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FOR THE RECORD

Identification of a common protease-sensitive region in D-alanyl-D-alanine and D-alanyl-D-lactate ligases and photoaffinity labeling with 8-azido ATP

GERARD D. WRIGHT AND CHRISTOPHER T. WALSH

Department of Biological Chemistry and Molecular Biology, Harvard Medical School, Boston, Massachusetts 02115

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Steps involved in the extracellular events in bacterial peptidoglycan synthesis provide killing sites for a number of clinically useful antibiotics such as the β-lactams (e.g., penicillin) and the glycopeptides (e.g., vancomycin) (Gale et al., 1981). The intracellular steps required for peptidoglycan biosynthesis are also targets for antibacterial agents. For instance, compounds such as D-cycloserine specifically target enzymes required for D-Ala biosynthesis (Gale et al., 1981; Walsh, 1989), a critical amino acid component of bacterial cell walls.

The D-alanine pathway includes the enzymes alanine racemase, D-Ala-D-Ala ligase, and the D-Ala-D-Ala adding enzyme (Walsh, 1989). D-Ala-D-Ala ligase catalyzes ATP-dependent amide bond formation between two D-Ala molecules (Neuhaus, 1962; Wright & Walsh, 1992). Two chromosomal D-Ala-D-Ala ligases, DdlA and DdlB, have been cloned, overproduced, and purified from *Escherichia coli* (Robinson et al., 1986; Zawadzke et al., 1991). In addition, a *Salmonella typhimurium* DdlA homologue has also been cloned and overproduced and is greater than 90% homologous to the *E. coli* enzyme (Daub et al., 1988; Knox et al., 1989). Ligase DdlA has a molecular mass of 39,000 and shows 35% primary sequence homology to the smaller (32,000-Da) DdlB, yet they both catalyze D-Ala-D-Ala formation with similar efficiencies (e.g., D-Ala_2 $k_{cat}/K_m \approx 8.2 \times 10^5$ M^{-1} min^{-1}). Recently, we have shown that high-level resistance to vancomycin in *Enterococcus faecium* requires an enzyme with D-Ala-D-Ala ligase activity, VanA, which shows 28–36% identity to Ddl enzymes, but preferentially catalyzes the formation of depsipeptides of the form D-Ala-O-D-X (K_m for X = D-lactate is 7.1 mM) over D-Ala-D-Ala (K_m for D-Ala_2 ≈ 40 mM) (Dutka-Malen et al., 1990; Bugg et al., 1991a,b). A predicted D-Ala-D-lactate terminus, resistant to vancomycin recognition, has been detected in these glycopeptide-resistant bacteria (Handwerger et al., 1992; Messer & Reynolds, 1992). Given the similarities in primary structure and the alteration in substrate specificity among Ddl and VanA ligases, we have sought to identify regions of the enzymes that are important to function. In this report we provide evidence to show that all three ligases have a proteolytically sensitive region approx. −10 kDa from the C-terminus, separating the enzymes into two distinct domains, and that the ATP-binding region, as determined by photoaffinity labeling with 8-azido ATP, is localized to the N-terminal domain.

Results and discussion

Limited proteolysis reveals a common hypersensitive region in D-Ala-D-Ala ligases and the related VanA ligase

Incubation of DdlB (molecular mass of 32 kDa) with proteases released a ~23-kDa fragment (Fig. 1). Chymotrypsin (CT), trypsin, and the nonspecific protease pronase all gave qualitatively similar results. Longer incubations with trypsin also gave a 22-kDa fragment derived from the 23-kDa peptide (not shown). The 23-kDa fragment ($DdlB_{CT23}$) was relatively stable to extended incubation with CT ($t_{1/2} \approx 60$ min). No complementary fragment of 9–10 kDa was generally observed, indicating that it is proteolytically labile. Similarly, DdlA (39 kDa) also released an ~10-kDa proteolytically labile peptide upon incubation with CT, but it accumulated transiently and could be detected (Fig. 1). Treatment of VanA (37 kDa) with CT likewise revealed two fragments, one at ~30 kDa and one at ~10 kDa, both with an estimated $t_{1/2}$ of 45 min.

Reprint requests to Gerard D. Wright at his present address: Department of Biochemistry, McMaster University, Hamilton, Ontario L8N 3Z5, Canada.

