Bacillus stearothermophilus TyrRS) (33), has led to questioning of the validity of an ordered sub-

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