Dipeptide Binding to the Extended Active Site of the *Streptomyces* R61 D-Alanyl-D-alanine-peptidase: The Path to a Specific Substrate†

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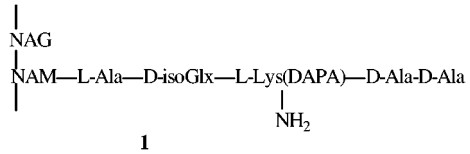
ABSTRACT: Bacterial cell walls are cross-linked in the final step of biosynthesis by specific D-alanyl-D-alanine(DD)-peptidases/transpeptidases. The natural substrates of these enzymes should therefore be segments of peptidoglycan, but high specificity for such structures has yet to be demonstrated. The binding of dipeptides to the extended substrate binding site of the *Streptomyces* R61 DD-peptidase has been studied by means of a fluorescent β-lactam probe. It was found that dipeptides of structure Gly-L-Xaa have affinity for a subsite adjacent to the β-lactam binding site. Hydrophobic peptides such as Gly-L-Met and Gly-L-aminocaprylic acid had the greatest affinity for this site, with dissociation constants in each case of 0.19 mM. A combination of this motif with the C-terminal D-alanyl-D-alanine moiety required of a DD-peptidase substrate yielded a new substrate, glycyl-L-α-amino-ϵ-pimelyl-D-alanyl-D-alanine. Steady-state kinetic measurements established this compound as the most specific peptide substrate yet discovered for a DD-peptidase by at least 3 orders of magnitude ($k_{cat} = 69\ s^{-1}$, $K_m = 7.9\ \mu M$, $k_{cat}/K_m = 8.7 \times 10^6\ s^{-1}\ M^{-1}$); acylation was rate-determining at saturation. This substrate, presumably not coincidentally, contains the acyl donor and acceptor moieties, appropriately separated, of the *Streptomyces* peptidoglycan structure. This general method of approach should be of value in the search for specific substrates and inhibitors (antibiotics) of other DD-peptidases.

The metabolism of bacterial cell walls has long provided other organisms, including, relatively recently, human chemists, with attractive targets for antibiotics (*1*, *2*). Probably the best known and most exploited of these targets are variants of the final enzymes in the murein biosynthetic pathway, the D-alanyl-D-alanine-transpeptidases or DD-peptidases that catalyze the final step of incorporation of the cell wall monomer unit into the polymer. It is these enzymes that are susceptible to covalent inhibition by β-lactams and are thus also known as penicillin-binding proteins. Each bacterium typically has several of this class of enzyme for optimal growth and maintenance of its cell wall (*3*). Much structural information is now available for these enzymes (*4*−*6*), but functional studies, rather counterintuitively, have not kept pace. It is known, of course, that the DD-peptidases catalyze the hydrolysis and aminolysis of certain small peptides and depsipeptides (*7*, *8*), but detailed kinetic studies of the turnover of natural substrates, or close analogues of these, have not, in general, yet been possible. The natural substrates of these enzymes are presumed to be the specific stem peptides of the cell wall monomer unit, of typical structure **1**, already incorporated into the cell wall by transglycosylation, i.e., the substrates are polymers. To date, the substrate specificity and turnover expected in the natural system to maintain bacterial growth rates have not been demonstrated in vitro, either in homogeneous solution assays or in membraneous or membrane-mimetic media (*9*−*11*).



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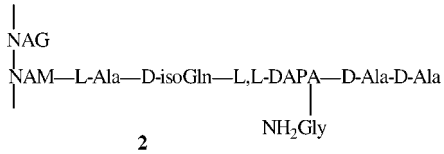
In Gram negative bacteria, the monomer unit is generally as shown in **1**, where the cross-linking acyl acceptor is the free amine of 2,6-diaminopimelic acid (DAPA) and the donor the penultimate D-Ala acyl group from an adjacent peptidoglycan strand. In Gram positive bacteria, however, the free amine of the acyl-acceptor is often extended by addition of a further amino acid or of amino acids (*12*). For example, the pentaglycine extension in *Staphylococcus aureus* is well-known. In a number of *Streptomyces* species, the extension is by a single glycine residue (*13*), **2**.



