Identification of Essential Residues within Lit, a Cell Death Peptidase of *Escherichia coli* K-12[†]

Nikki A. Copeland, Ryan Bingham,[‡] Theonie Georgiou, Peter Cooper,[§] and Colin Kleanthous*

Department of Biology, P.O. Box 373, University of York, York YO10 5YW, U.K.

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ABSTRACT: Bacteriophage exclusion is a suicide response to viral infection. In strains of *Escherichia coli* K-12 infected with T4 phage this process is mediated by the host-encoded Lit peptidase. Lit is activated by a unique sequence in the major head protein of the T4 phage (the Gol sequence) which then cleaves site-specifically the host translation factor EF-Tu, ultimately leading to cell death. Lit has very low sequence identity with other peptidases, with only a putative metallopeptidase motif, $H^{160}EXXH$, giving an indication of its catalytic activity. The aim of the present study was to ascertain if Lit is a metallopeptidase, identify residues essential for Lit activity, and probe the involvement of the Gol sequence in the activation of enzymatic activity. Lit activity was inhibited by the zinc chelator 1,10-phenanthroline, consistent with the suggestion that it is a metallopeptidase. Preliminary covalent modification experiments found that Lit was susceptible to inactivation by diethyl pyrocarbonate, with about three histidines reversibly modified, one of which was found to be essential for proteolytic activity. Subsequently, 13 mutants of the Lit enzyme were constructed that included all 10 histidines as well as other residues within the metallopeptidase motif. This demonstrated that the residues within the HEXXH motif are required for Lit activity and further defined the essential catalytic core as $H^{160}EXXHX_{67}H$, with additional residues such as His169 being important but not essential for activity. Kinetic analysis of Lit activation by a synthetic Gol peptide highlighted that elevated concentrations of the peptide (>10-fold above activation K_M) are inhibitory to Lit, with this effect also seen in partially active Lit mutants. The susceptibility of Lit to inhibition by its own activating peptide suggests that the Gol sequence may be able to bind nonproductively to the enzyme at high concentration. We discuss these data in the context of the currently understood models for Gol-mediated activation of the Lit peptidase and its mechanism of action.

Bacteriophage exclusion is an altruistic response to viral infection that is usually mediated by a prophage-encoded toxin. During bacteriophage infection the bacteriocidal toxin becomes activated by the expression of a viral product leading to cell death, thus preventing the propagation of phage and preserving prophage DNA within the population (*1*). Bacteriophage exclusion is poorly understood at the molecular level, although there have been a number of mechanisms described in both Gram-positive and Gram-negative bacteria. In *Escherichia coli*, these include Rex, a λ phage-encoded two-component sensor/pore-forming toxin pair that is active against some strains of T4, T5, and T7 phage (*2*); PrrC, a tRNA-directed ribonuclease activated by a small peptide expressed during T4 infection (*1*); and Lit, a proteolytic toxin that cleaves an essential translation factor. Both Lit and PrrC prevent protein synthesis (*3*, *4*).

Lit is a constitutively expressed peptidase that mediates bacteriophage exclusion in *E. coli* K-12 strains, encoded by a latent prophage-like element called e-14 (*3*, *5*). Lit, which has a molecular mass of ∼34 kDa, is thought to associate with the inner membrane (*3*). Activated late in the T-even phage replication cycle upon the appearance of the major capsid protein gp23, Lit hydrolyzes a single peptide bond within the universally conserved switch region ($R^{58}G\downarrow ITI$ motif) of the host translation factor EF-Tu[1] (*4*, *6*). This region plays a critical role in the coordination of the Mg^{2+} ion and the γ-phosphate of GTP (*7*), with cleavage by Lit blocking protein biosynthesis presumably due to the impaired functioning of the translation factor (*4*). EF-Tu forms a weak complex with a synthetic 29-residue region of gp23 (corresponding to residues 94−122) called the Gol peptide (*8*). This binary complex (K_i ∼0.3 mM; *8*), thought to serve as the signal for viral infection, forms the substrate for the Lit enzyme (*9*).

