Effects of Substrate and Inhibitor Binding on Proteolysis of Isoleucyl-tRNA Synthetase from Staphylococcus aureus*

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Binding of ligands to isoleucyl-tRNA synthetase (IleRS; E) from Staphylococcus aureus was investigated through effects on proteolytic digestion. Approximately 50-fold higher concentrations of protease (trypsin or chymotrypsin) were required to inactivate IleRS after incubation with substrates and formation of the E-Ile-AMP intermediate compared with free E. Binding of pseudomonacic acid A (PS-A) or isoleucyl adenylate (Ile-ol-AMP) also induced resistance to proteolysis and altered the patterns of IleRS cleavage fragments in an inhibitor-class specific manner. The determinants for PS-A binding were investigated via proteolysis of E-[3H]PS-A. Limited proteolysis of E-[3H]PS-A (excising residues 186–407) could be achieved without significant loss of bound inhibitor, eliminating this region as contributing to inhibitor binding. Assays were developed which allowed IleRS proteolysis to be readily followed using fluorescence polarization. Inhibitor-protected IleRS was labeled with fluorescein isothiocyanate with only a small effect upon catalytic activity (Fl-IleRS). The (pseudo) kinetics of proteolytic cleavage of Fl-IleRS could be measured at low nanomolar Fl-IleRS concentrations in 96/384-well microtiter plates, allowing real-time monitoring of dose-dependent protection from proteolysis. Thus, inhibitor (and substrate) binding could be reproducibly assessed in the absence of measurements of catalytic activity. This could potentially form the basis of novel screening assays for ligands to other proteins.

Pseudomonacic acid A (PS-A) is a potent antibiotic that acts through inhibition of bacterial isoleucyl-tRNA synthetase (IleRS, E) (1, 2). As part of an effort to understand the precise mechanism of the interaction of PS-A and its analogues with IleRS we have undertaken a detailed characterization of the kinetics and thermodynamics of substrate and inhibitor binding by this enzyme (3, 4). These experiments showed that PS-A analogues are reversible, slow tight-binding inhibitors that form an highly stabilized inhibitor complex with overall dissociation constants in the picomolar range. Comparison of PS-A analogues with inhibitors that were non-hydrolyzable analogues of the normal activated enzyme intermediate, Ile-AMP, showed that both classes of inhibitor bound to IleRS in a manner that was mutually exclusive with each other and with the substrates, L-isoleucine (Ile) and MgATP, but that very different enzyme conformations were induced upon binding (as shown by differences in IleRS tryptophan fluorescence) (4). In this report, we describe experiments in which we attempted to gain additional information on the binding of substrates and inhibitors to IleRS using different approaches to the more classical techniques which we had previously employed (e.g. state-state and transient kinetics (3, 4)).

One technique for investigating changes in the conformation of proteins as a result of ligand binding is to test their effect upon proteolysis (e.g. Ref. 5), and this has previously been shown to yield useful information on several aminoacyl-tRNA synthetases (e.g. Refs. 6–9). Here, we used this approach in two distinct ways. First, the effect of substrate and inhibitor binding on IleRS proteolysis patterns was investigated in an attempt to identify protein determinants for binding. Second, a homogeneous real-time assay capable of monitoring ligand protection from IleRS proteolysis using fluorescence polarization was developed which could be potentially used to screen for new inhibitors.

MATERIALS AND METHODS

IleRS and Other Reagents—IleRS from S. aureus was purified (to >98% purity as judged by SDS-PAGE or reverse phase HPLC) from Escherichia coli DH1 cells carrying the pDB575 plasmid (10). Fluorescein isothiocyanate (FITC) was from Molecular Probes (Leiden, Holland). Inhibitors and [3H]labeled PS-A were prepared at SmithKline Beecham. Except where otherwise stated, reagents were of the highest grade available and purchased mainly from Sigma (Poole, Dorset, United Kingdom).

IleRS Activity Measurements—tRNA aminoacylation and PP/ATP exchange assays were performed using modifications to previously published methods (11) as described by Pope et al. (3, 4). All assays were performed at 22 °C in 50 mM Tris-HCl, pH 7.9, 10 mM MgCl2, 50 mM KCl and 2 mM dithiothreitol.

