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Studying a Cell Division Amidase Using Defined Peptidoglycan Substrates

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The bacterial cell wall (murein sacculus) is an essential cellular component that maintains cell shape and protects against lysis due to high internal pressure. It is composed of cross-linked strands of peptidoglycan (PG) that form a meshlike structure around the inner membrane (Figure 1a).[1] Although it provides stability, the cell wall is a dynamic structure; it is continuously modified by a number of synthetic and degradative enzymes that are finely tuned to allow the sacculus to grow and divide without lysing. The action of degradative enzymes known as murein hydrolases is especially critical during the late stages of cell division. In *Escherichia coli*, three periplasmic *N*-acetylmuramoyl-L-alanine amidases, AmiA, AmiB, and AmiC, have been shown to be particularly important for hydrolysis of septal PG, which enables cell separation.[2] The amidases cleave the amide bond between the lactyl group of muramic acid and the amino group of L-alanine to release a peptide moiety (Figure 1b). Cell division amidases remain largely uncharacterized because substrates suitable for studying them have not been available.[3] Existing assays for monitoring amidase activity are mainly based on in-gel degradation of isolated murein sacculi, which are heterogeneous, insoluble, and contain peptide chains of varying length, composition, and degree of cross-linking.[1a,b] This variability complicates any attempts to analyze amidase substrate preferences and possible modes of activation.

We have developed methods to synthesize the PG precursors Lipid II (**1**)[4a,b] and Lipid IV (**2**)[4c] and established enzymatic methods to convert these substrates to longer glycan strands (nPG, **3**) that vary only in chain length.[4c−e] Here, we use these defined PG substrates to characterize the *E. coli* cell division amidase AmiA.[5] The approach described, which is applicable to many other amidases and cell wall hydrolases, enables a more detailed understanding of the activity and regulation of this important class of enzymes.

AmiA is a member of the zinc-dependent amidase 3 family, of which one member, *Bacillus polymyxa* var. *colistinus* CwlV, has been crystallized (PDB entry 1JWQ), allowing us to predict the active-site residues involved in metal binding (Figure 2a).[6] We overexpressed and purified C-terminally His-tagged AmiA[7] and incubated it with nPG **3** containing a radiolabel on the pentapeptide in the presence and absence of zinc. Reactions were analyzed using paper chromatography.[8] Unreacted nPG remained at the baseline, cleaved peptides migrated, and conversion was quantified by scintillation counting. The peptide product was verified using an authentic standard. Control experiments using lysozyme confirmed that **3** could be cleaved by a PG hydrolase and that the reaction could be analyzed by paper chromatography (Figure S1 in the Supporting Information). Without added zinc, AmiA exhibited no initial activity; in the presence of Zn^{2+}, the radiolabeled peptide was cleaved from **3** in a concentration-dependent manner (Figure 2a, right, wt rates). Adding zinc chelators such as EDTA and 1,10-phenanthroline prevented hydrolysis, but the reaction could be rescued by supplying an excess of zinc (Figure S2).

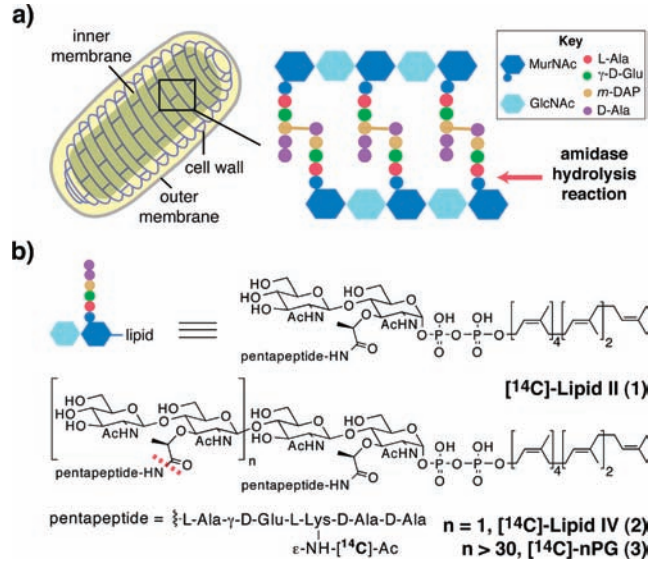

Figure 1. *E. coli* amidases cleave peptide cross-links from peptidoglycan (PG). (a) Schematic representation of an *E. coli* cell. Amidase activity is required for cell separation to occur during division. (b) Chemical structures of synthetic labeled PG fragments Lipid II (**1**) and Lipid IV (**2**) and non-cross-linked (nascent) PG (**3**).[1c]

We also examined the initial rates of several mutants made on the basis of comparisons of amidase family 3 catalytic domains. E242 is predicted to act as a general base catalyst, and H65, E80, and H133 are proposed to coordinate zinc to form the active site (Figure 2a). Mutation of any of these residues results in a full loss of activity, except for E80A, which suffered a 100-fold decrease in rate compared to the wild-type (wt) (Figure S3). Altering residues D69 and D109, which are also highly conserved but are not predicted to be essential for activity, produced more modest decreases in reaction rate (~10-fold less than the wt). We have concluded that AmiA is a zinc-dependent metalloprotease with an active-site configuration similar to that of CwlV.