Gol was first identified genetically by Champness and Snyder (*10*) as a region within the major head protein of T4, where mutations enabled T4 phage to replicate efficiently in Lit-containing cells. This region was shown to be the minimum sequence required to activate Lit in vivo (*6*) and later using the synthetic Gol peptide in vitro (*9*). Lit is highly specific for folded EF-Tu with neither the homologous translation factor EF-G or denatured EF-Tu serving as the

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* Corresponding author. Tel: (+44) 1904 328820. Fax: (+44) 1904 328825. E-mail: ck11@york.ac.uk.

[‡] Present address: GlaxoSmithKline, New Frontiers Science Park, Third Avenue, Harlow Essex CM19 5AW, U.K.

[§] Present address: 25 Thornton Hill, Exeter EX4 4NN, U.K.

[1] Abbreviations: EF-Tu, elongation factor Tu; CD, circular dichroism; DEPC, diethyl pyrocarbonate; EDTA, ethylenediaminetetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

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substrate for the enzyme. Hence, it is a combination of both sequence and conformation of the EF-Tu/Gol complex that are necessary for recognition and cleavage by Lit (*8*, *9*).

Very little is known about the mechanism of action of Lit, and so we undertook a study to identify essential residues. Sequence alignments with genomic databases reveal that Lit shares very little global similarity with other peptidases. The only sequence similarity thus far identified is a putative Zn^{2+} binding motif, $H^{160}EXXH$, suggesting that Lit may be a metallopeptidase (*1*). In the present work we have used 1,10-phenanthroline to show the likely requirement of a metal ion for catalysis. We used chemical modification of the peptidase using a histidine-specific reagent to show the importance of histidine residues for Lit activity, with mutagenesis experiments identifying those that are essential for catalysis. Using steady-state kinetics, we determined the activation parameters for the Gol peptide, in the process demonstrating that although this is the activating sequence for Lit, it can also inhibit the enzyme.

EXPERIMENTAL PROCEDURES

Molecular biological reagents were obtained from Stratagene (La Jolla, CA). Diethyl pyrocarbonate (DEPC) and hydroxylamine were purchased from Sigma (Poole, Dorset, U.K.) and stored desiccated at 4 °C. *E. coli* EF-Tu was prepared as described previously (*13*). Synthetic Gol peptide (*9*) was prepared chemically by Affiniti Research Products Limited (Manhead, U.K.) and purified to >95% purity by HPLC.

Expression and Purification of Lit. The overexpression and purification of wild-type and mutant Lit peptidases were performed as described previously (*9*). For the comparisons of Lit mutants with wild type, the protocol was modified with EDTA omitted from all purification buffers. For all preparations, the Lit protein was >95% pure as judged by SDS−PAGE. Protein concentrations were determined by the Bradford method (*14*). The yield for each preparation was typically 10 mg from 1 L of culture. For the 1,10-phenathroline inhibition experiments the purification of Lit was modified, which will be described in detail elsewhere, and involved the removal of sarkosyl and use of urea for the refolding of Lit from inclusion bodies.

EF-Tu Cleavage Assay. Cleavage reactions were performed essentially as described by Georgiou et al. (*9*) under pseudo-first-order conditions with a 10-fold excess of substrate to enzyme (2 μM EF-Tu·GDP, 0.2 μM Lit, and 10 μM Gol). All reactions were performed in 50 mM Tris-HCl, pH 8.0, 10 mM β-mercaptoethanol, 2 mM $MgCl_2$, and 10 μM GDP at 30 °C and initiated with the addition of the activating Gol peptide. At time points between 0 and 60 min an aliquot was removed and the reaction quenched by the addition of EDTA to a final concentration of 20 mM. SDS−PAGE gels (12%) were then used to separate the products, and the relative abundance of uncleaved EF-Tu to the smaller ~37 kDa EF-Tu fragment was determined by densitometry. Pseudo-first-order rates were determined as described by Georgiou et al. (*9*). Cleavage assays in the presence of 1,10-phenanthroline were performed as above except the reaction included 2 mM 1,10-phenanthroline as indicated in the figure. Time points were taken, and the percent cleaved EF-Tu was plotted against time. Observed first-order rates were determined by fitting to a single exponential equation in the absence of 1,10-phenanthroline and by linear regression in its presence using Sigmaplot (SPSS).