Protein Purification and Analysis—SDS-PAGE was performed using pre-cast (4–20%) gradient gels obtained from Novex (San Diego, CA). Column chromatography was performed on a Pharmacia FPLC system (Pharmacia, St. Albans, Herts, UK). Sephacryl S-100 was obtained from Pharmacia as was the ResourceT™Q and Fast-desalting columns. Microbore reverse-phase HPLC columns were packed in house as described previously (12) with Polymer Laboratories reverse phase material, 8 μm particle size in green peak tubing. The gradient used was 0–80% acetonitrile with 0.1% trifluoroacetic acid over 10 min at a flow rate of 0.3 ml/min. Laser desorption mass spectrometry analysis was performed on a Finnigan Matt Lasermat. N-terminal amino acid sequencing was performed on an Applied Biosystems 477A protein sequencer using standard Edman chemistry.

FITC Labeling of IleRS and Fluorescence Measurements—IleRS (complexed with Ile-ol-AMP) was labeled with FITC in 100 mM bicarbonate buffer, pH 8.5, using a 200-fold (w/w) ratio of FITC to IleRS and 1 h incubation at room temperature. Excess unreacted FITC was removed by desalting into 50 mM Hepes, pH 7.8, 20% glycerol. The IleRS fluorescence labeling stoichiometry was calculated using the extinction coefficient of fluorescein at 494 nm (7.2 × 104 M−1 cm−1) and IleRS concentrations from amino acid analysis. FITC-labeled IleRS (Fl-IleRS) was stored at −70 °C, following flash freezing in N2. Fluorescence...
RESULTS AND DISCUSSION

Effect of Substrates on Proteolysis of IleRS
Monitored by Catalytic Activity

The catalytic activity of IleRS can be monitored two ways: either through tRNA aminoacylation or via isotopic exchange from $[^{32}P]PP_i$ into ATP, which is independent of tRNA$^{Ile}$ binding (e.g. Ref. 14). The effects of protease digestion, using trypsin or chymotrypsin, were first examined on both of these activities where the digestion was performed in the absence of any substrates. This showed, unsurprisingly, that identical conditions were required for inactivation of both activities. Representative data showing the results obtained from trypsin treatment of IleRS are shown in Fig. 1. PPi/ATP exchange activity (○), or PP/ATP exchange activity (●), in the presence (■) or absence (○, ●) of 1 mM Ile and 5 mM Mg$\text{ATP}$. Other conditions were as described under “Materials and Methods.”

Polarization measurements were made on a Polastar 96-well plate reader (BMG Instruments GmbH) or FPM-1 or FPM-2 Instruments (Jolley Consulting Ltd). Stopped-flow measurements were made on a Hi-Tech Instruments Model SF-51 Instrument, with excitation at 290 nm for tryptophan fluorescence and 488 nm for fluorescein. Fluorescence emission was observed using 300- and 500-nm cut-on filters, respectively. Traces shown are the average of four independent experiments. All least-squares fitting was performed using Grafit (13).

Effect of Inhibitors on Patterns of Proteolysis—Protection of IleRS from proteolysis by inhibitor binding was examined using representative compounds from two classes of competitive IleRS inhibitor, PS-A (1, 2, 4) and its semi-synthetic analogue, SB 205952 (4, 15), are slow tight-binding competitive IleRS inhibitors with overall dissociation constants in the picomolar range. Ile-ol-AMP and Ile-NHSO$_2$-AMP are non-hydrolyzable analogues of the IleRS reaction intermediate, Ile-AMP, and are simple competitive inhibitors, with $K_i$ values of 30 and 1 nM, respectively (3, 4). Both classes of inhibitor protected IleRS from proteolysis (compare the control lane in Fig. 2B with the lanes containing inhibitors). Moreover, the pattern of proteolytic fragmentation observed upon digestion of E-Ile inhibitor complexes appeared to be inhibitor class specific. This was shown by the fact that the digestion pattern observed by addition of PS-A and SB 205952 and of Ile-ol-AMP and Ile-NHSO$_2$-AMP, respectively, were identical (Fig. 2B). In particular, digestion of the IleRS complex with PS-A or SB 205952 yielded a pattern that was most similar to that observed upon digestion of the E-Ile binary complex (compare Fig. 2, A and B), yielding around 8 major fragments sized between 10 and 60 kDa, two of which were most predominant (Figs. 2 and 3). The proteolytic patterns observed with the two analogues of Ile-AMP were clearly different, confirming that the conformation of the inhibitor complex was distinct when formed with PS-A or Ile-AMP analogues, respectively (4).