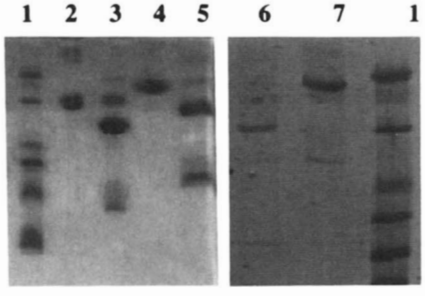

Fig. 1. Chymotryptic cleavage of D-Ala-D-Ala ligases. Ligases were treated with CT (1:100, w/w) for 15 min, quenched with phenylmethylsulfonylfluoride, and run on a 20% SDS-polyacrylamide gel. Lane 1, molecular weight markers (masses: 97.4, 67.8, 43, 29, 18.4, 14.3 kDa); lane 2, DdlB; lane 3, DdlB + CT; lane 4, VanA; lane 5, VanA + CT; lane 6, DdlA + CT; lane 7, DdlA.

N-terminal sequencing of the large fragments of all three proteins demonstrated that these were derived from their respective N-termini of the enzyme, all fragments having lost Met 1 (Table 1). N-terminal sequencing of $VanA_{CT10}$ revealed a peptide of C-terminal origin with a predicted molecular weight of 11.2 kDa cut at the CT cleavage site: F240–R241 (Table 1), consistent with our estimate of the size of the fragment determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Loss of a C-terminal 10-kDa fragment from all three ligases suggests a common structural motif. In the absence of N-terminal sequence analysis on the 10-kDa fragments of DdlB and DdlA, we sought to determine the exact size of the N-terminal fragments and thereby the region susceptible to proteolysis in the primary sequence. Chymotryptic peptides from all three ligases were purified by high-performance liquid chromatography (HPLC) and analyzed by mass spectrometry (Chait & Kent, 1992). Matrix-assisted laser desorption mass spectrometry (MALDMS) gave molecular weights accurate to within approx. 0.1% and was performed successfully with DdlA and DdlB, but no data could be obtained from the VanA fragments using a variety of matrices. Ligase DdlA gave peptides corresponding to full-length enzyme (expected molecular mass 39,362; found 39,399), as well as two fragments at 27,361 and 12,115 (Table 1). Inspection of an overlap of the primary sequences of the three ligases (see supplementary material in the Diskette Appendix) reveals a potential chymotryptic site in DdlA, Y256, in the immediate vicinity of F240 of VanA. A DdlA fragment from A2 to Y256 has a predicted molecular weight of 27,422, within 0.2% of the observed 27,361, suggesting it is a reasonable candidate for the site of CT cleavage. Similarly, DdlB shows a fragment with a molecular mass of 22,473 by MALDMS (Table 1). This sample was further analyzed by electrospray-ionization mass spectrometry from which an accurate mass of 22,193.1 was determined. Inspection of potential DdlB chymotryptic fragments indicates that the region consisting of amino acids T2 through Y210 has a predicted mass of 22,193.9. Tyr 210 of DdlB is in the same region (predicted by overlap) as Y256 of DdlA and F240 of VanA, suggesting that all three ligases share a common protease-accessible region (Fig. 2). This region does not contain a significant degree of primary sequence homology, therefore it is likely the tertiary structure that is conserved.

Table 1. *Chymotryptic D-Ala-D-Ala and D-Ala-D-lactate ligase fragments*

Ligase	Major MALDMS fragments	HPLC-isolated fragments	Size[a] (kDa)	N-terminal sequence
DdlB	22,473, 12,947, 9,558	$DdlB_{CT23}$	23	T2DKIAVLLGG
DdlA	39,399, 27,361, 12,115	$DdlA_{CT30}$	30	A2KLRVGIVFG
VanA	No fragments were observed	$VanA_{CT30}$	30	N2RIKVAILF[b]
		$VanA_{CT10}$	10	R241IHQEVEPEK

[a] Determined by electrophoresis on 20% SDS-polyacrylamide gels.
[b] Some minor contaminating partial sequences were noted upon Edman degradation of $VanA_{CT30}$ that are not derived from VanA.

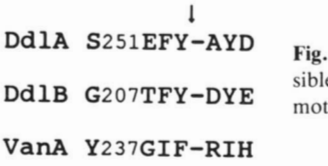

Fig. 2. Predicted ligase protease-accessible loop region. Arrow indicates chymotrypsin cleavage site.