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Such extensions may contribute as secondary specificity elements in the binding of substrates to the relevant DD-peptidases, where the primary specificity might be expected to lie with the stem pentapeptide itself. One would expect that a DD-peptidase would have an extended substrate binding region, with specific subsites for several of the component amino acid side chains, both on the acyl donor side [S sites, according to the convention of Schechter and Berger (*14*)]

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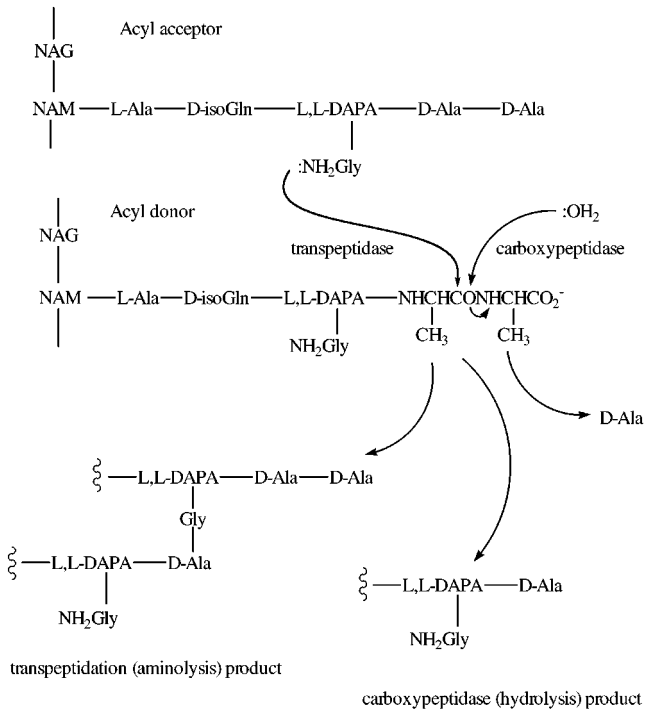
Scheme 1



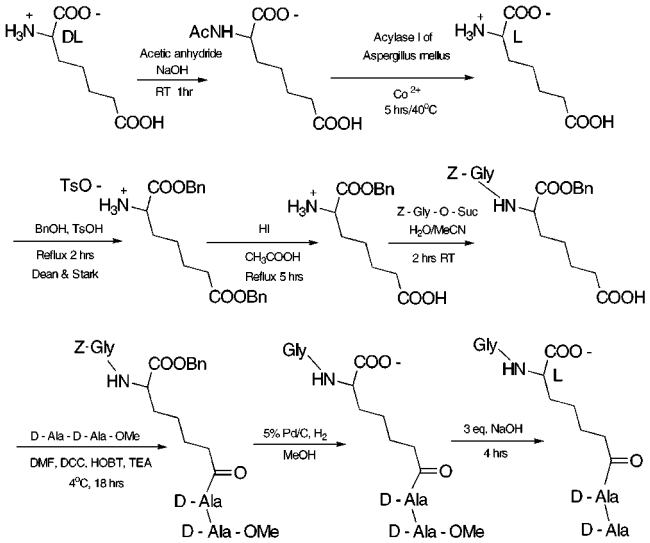
and acyl acceptor side (S′ sites). The transpeptidation and carboxypeptidase reactions (the latter are also catalyzed by these enzymes) in *Streptomyces* species would therefore be as shown in Scheme 1. The acyl donor and acyl acceptor peptides are labeled in this scheme. The presence of specific subsites, contributing to substrate specificity, in classical proteases is well-established (*15*) and much exploited in inhibitor design. The contribution to substrate specificity anticipated from the secondary structural heterogeneity has not yet been clearly and generally demonstrated in DD-peptidases, although the strong preference of these enzymes for the C-terminal and invariant D-Ala-D-Ala has been observed (*7*, *9*). Small, nonspecific D-Ala-D-Ala terminating peptides are not, however, good substrates ($k_{cat}/K_m \leq 3000$ s^{-1} M^{-1}).

This paper describes how we have explored and exploited the secondary specificity of a particular bacterial DD-peptidase, the soluble enzyme of *Streptomyces* R61. First, we investigated the specificity of small peptide binding, probably at a particular subsite of the donor peptide. On finding significant binding strength, we combined this fragment with the primary D-Ala-D-Ala element to obtain the most specific (k_{cat}/K_m) peptide substrate for a DD-peptidase yet described.

EXPERIMENTAL PROCEDURES

Materials. The DD-peptidase of *Streptomyces* R61 was generously supplied by Dr. J.-M. Frére of the University of Liège (Liège, Belgium). Dansylpenicillin (**3**), dansylcephalexin (**4**), dansylcephalothin (**5**), and *m*-[[(phenylacetyl)-D-alanyl]oxy] benzoic acid (**6**) were prepared previously in this laboratory (*16*−*18*). The following ligands were also available from previous studies (*19*): *N*-(phenylacetyl)glycyl-D-phenylalanine, *N*-(phenylacetyl)glycine, *N*-(phenylacetyl)-glycyl-D-alanine, disodium penicilloate, *N*-acetyl-D-alanyl-D-alanine, and *N*-benzyloxycarbonyl-D-alanyl-D-alanine.