Although the digestion patterns observed in these experiments yielded some interesting information, they were of fairly limited use as the precise stoichiometries and patterns of proteolytic fragmentation were difficult to characterize quantitatively and varied considerably with exposure of the IleRS to proteolysis. This made it extremely difficult to make comparisons between independent experiments, as the overall extent of proteolysis could not easily be controlled. For example, this may well be the reason why the fragmentation pattern observed for cleavage of the catalytic E-Ile-AMP complex (i.e. E + Ile + Mg$\text{ATP}$) appears dissimilar to that when the E-Ile-ol-AMP or E-Ile-NHSO$_2$-AMP complexes are digested (e.g. compare the extent of proteolysis in Figs. 2, A, lane 9 (+Ile-AMP, +Ile-NHSO$_2$-AMP)).

Analysis of Ligand-induced Stabilization of IleRS by SDS-PAGE

Effects of Substrates on Patterns of Proteolysis—The effect of a range of ligands on IleRS proteolysis was surveyed by characterizing the digestion products by SDS-PAGE. Fig. 2A shows representative results obtained when IleRS was preincubated with saturating concentrations of each of the individual substrates of the tRNA aminoacylation reaction (i.e. Ile, ATP, and tRNA$^{Ile}$) either alone or in combination, and then subjected to proteolysis using chymotrypsin. The control lane in Fig. 2B shows that, in the absence of substrates, IleRS was extensively digested under these conditions, with the most predominant product being a single band at around 60 kDa. In contrast, each of the substrates protected IleRS from digestion by chymotrypsin to some extent (as shown by the residual undigested IleRS; Fig. 2B), and produced a distinct pattern of proteolysis. Protection from proteolysis was much more pronounced with Mg$\text{ATP}$ and tRNA$^{Ile}$ than with Ile (Fig. 2A) and, interestingly, the major product of digestion of E-Ile was similar to that from free enzyme. This product was almost completely absent in the presence of other substrates. Adding combinations of substrates produced slightly more protection still. Interestingly, the presence of ATP and tRNA$^{Ile}$ appeared to produce the highest level of protection of any of the conditions, even compared with all three of the substrates added together (Fig. 2A). Digestion patterns of similar complexity, and showing more or less the same pattern of substrate protection were obtained when IleRS complexes were digested with other proteases (e.g. trypsin, thrombin, V8, thermolysin; data not shown).

Fig. 1. Protection of IleRS from proteolysis by substrates. Effect of digestion with varying concentrations of trypsin for 60 min at 37 °C on residual IleRS activity measured by tRNA aminoacylation (●) or PP/ATP exchange activity (○). Data are shown for all substrates in combination (solid line), and for each individually (dashed lines with different symbols). Proteolysis data are given as the mean ± S.D. (n = 4), with the average ± S.D. shown as a bar. The differences were significant at each concentration of trypsin (P < 0.05).

Effect of Substrates on Proteolysis of IleRS

Protection from proteolysis by substrates.
suggested a similar enzyme conformation in both cases (3, 4).

For this reason, we focused on trying to gain some further information on the distinct fragmentation pattern observed when binding PS-A to IleRS, and simultaneously developed alternative methods to allow proteolysis to be characterized in a more quantitative manner.