Proteolysis was associated with loss of ligase activity in all three enzymes, demonstrating that structural integrity is required for catalysis. Preincubation with saturating amounts of ATP, ADP, D-Ala, D-Ala-D-Ala, D-cycloserine, or (1-aminoethyl)(2-carboxy-2-methyl-1-ethyl)phosphinic acid (Fig. 3), an ATP-dependent tight binding amino alkylphosphinate mimic of a D-Ala-D-Ala tetrahedral intermediate, failed to provide significant protection against proteolysis in DdlA, DdlB, and VanA. Addition of ATP and D-Ala in tandem also showed no protection; however, addition of ATP and the amino alkylphosphinate inhibitor *together* conferred complete protection to DdlA, DdlB, and VanA (not shown) against chymotrypsinolysis. Previous experiments have shown that (1-aminoethyl)(2-carboxy-2-methyl-1-ethyl)phosphinic acid is a time- and ATP-dependent, tight-binding inhibitor of ligases (McDermott et al., 1990; Bugg et al., 1991a) with a half-life for the regain of activity of 17 days for DdlA (McDermott et al., 1990). Solid-state ^{31}P-NMR analysis on the ATP·phosphinate·DdlA complex indicated that the long-

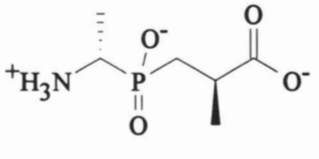

Fig. 3. Structure of (1-aminoethyl)(2-carboxy-2-methyl-1-ethyl)phosphinic acid.

lived structure was a phosphoryl-phosphinate adduct, which is highly homologous to the proposed phosphorylated tetrahedral adduct for D-Ala-D-Ala formation (McDermott et al., 1990). If we consider the phosphoryl-phosphinate adduct to be a close mimic of the tetrahedral intermediate, then the ATP-dependent protection against proteolysis conferred by the phosphinate provides evidence for segmental motion during catalysis. We propose that the protection is due to "freezing" of an ES (or in this case E*I, the enzyme-derived phosphoryl-phosphinate adduct) closed enzyme conformation (loop is inaccessible). This suggests that D-Ala-D-Ala and D-Ala-D-lactate ligases each require a similar conformational change to effect catalysis, presumably to properly position important catalytic residues. Such "induced-fit" types of mechanisms, from open to closed structures, have been noted in kinases such as hexokinase (Benet & Steitz, 1978) and phosphoglycerate kinase (Pickover et al., 1979), possibly to ensure efficient transfer of the γ-phosphate group to cosubstrate rather than solvent. In terms of ATP cleavage to ADP, D-Ala-D-Ala, and D-Ala-D-lactate ligase involves a kinase reaction with formation of an enzyme-bound acyl-phosphate intermediate, so that if the closed conformation is along the normal catalytic pathway, this movement could likewise provide protection of the hydrolytically labile, thermodynamically activated acyl-phosphate from premature hydrolysis and ensure its capture by the cosubstrate (D-Ala or D-lactate).

8-Azido-ATP inactivates and covalently labels DdlB and VanA

All three ligases bind the common substrates D-Ala and ATP. Given that two D-Ala sites are predicted per ligase, a high-affinity N-terminal site and a low-affinity C-terminal site, for which the K_m for D-Ala varies roughly 100-fold between sites and 40-fold between ligases (Bugg et al., 1991a), we chose first to attempt to delineate the location of the ATP-binding region, which we suspected to be common to all three ligases because the K_m for ATP varies only fourfold (Bugg et al., 1991a).