Modification of Lit Peptidase with Diethyl Pyrocarbonate (DEPC). Solutions of DEPC were freshly prepared for each experiment by dilution into 50 mM MOPS, pH 7.0, 2 mM EDTA, and 10% anhydrous ethanol. The concentration of stock DEPC was determined by the addition of a small aliquot into 10 mM imidazole (in 50 mM MOPS, pH 7.0, 2 mM EDTA, and 0.005% sarkosyl), followed by absorbance measurements at 242 nm. Similarly, the stoichiometry of modification was determined by the increase in the absorbance at 242 nm, due to the formation of *N*-carbethoxyhistidine (*15*) using an extinction coefficient of 3200 M^{-1} cm^{-1}. For [^{3}H]DEPC experiments, Lit modification was followed by the molar ratio of protein to incorporated radioactivity. All reactions were initiated by the addition of DEPC and quenched by the addition of excess imidazole when an absorbance maximum at 242 nm was obtained. To relate stoichiometry of modification to the activity of Lit, samples were periodically removed, quenched, and assayed for Lit activity.

Mutagenesis of the Lit Peptidase. All mutations of Lit peptidase were performed using the Stratagene QuickChange method. Overlapping primers were generated containing the desired mutations, and these were used in thermocycling reactions with pIJ2091 (pET11c containing the Lit gene). Generated constructs were sequenced to confirm the presence of the desired mutations and overexpressed, and the proteins were purified as described above.

Gol Concentration Dependence for Activation of Lit and Its Mutants. The cleavage reactions were performed as described above except that the concentration of the Gol peptide was varied between 0.25 and 300 μM. For each concentration of Gol peptide the observed pseudo-first-order rate was determined, and this value was plotted against the concentration of Gol peptide for each Lit mutant and wild-type enzyme. Lit-mediated catalysis of EF-Tu was found to fit well to a minimum mechanism for substrate inhibition (eq 1). Values for the K_M, K_i, and an arbitrary value for k_{cat} were determined from eq 1 (*16*−*18*).

$$v = (k_{cat}[E][Gol])/(K_M + [Gol] + ([Gol]^2/K_i)) \quad (1)$$

RESULTS AND DISCUSSION

Lit Is Likely To Be a Metallopeptidase. The proteolytic activity of Lit has yet to be definitively assigned, although sequence alignments suggest that it may be a metallopeptidase. We have however been unable to demonstrate stoichiometric levels of zinc within the purified protein. We find that zinc is routinely associated with Lit but at substoichiometric levels, between 0.1 and 0.25/mole protein as deduced by atomic absorption spectroscopy (data not shown). This finding is reproducible and may be related to the purification method used for overexpressed Lit, requiring the enzyme to be refolded from inclusion bodies and solubilized using either detergents or urea (*9*; N. Copeland and C. Kleanthous, unpublished results).

In the absence of definitive metal analysis data we investigated the susceptibility of Lit activity to inhibition by 1,10-phenanthroline, a divalent transition metal ion-specific

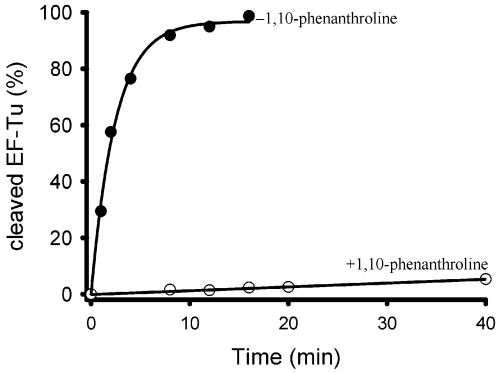

FIGURE 1: 1,10-Phenanthroline inhibits Lit-mediated EF-Tu cleavage. The ability of Lit to cleave EF-Tu was determined under standard cleavage conditions except that the reaction contained 2 mM 1,10-phenanthroline. The data are plotted as the percentage of EF-Tu cleavage versus time. The rate of EF-Tu cleavage in the presence and absence of 1,10-phenanthroline was determined as described in Experimental Procedures. In the presence of 1,10-phenanthroline Lit activity is reduced to ~6% of that of the native control reaction.