The relatively complex patterns of proteolytic digestion observed here contrast with the results of Yanigasawa et al. (9) who found that tryptic digests of the Escherichia coli IleRS complexed with ATP or PS-A resulted in a specific cleavage at a single site at lysine 605. The same authors observed no protection from this proteolytic event by saturating concentrations of Ile or tRNAIle. Yanigasawa et al. (9) used similar amounts of protease (~1:50, w/w, protease:IleRS) but very different exposure conditions (overnight on ice, 37 °C for 1 h in the present study). It is therefore difficult to conclude whether the very simple proteolytic patterns observed by these workers reflected differences in the structure of the E-PS-A complex between E. coli and Staphylococcus aureus IleRS, or the kinetics/extent of the proteolysis conditions.

**Proteolysis of the E[^3]H]PS-A Complex—**To test if the cleaved IleRS is competent to bind PS-A, proteolytic digestions were performed on the complex of IleRS with[^3]H-labeled PS-A. Digestion of E[^3]H]PS-A with chymotrypsin under the conditions shown in Fig. 2B did not lead to any significant loss of bound[^3]H]PS-A. Using[^3]H]PS-A as a marker, the fragments that retained inhibitor binding were purified under non-denaturing conditions. However, chromatographic separation of the digested material using a range of different techniques (e.g. ion exchange (Fig. 3A, lane 5, Resource Q), size exclusion (Fig. 3A, lane 6, Sephacryl S-200), hydrophobic interaction (Phenyl-Sepharose; data not shown)), including running multiple separation steps in tandem, always resulted in elution of[^3]H]PS-A in a single peak, which on SDS-PAGE (Fig. 3A) or reverse-phase-HPLC (Fig. 3B, inset) was shown to consist of two components. Fig. 3B shows a typical ion-exchange chromatographic purification of the digested E[^3]H]PS-A complex. The fact that these two enzyme fragments always co-purified together suggests strongly that they were tightly associated with each other and with[^3]H]PS-A.

Laser desorption mass spectrometry and N-terminal sequencing of the product(s) from several purifications showed that they were always the same two peptides comprising amino acids 1–185 (21,762 kDa; N-terminal amino acid sequence, MDYKETLLM) and 407–918 (59,073 kDa; N-terminal amino acid sequence, RATPQWFASISK). These fragments therefore correspond to the two major products observed from digestion of the complex of IleRS with PS-A or SB 205952 (e.g. Figs. 2B and 4A). Therefore, chymotryptic digestion of IleRS complexed with this class of inhibitor leads to excision of a central portion of the enzyme between residues 186 and 406, with the remaining portions still tightly associated with each other and inhibitor. These enzyme domains contain all of the conserved sequences implicated in catalysis (e.g. HIGH and KMSKS; see Ref. 14). Kinetic experiments to determine the stability of the E[^3]H]PS-A complex after proteolysis (e.g. by “chase” off rate experiments; see Ref. 4), showed no significant effect of proteolysis on the kinetics of PS-A dissociation (data not shown). Residues 186–406 therefore appear to play no role in PS-A binding. Using similar purification procedures to those shown in Fig. 3, the truncated E[^3]H]PS-A complex was successfully purified to >99% purity yielding sufficient material (>10 mg) to initiate crystallography for x-ray diffraction experiments.

**Fluorescence Polarization Assay for Monitoring Ligand-induced Stabilization of IleRS**

Fluorescein Labeling of IleRS—Fluorescence polarization provides a straightforward and convenient method for monitoring the proteolysis of large fluorescently labeled protein substrates (e.g. Refs. 16 and 17). This approach is based upon the differences in the molecular rotational correlation time and hence polarization of a fluorphore when attached to a large protein substrate compared with that of small proteolytic fragments. Hence, proteolysis leads to a reduction in overall fluorophore fluorescence polarization that allows enzymatic cleavage to be followed in real-time. To facilitate this approach, we labeled IleRS with FITC in the presence of saturating concentrations of Ile-ol-AMP to protect the IleRS active site. This inhibitor was chosen because it has a relatively fast off-rate (k~ = ~0.1 s⁻¹ (4)) and could readily be removed by gel filtration or dialysis. By optimizing the conditions for labeling we were able to reproducibly produce fluorescein-IleRS (Fl-IleRS) that had a labeling stoichiometry of ~1:1 mol/mol and retained around 75% of the catalytic activity of unlabeled IleRS, as measured by tRNA aminoaclation or PP/ATP exchange. Higher labeling stoichiometries led to a greater loss of catalytic activity.