The substrate requirements of CwlV, AmiA, and related autolytic amidases have not been determined: to date, the murein sacculus has been the only reported substrate for these enzymes. In order to assess the substrate requirements of AmiA, we tested defined PG fragments of different glycan chain lengths that represent various stages of PG synthesis in the periplasm (Figure 2b). The substrates

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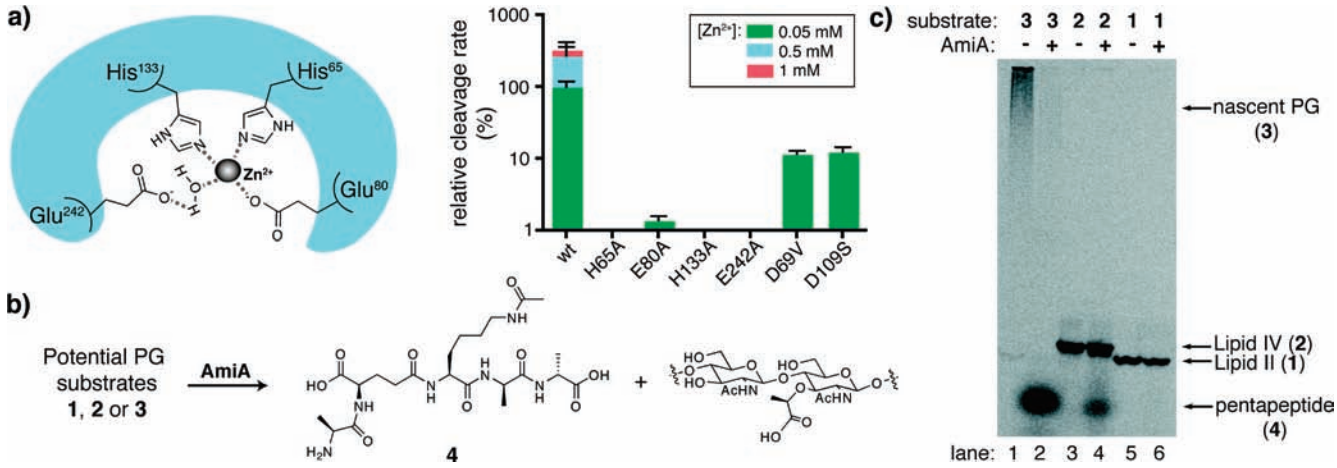

Figure 2. Analysis of AmiA substrate preferences and catalytic features. (a) (left) Predicted zinc-binding site of AmiA based on alignment with *B. polymyxa* var. *colistinus* CwlV; (right) comparison of relative cleavage rates of **3** (7.2 μM) by mutants of AmiA (4.0 μM). (b) Reaction scheme for AmiA cleavage to produce [^{14}C]-pentapeptide **4** from potential PG substrates that differ in length. (c) Gel electrophoresis of **3** (lanes 1 and 2), **2** (lanes 3 and 4), and **1** (lanes 5 and 6) without and with AmiA addition, respectively, under similar reaction conditions. AmiA cleaves **3** and **2** (but not **1**) to produce a new band that represents **4**.

were incubated with purified enzyme under similar conditions, and the reactions were analyzed by gel electrophoresis (Figure 2c).[9] AmiA cleaves substrates **2** and **3** to produce a common low-molecular-weight band, which was identified as the released pentapeptide **4** by correlation to an authentic standard using HPLC and electrophoretic mobility (Figure S4). AmiA does not cleave the peptide from **1** (compare lanes 2 and 4 with lane 6), and a maximum of 50% of the radiolabeled peptide can be cleaved from **2** (data not shown). These results show that Lipid II (**1**) is not a substrate and suggest that AmiA contains an extended binding pocket that recognizes sugars on either side of the glycopeptide substrate. Consistent with this idea, disaccharide−peptide fragments obtained by treating **3** with lytic transglycosylases also were not cleaved by AmiA (Figure S5). Hence, our results show that AmiA requires at least a tetrasaccharide as a substrate.

The use of compositionally well-defined PG polymer substrates has allowed us to characterize a cell wall amidase, *E. coli* AmiA, involved in PG degradation during division. The turnover number for cleavage of the polymer is 0.05 min^{-1} (Figure S3). Since cleavage rates from sacculi by similar purified enzymes have not been reported, there are no in vitro data for comparison.[3d] Although this rate is lower than estimates required to support bacterial growth,[10] low rates are typically observed in vitro for the enzymes that synthesize glycan strands.[11] A possible explanation is that cell wall synthetic and degradative enzymes are proposed to operate as components of multiprotein machines with tightly coordinated activities. Higher in vitro rates may be observed when other important components of the system are reconstituted. Some protein candidates for amidase regulation have been suggested, but it is unclear whether they simply recruit amidases to the appropriate cellular location or influence activity through direct interaction with the amidase or its substrate.[1a,b,12] Access to homogeneous substrates rather than crude cell wall fractions provides the capability to evaluate amidase kinetics in response to prospective activators and could, in turn, help illuminate the nature of these complex interactions.

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Supporting Information Available: Experimental procedures, including synthesis of peptide standard, cloning and purification of proteins, and rate and gel electrophoresis analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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