8-Azido-NTPs have been used to map the nucleotide-binding regions of a number of proteins (Haley & Hoffman, 1974; Potter & Haley, 1983; Lewis et al., 1989; Rush & Konigsberg, 1990; Haley, 1991; Knoll et al., 1992). 8-Azido-ATP (Z-ATP) is a D-Ala-dependent substrate for DdlB with a K_m of 1.2 ± 0.3 mM, some 30-fold higher than ATP. We were unable to determine an accurate K_m for Z-ATP and VanA due to high background levels using the molybdate/malachite green Pi release assay (Lanzetta et al., 1979), although Z-ATP was accepted as a low-affinity substrate. Neither enzyme was significantly inhibited by Z-ATP at concentrations of 0.5 mM or lower. Nonetheless, Z-ATP inactivated both DdlB and VanA at μM concentrations *only after photolysis*. Inactivation was substantially prevented by the addition of ATP, suggesting labeling was occurring at the ATP-binding site. We therefore prepared [γ^{32}P]Z-ATP and demonstrated that this reagent covalently modified both DdlB (concentration for half-maximal incorporation of ^{32}Pi = 11 ± 4 μM) and VanA (concentration for half-maximal incorporation of ^{32}Pi = 44 ± 27 μM), and that unlabeled ATP protected against inactivation and ^{32}P incorporation into the enzyme. Incorporation of ^{32}P was maximal with an irradiation time of 5 min at a concentration of approx. 40 μM [γ-^{32}P]Z-ATP for DdlB and 100 μM for VanA and was decreased by the presence of ATP (Fig. 4). We determined stoichiometry of covalent ^{32}P incorporation by photolysis followed by extensive dialysis against HEPES buffer; under these conditions VanA

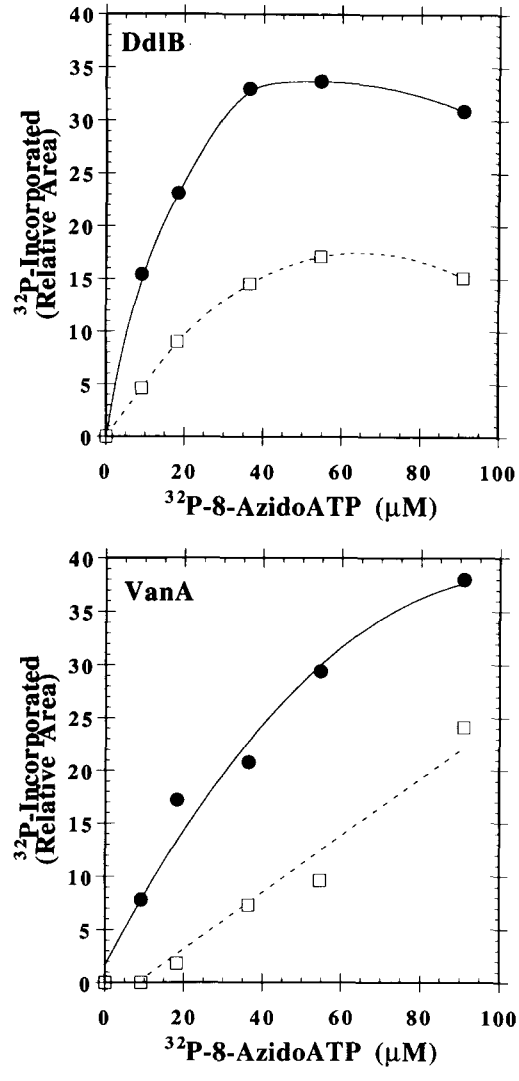

Fig. 4. Concentration dependence of [γ^{32}P]-Z-ATP labeling of DdlB and VanA. [γ^{32}P]-Z-ATP was added to purified ligases in a solution of 10 mM $MgCl_2$, 10 mM KCl, 50 mM HEPES, pH 7.8, to a final volume of 10 μL. Samples were irradiated at 0 °C for 5 min at a distance of 10 cm with a Mineralight UVGL-25 model lamp on the UV 254 setting operating at a flux of 1.9 mW/cm^2. Samples were run on 20% SDS-polyacrylamide gels and subjected to autoradiography. Label incorporation was quantitated by integration of a densitometric scan of the autoradiogram. Closed circles, ligase + [γ^{32}P]-Z-ATP; open squares, ligase + [γ^{32}P]-Z-ATP + 0.9 mM ATP.

showed 7.1 ± 2.7% label incorporation while DdlB showed 1.5 ± 0.5% incorporation. Inhibition and loss of activity was not reversible and greater than 50% for both enzymes. Therefore, the low stoichiometry, as assessed by ^{32}P labeling, probably reflects the instability of the γ-phosphate group or phosphoribosyl moiety on the enzyme–[γ-^{32}P]Z-ATP complex after irradiation, and this lability causes problems for localization of ^{32}P-labeled peptides. Despite several attempts to purify and N-terminal sequence ^{32}P-labeled tryptic peptides of [γ-^{32}P]Z-ATP-labeled DdlB and VanA (using >200 nmol quantities of enzymes), no unambiguously labeled amino acids were detected. This instability of ^{32}P label has been noted in other work with Z-ATP (Haley, 1991). The nature of the labile adduct(s) has not yet been determined. Model studies on the photolysis of arylazides have indicated that highly electrophilic ring expansion products predominate after photolysis (Liang & Schuster, 1987; Li et al., 1988), which, although highly reactive in themselves, may not yield stable covalent complexes with nearby amino acid residues suitable for identification by peptide mapping and sequencing.