To establish whether fluorescein labeling affected inhibitor binding to IleRS we performed stopped-flow mixing experiments between Fl-IleRS and SB 205952, a pseudomonic acid analogue that possesses a chromophore that induces a large change in enzyme tryptophan fluorescence upon binding via radiationless energy transfer (4). The kinetics of binding of SB 205952 to Fl-IleRS and unlabeled enzyme were almost identical. In the example shown in Fig. 4A, in which 0.2 μM Fl-IleRS was mixed with 75 μM SB 205952 (final concentrations), the rate constant for binding (kobs) was 125.1 ± 1.75 s⁻¹ and
126.5 ± 2.9 s⁻¹ for native and Fl-IleRS, respectively. However, the amplitude of the fluorescence transient was smaller with Fl-IleRS (27% of total enzyme tryptophan fluorescence, cf. 40% for native IleRS). This was consistent with the measurements of catalytic activity which suggested that fluorescent labeling of IleRS resulted in loss of about a quarter of its catalytic activity, possibly by labeling at lysine residues in the region of the active site. As the overall stoichiometry of labeling was close to unity, one possible interpretation was that only a proportion of the IleRS was labeled with fluorescein (at multiple stoichiometry), and that the catalytic activity and stopped-flow transients were arising from residual unlabeled enzyme. However, this was not the case because Fl-IleRS was capable of binding inhibitor since a transient change in the fluorescence emission could also observed upon binding SB 205952 (Fig. 4A). This could only originate from labeled enzyme.

Subsequent proteolysis protection experiments suggested that Fl-IleRS was competent to bind substrate and inhibitors (see below), so the reduction in catalytic activity seems most likely to be a general effect of IleRS labeling, rather than the presence of populations of active and inactive (and unlabeled) IleRS forms. SDS-PAGE analysis of Fl-IleRS digestion patterns in the presence and absence of inhibitors showed similar patterns to those observed with unlabeled enzyme (e.g. Fig. 1). Despite the fact that IleRS was labeled at an average stoichiometry of close to 1, virtually all of the Fl-IleRS digestion fragments were fluorescently-labeled (as visualized using an imaging system). It therefore seemed that FITC treatment resulted in the labeling of Fl-IleRS at many different surface lysine residues.

**Real-time Measurements of Fl-IleRS Proteolysis Monitored by Fluorescence Polarization**—Measurements of the fluorescence properties of Fl-IleRS suggested that it would be a useful system with which to monitor IleRS proteolysis. The fluorescence intensity of Fl-IleRS was sufficiently bright to monitor ~1 nM concentrations with a coefficient of variation of <5% in commercial fluorescence plate readers. However, as is commonly found with fluorescein (18), the Fl-IleRS conjugate fluorescence was highly quenched (<30% of the brightness of unreacted FITC). As would be expected from the large size of the IleRS monomer (106 kDa), Fl-IleRS fluorescence was highly polarized (~250 mP). These observations suggested that the generation of proteolytic fragments of Fl-IleRS could be monitored either via relief of fluorescence quenching or changes in overall fluorescence polarization. Indeed, mixing Fl-IleRS with protease resulted in a time-dependent increase...
in overall fluorescence intensity (data not shown) and a decrease in fluorescence polarization (e.g., Fig. 4B). The rate of fluorescence change showed a first-order dependence in protease concentration up to around 25 µg/ml (or trypsin or chymotrypsin), yielding a rate of polarization decrease of 3–4 mP/min. This was sufficient to measure proteolysis rates with reasonable precision at Fl-IleRS concentrations of around 1 nM and using 30–60-min time courses. In the absence of protease, the fluorescence signal from IleRS was stable over this period (e.g., Fig. 4B). Although measurements of the changes in fluorescence intensity and polarization could be used more or less interchangeably, fluorescence polarization, which is a metric technique, was found to be intrinsically more reliable and less prone to interference and so was used routinely.