chelator that has low affinity for Mg^{2+} ions (which are required for nucleotide binding in EF-Tu; *25*). Lit activity was determined in both the presence and absence of 2 mM 1,10-phenanthroline (Figure 1). In the presence of the metal chelator Lit activity was severely reduced, exhibiting only ~6% of the wild-type activity. This reduced level of activity was also seen where Lit was dialyzed against 1 mM 1,10-phenanthroline prior to analysis in the standard cleavage assay (data not shown). As a further control, we also investigated the effect of other peptidase inhibitors on Lit activity, including inhibitors of serine/threonine (AEBSF, T-PCK), cysteine (leupeptin), and aspartate (pepsinostreptin and pepsinostatin) proteases. None were found to affect Lit activity (data not shown). Taken together, these data suggest that Lit is most likely a zinc metallopeptidase.

DEPC Inactivates Lit. As an initial indicator of what amino acids are required for Lit activity, we investigated the susceptibility of Lit to the histidine-specific reagent DEPC. Figure 2a demonstrates that Lit is sensitive to DEPC modification, with the observed first-order rate for enzyme modification proportional to the concentration of DEPC. This modification was found to be reversible by the addition of hydroxylamine, consistent with histidine-specific modification (data not shown; *19*−*22*). The stoichiometry of histidine modification by DEPC was determined using both spectrophotometric and radioactive methods (see Experimental Procedures) and identified that about three histidines were alkylated during inactivation (Figure 2b). These data were fitted by the statistical method of Tsou (*23*) to determine the number of essential residues modified by DEPC (eq 2). This assumes that the modification of any essential residue leads to a complete loss of activity and that the fraction of unmodified histidines, denoted as A/A_0, will be equal to the fraction of activity remaining. According to this, the number of residues modified per molecule (m) can be determined as follows (*23*, *24*):

$$m = n - p(A/A_0)^{1/i} - (n - p)(A/A_0)^{1/i} \qquad (2)$$

where n is the number of modifiable residues, i is the number of essential residues, and p is the number of nonessential