The N-terminal domain is labeled by [γ-^{32}P]Z-ATP

Photolabeling of DdlB and VanA with [γ-^{32}P]Z-ATP followed by limited proteolysis with CT and resolution of the two fragments by SDS-PAGE revealed that only $DdlB_{CT23}$ and $VanA_{CT30}$ were covalently labeled (Fig. 5). Identical results were obtained by first treatment with CT followed by labeling of the two fragments with [γ-^{32}P]Z-ATP. These results are consistent with localization of the ATP-binding region to the N-terminal ligase domain and, because labeling also occurred with CT-cleaved ligases, the ATP-binding region is probably contiguous and remains intact within this domain.

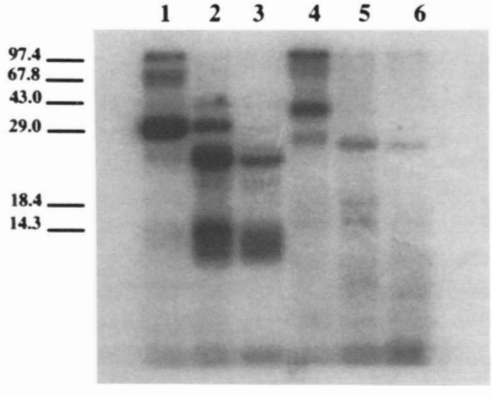

Fig. 5. Photolabeling of DdlB and VanA and chymotryptic fragments by [γ^{32}P]-Z-ATP. Photolabeling was performed as described in the legend to Figure 4. The [γ^{32}P]-Z-ATP was added to a final concentration of 96 μM and the reaction mixes run on a 20% SDS-polyacrylamide gel. Positions of the molecular weight markers are indicated beside the autoradiogram. Lane 1, DdlB + [γ^{32}P]-Z-ATP; lane 2, DdlB + [γ^{32}P]-Z-ATP followed by the addition of CT; lane 3, DdlB + CT followed by the addition of [γ^{32}P]-Z-ATP and irradiation; lane 4, VanA + [γ^{32}P]-Z-ATP; lane 5, VanA + [γ^{32}P]-Z-ATP followed by the addition of CT; lane 6, VanA + CT followed by the addition of [γ^{32}P]-Z-ATP and irradiation.

Two potential ATP-binding regions in ligase have been noted based on primary sequence analysis (Dutka-Malen et al., 1990; Zawadzke et al., 1991). One site, G179XP XXVKP (using numbering indicated in the sequence alignment on the Diskette Appendix) located in region III, shows marked similarity to the proposed ATP-binding domain of carbamoyl phosphate synthase (Post et al., 1990; Zawadzke et al., 1991). Another site in region IV, G294CXGX(AG)R, shows similarity to a consensus sequence for the putative ATP-binding regions of a series of kinases and other ATP-binding proteins, GXXXX(AG)(KR) (Frye et al., 1986; Dutka-Malen et al., 1990). Whereas these ATP-binding motifs are implicated in triphosphate binding and not expected to interact with position 8 of the adenine base, the fact that $DdlB_{CT23}$ and $VanA_{CT30}$ are efficiently labeled with Z-ATP suggests that the nucleotide-binding region is contiguous within the N-terminal domain. Therefore, this argues against the G294–R300 sequence in region IV from consideration as the ATP-binding region and supports identification of the G179–P186 in region III as the nucleotide-binding site, a prediction that can be approached by mutagenesis.

These results provide the first structural information for this class of antibiotic target proteins. The fact that D-Ala-D-Ala and the vancomycin resistance *vanA*-encoded D-Ala-D-lactate ligases show significant primary sequence homology, and a common domain structure predicts that an experimentally determined three-dimensional structure of one ligase by X-ray analysis will permit modeling of the structure of other ligase proteins.

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