The recent availability of commercial microtiter plate fluorescence polarization instruments meant that such measurements could be made in a highly automated manner and using relatively small sample volumes (30–100 µl). Typical time courses measured on such a system are shown in Fig. 4B, which shows chymotrypsin digestion of Fl-IleRS in the presence and absence of Ile and Mg-ATP. In this example, the rate of decrease of fluorescence polarization was 3.6 mP·min⁻¹ in the absence of substrates and 1.1 mP·min⁻¹ in the presence of Mg-ATP and Ile, so the rate of digestion was therefore reduced by around one-third when Fl-IleRS formed the Fl-E-Ile-AMP intermediate. This method therefore provided at least semi-quantitative data on the rates of proteolysis and the susceptibility to proteolysis of different Fl-E-ligand complexes. Table I summarizes the results of experiments to measure the protection from proteolysis by each of the individual substrates using this method. The results are in reasonable accordance with measurements of substrate binding using conventional methods (K_d,ATP = 2.5 mM; K_d,Ile = 100 µM, K_m,tRNA = <0.1 µM (3)). Interestingly, the maximum decrease in proteolytic rate observed in the presence of substrates was around one-third of the control values. This may represent the limit of proteolytic protection for Fl-E-S (or reaction intermediate) complexes, at least under the conditions used here.

This method was also capable of detecting inhibitor binding in a dose-dependent manner. Fig. 4C shows dose-response data for protection from proteolysis by Ile-ol-AMP and PS-A. PS-A has an overall dissociation constant of ~50 µM, far below the detection limit for Fl-IleRS fluorescence. Thus, the experiment shown in Fig. 4C was conducted at an Fl-IleRS concentration of 1 nM. Under these conditions, tight binding kinetics dominate and the inhibitor dose-response curve merely titrates the enzyme concentration (e.g., Refs. 4 and 19), thus yielding in this case an IC₅₀ value close to 1 nM. However, almost identical results are obtained when attempting to measure the potency of this compound using catalytic assays (i.e., tRNA aminoacylation or PP/ATP exchange) since these require enzyme concentrations of close to 1 nM (4).

In cases where the dissociation constant for inhibitor binding was above the Fl-IleRS concentration, the protease digestion method yielded IC₅₀ values which seemed to directly reflect inhibitor potency. One such example is shown for Ile-ol-AMP in Fig. 4C. This compound has a K_d for binding to IleRS of ~30 nM (3, 4), and experiments similar to those in Fig. 4C yielded a mean IC₅₀ value of 7 ± 2 nM (mean ± S.E., n = 3). The data obtained from proteolysis protection experiments are therefore in reasonable accord with that from other, more conventional methods (3, 4). However, the data shown in Fig. 4C illustrate one important limitation of this method, which is that the ratio of protease to target protein needs to be carefully optimized in order to see dose-dependent protection, and this may vary from one class of ligand to another (e.g., substrates versus inhibitors). This is because the stabilization to proteolysis by ligand binding can normally be surmounted by high levels of protease (e.g., see Fig. 1). Performing experiments at too high concentrations of protease therefore either results in apparently incomplete dose-response curves (where the asymptotic digestion rate in the presence of inhibitors is a significant proportion of that in the absence of any ligands) or, in the most extreme case, complete loss of any apparent protection by ligand binding. Therefore, care would be required if using this model to screen for new interactions, e.g., in high-throughput screening of compound libraries or “ligand fishing,” for example, for the natural partner for a new gene product of unassigned function. However, provided a positive control is available, the conditions can be optimized easily. The availability of a known ligand to the target protein is also useful as a means of protecting crucial residues during labeling. This approach therefore seems to have considerable promise as a general screening method.

The work described here shows that limited proteolysis provided useful information about the conformation of IleRS-ligand complexes, including the identification of a central portion of the enzyme that plays no role in inhibitor binding. The production of Fl-IleRS and real-time monitoring of proteolysis in microtiter plate allowed the extension of this approach into a method for at least semi-quantitatively screening for IleRS ligands that had equivalent sensitivity to catalytic assays but avoided separation steps, expensive reagents such as tRNA and the hazards associated with the use of radioisotopes.

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