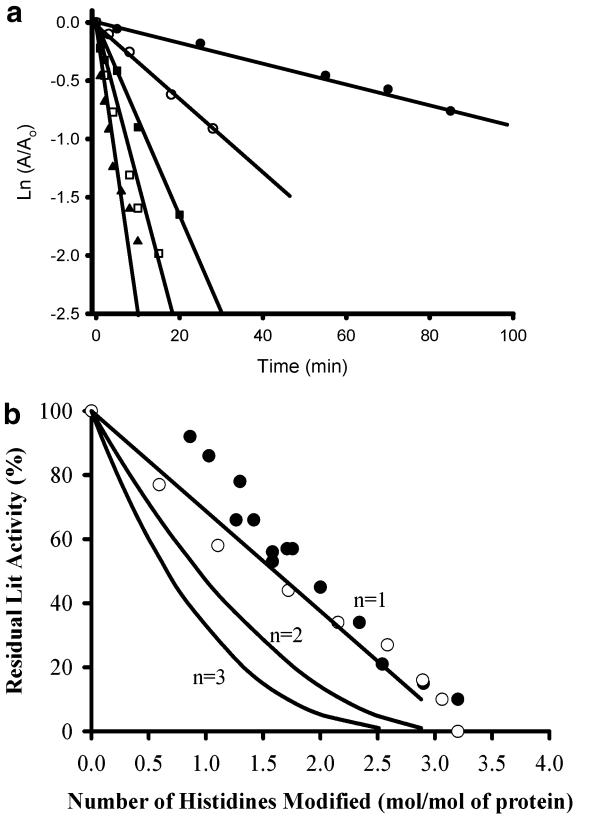

FIGURE 2: Kinetics of Lit inactivation by diethyl pyrocarbonate. (a) Determination of pseudo-first-order rates of Lit inactivation by DEPC. Lit (10 μM) was incubated with various concentrations of DEPC at pH 7.0 and 4 °C. Data were plotted according to the integrated rate equation $-\ln(A/A_0) = k_{obs}t$, and the curves were fitted using least-squares analysis. The concentrations of DEPC used were 0.1 mM (solid circle), 0.2 mM (open circle), 0.5 mM (closed square), 0.75 mM (open square), and 1 mM (closed triangle). (b) Stoichiometry of Lit inactivation by DEPC. 10 μM Lit was incubated with either tritiated (open circles) or nontritiated DEPC (closed circles), and the stoichiometry of labeling was determined by radioactive incorporation and difference spectroscopy, respectively (see Experimental Procedures for details). Residual Lit activity was plotted against the number of histidine residues modified. Tsou analysis (see text) was used to give the number of catalytically essential histidine residues, with $n = 1$ most closely fitting the data. Theoretical fits for $n = 2$ and $n = 3$ are also highlighted.

residues. The theoretical fit for one, two, and three essential residues for the DEPC inactivation by Lit is shown in Figure 2b, with the data most clearly defined by the fit for a single essential histidine residue.

Mutagenesis of Lit Identifies Essential Residues. The Lit sequence contains 10 histidines, two of which reside within the putative metallopeptidase motif. To identify those required for catalytic activity and likely targeted by DEPC modification, all 10 histidines were mutated to alanine. In addition, other residues within the HEXXH motif itself were also investigated. Thirteen alanine mutants of Lit were overexpressed and purified, and their activities were determined relative to the wild-type enzyme (Figure 3b). We identified five residues as being required for Lit activity, three of which are present within the H^{160}EXXH motif: H160, E161, H164, H169, and H232. Far-UV circular dichroism spectroscopy was performed for each of the inactivating mutants and indicated that there were no significant changes in secondary structure (data not shown). Further analysis of

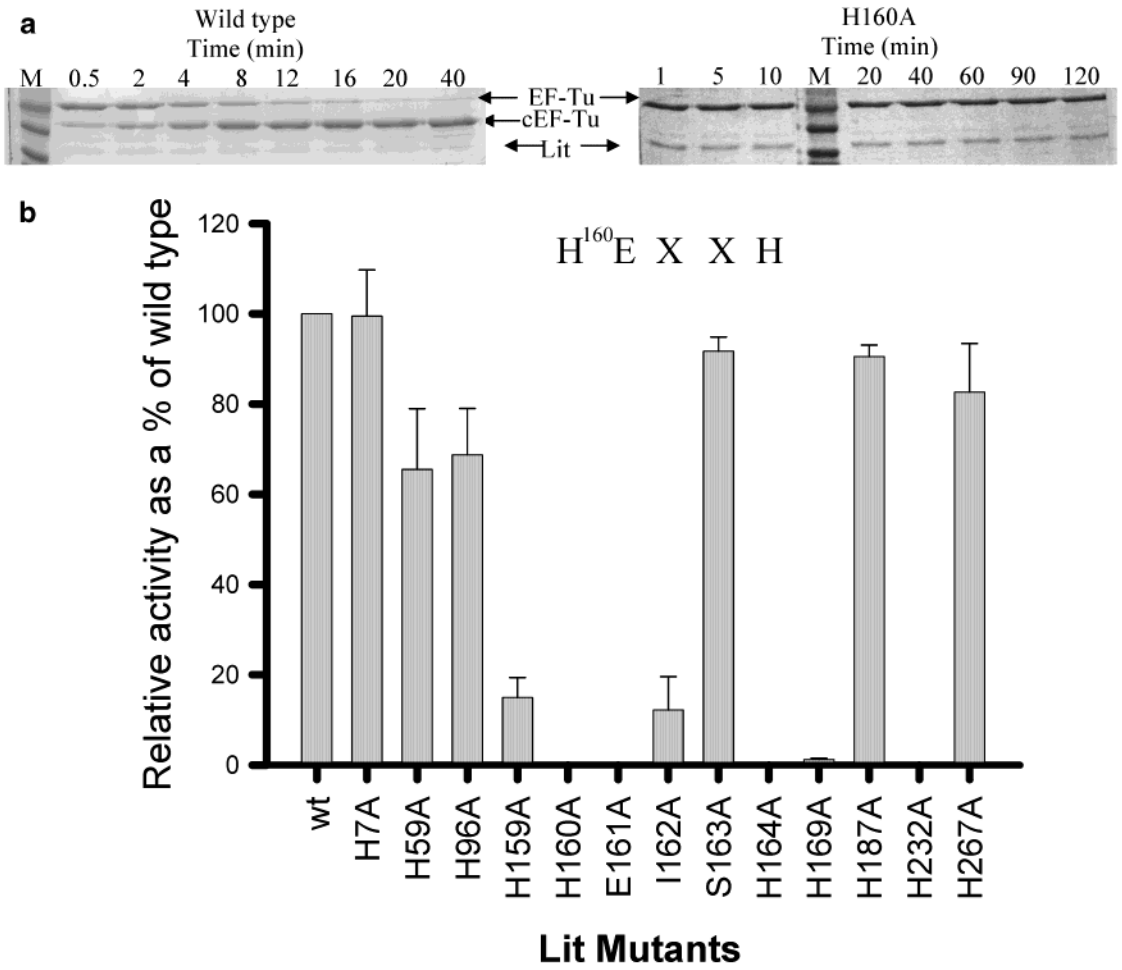

FIGURE 3: Site-directed mutagenesis identifies essential residues within Lit. (a) Comparison of a typical wild-type Lit EF-Tu cleavage assay with an inactive Lit mutant (H160A). For the wild-type enzyme (0.2 μM) the reaction is complete after 40 min (formation of cleaved EF-Tu, cEF-Tu) whereas the inactive mutant has no detectable activity even after extended incubation (120 min) and with increased enzyme (2 μM). (b) Mutagenesis reveals that three histidines and a glutamate are essential for catalysis. The histogram shows the mean pseudo-first-order rate for each Lit mutant with the bars representing the standard deviation ($n = 3$). This shows that alanine mutations at positions H160, E161, H164, and H232 inactivate Lit. H169A is partially active with ~3% of wild-type activity. These mutations are contained for the most part within the HEXXH motif as illustrated in the figure.

the histidine mutants at elevated protein concentrations (2 μM) and assayed over an extended time period indicated that H160A, H164A, and H232A were completely devoid of activity, while H169A showed partial activity (~3%) relative to wild-type Lit (Figure 3a).

Lit Is Inhibited by High Concentrations of the Gol Peptide. Previous studies have demonstrated that a binary complex of EF-Tu and Gol peptide, covalently attached through chemical cross-linking, is a substrate for the Lit enzyme (*8*). Cross-linking failed however to identify any interaction between Gol and the Lit enzyme in this study. The affinity of the noncovalent EF-Tu/Gol complex was detemined by the inhibition of the intrinsic EF-Tu GTPase activity in the presence of Gol, giving a value for K_i ~0.3 mM (*8*). It is unknown how the weak affinity of the EF-Tu/Gol complex relates to the activation kinetics of the Gol-mediated cleavage of EF-Tu in the three-component system. Hence, we investigated the Gol concentration dependence of EF-Tu cleavage (see Experimental Procedures). The resulting plot of pseudo-first-order rate for EF-Tu cleavage as a function of Gol peptide concentration is shown in Figure 4. The data illustrate that between 0.2 and 10 μM there is a hyperbolic increase in activity, but at concentrations >10 μM there is a marked decrease in Lit activity reminiscent of "substrate inhibition". The kinetic constants K_M, K_i, and an arbitrary value for k_{cat} were determined by fitting the data to a substrate inhibition mechanism (eq 1, Experimental Procedures). This yielded values of 1 μM, ~100 μM, and 5.6 min^{-1} for K_M, K_i, and k_{cat}, respectively. We also investigated the activation kinetics of partially active Lit mutants (H159A and I162A) and found that these also exhibited severe inhibition kinetics at concentrations of Gol >10 μM (Figure 4).

CONCLUSIONS

Lit is a novel peptidase that shares little sequence identity with any peptidase identified thus far. It mediates bacteriophage exclusion in *E. coli* K-12 where it performs an immune system like role. Lit recognizes the presence of viral infection in the host cell and mediates a response that ultimately leads to the arrest of protein synthesis and cell death before phage maturation. The work presented here provides the first steps toward understanding how Lit catalyzes the cleavage of the essential translation factor EF-Tu.

Here we have shown that Lit likely requires a metal ion for activity since 1,10-phenanthroline greatly reduces Lit

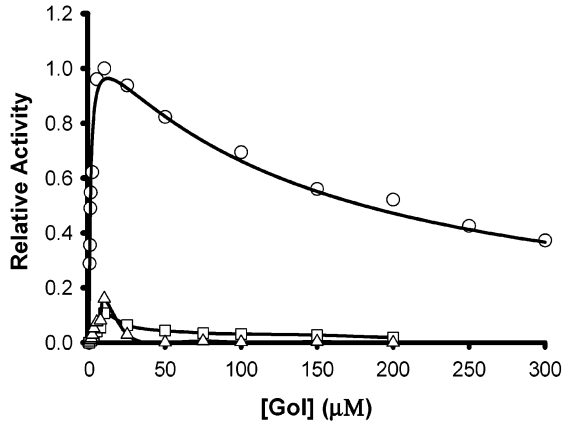

FIGURE 4: GoI peptide concentration dependence of Lit-catalyzed EF-Tu cleavage. Observed first-order rates for EF-Tu cleavage by Lit were determined under standard assay conditions for a range of GoI concentrations (0.25−300 μM). Data for wild-type Lit (circles) were fitted to eq 1 (see Experimental Procedures) by nonlinear least-squares curve fitting, giving the kinetic constants of K_M, K_i, and V_{max} as 1 μM, 100 μM, and 5.6 min^{-1}, respectively. Activation/inhibition data are also shown for the partially active Lit mutants H159A (squares) and I162A (triangles).

activity. Preliminary work using DEPC modification identified that Lit possesses several reactive histidine residues. Statistical analysis revealed that one histidine appears essential for catalytic activity and which pH measurements show has a pK_a of 6.1 (data not shown), consistent with the imidazole side chain of histidine. Subsequently, through alanine mutagenesis, four essential residues were identified. Three are histidines (His160, His164, and His232) that likely make up a metal-ligating center, and the fourth is Glu161 that, by analogy to other metallopeptidases, is likely involved in the coordination of the hydrolytic water molecule. Our data suggest therefore that the metallopeptidase motif of Lit is more accurately depicted as $H^{160}EXXHX_{67}H^{232}$.

Lit activity is dependent upon the presence of the GoI peptide sequence, which mimics in vitro the viral coat protein gp23 in vivo. The role of GoI in Lit activation is not well understood although previous work has shown that the peptide can form a binary complex with EF-Tu and that this complex is the substrate for Lit (*8*). EF-Tu is thought to have chaperone-like properties. This led Bingham et al. (*8*) to speculate that the interaction between GoI and the translation factor is involved in the maturation of the phage capsid. Here we establish the kinetic parameters for GoI peptide activation. The apparent K_M for GoI activation of wild-type Lit activity (in the three-component system) is 1 μM, which is over 2 orders of magnitude lower than the apparent binding constant for the GoI/EF-Tu binary complex, implying that the peptide is making additional contacts in the ternary GoI/EF-Tu/Lit complex. If GoI does form contacts with Lit, then this might also explain why at higher concentrations it is inhibitory. As with the classic interpretation of substrate inhibition, the peptide may be only partially occupying its binding site on the enzyme, thereby blocking access of the EF-Tu-bound GoI peptide. The precise role of GoI in the cleavage of EF-Tu by the Lit enzyme is unknown. GoI could be providing active site groups to complete the catalytic machinery of the enzyme, acting as an allosteric activator or simply acting as a platform for specific substrate recognition. Differentiating between these possibilities is the subject of ongoing work.

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