Scheme 2: Synthesis of **7**



Glycyl-D-phenylalanine was purchased from ICN Biomedical and meso-diaminopimelic acid from Calbiochem. Glycyl-D,L-aminocaprylic acid, glycyl-ϵ-*N*-acetyl-L-lysine, and glycyl-L-phenylglycine were prepared by coupling benzyloxycarbonyl-glycine *N*-hydroxysuccinimide ester (Bachem) with the appropriate amino acid by the method of Anderson et al. (*20*). The dipeptides were then obtained by catalytic hydrogenation over 10% Pd/C in methanol. The same procedure was used for the preparation of glycyl-L-naphthylalanine and glycyl-L-2-naphthylalanine except that the solvent system of Mitin (*21*), 1/0.25/0.1 DMF/pyridine/-trifluoroacetic acid, was employed for the initial coupling. The remaining ligands employed and the substrate *N*,*N*′-diacetyl-L-lysyl-D-alanyl-D-alanine were purchased from Sigma Chemical Co. and used as received.

Glycyl-L-α-amino-ϵ-pimelyl-D-alanyl-D-alanine (7). This compound was prepared by classical solution peptide chemistry as shown in Scheme 2. Thus, D,L-α-aminopimelic acid (Sigma) was *N*-acetylated as described by Wade et al. (*22*). Enzymatic resolution of the enantiomers was achieved by means of acylase I of *Aspergillus melleus* (Fluka) (*22*, *23*), yielding a mixture of *N*-acetyl-D-α-aminopimelic acid and L-α-aminopimelic acid which was separated by cation exchange chromatography (*22*). The dibenzyl ester of L-α-aminopimelic acid was then prepared as described by Zervas et al. (*24*) and selectively hydrolyzed to the α-monobenzyl ester by the method of Bryant et al. (*25*). In the next step, L-α-aminopimelic acid α-benzyl ester was *N*-acylated by benzyloxycarbonylglycine *N*-hydroxysuccinimide ester (*20*), and the product, benzyloxycarbonylglycyl-L-α-aminopimelic acid α-benzyl ester, coupled to D-alanyl-D-alanine methyl ester in anhydrous DMF, employing dicyclohexylcarbodiimide, L-hydroxybenzotriazole, and triethylamine as described by Blaakmeer et al. (*26*). The required product, **7**, was then achieved by catalytic hydrogenation in methanol followed by alkaline hydrolysis of the methyl ester in sodium hydroxide. Final purification of the product as a zwitterion was accomplished by Sephadex G-10 chromatography. The product was characterized by ^{1}H NMR spectroscopy [(^2H_2O) δ 1.36 (3H, d, $J = 7$ Hz, CH_3), 1.39 (2H, m, γ CH_2), 1.41 (3H, d, $J = 7$ Hz, CH_3), 1.61 (2H, m, δ CH_2), 1.80 (2H, m, β-CH_2), 2.29 (2H, t, $J = 7$ Hz, ϵ CH_2), 3.87 (2H, AB, $J =$

19 Hz, gly CH_2), 4.31 (3H, m, 3 CH)], and ES/MS [*m*/*e* 375.2, M + 1].

The synthesis of glycyl-γ-L-glutamyl-D-alanyl-D-alanine (**8**) was achieved by the same general method, beginning with the commercially available α-monobenzyl L-glutamate (Bachem), and the product characterized in the same way: ^{1}H NMR [(^2H_2O) δ 1.36 (3H, d, J = 8 Hz, CH_3), 1.41 (3H, d, J = 8 Hz, CH_3), 2.01 (1H, dt, J = 14, 7.5 Hz, β-CH), 2.18 (1H, dt, J = 14, 7.5 Hz, β-CH), 2.38 (2H, t, J = 7 Hz, γ CH_2), 3.86 (2H, AB, J = 21.5 Hz, gly CH_2), 4.30 (3H, m, 3 CH)], and ES/MS [*m*/*e* 347.2, M + 1]].

Electrospray mass spectra were obtained from the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois. The Quattro mass spectrometer was purchased in part with a grant from the Division of Research Resources, National Institutes of Health (RR 07141).

Preparation of Dansyl β-Lactam-Labeled DD-Peptidase. The following procedure was employed for each of the dansyl β-lactams, **3**−**5**. Freeze-dried enzyme (0.5 mg) was dissolved in 0.5 mL of buffer (20 mM MOPS, pH 7.5; all experiments described below were carried out in the same buffer at 25 °C) and the β-lactam added to a final concentration of 0.1 mM. After ca. 10 min, enzyme activity, as monitored by turnover of the depsipeptide **6**, was zero. The labeled protein was then separated from small molecules by passage through a BioGel P-6DG column at 5 °C. Fractions containing the fluorescent proteins were stored frozen at −70 °C.

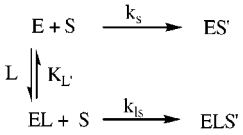
Titration of Labeled DD-Peptidase with Ligands. Titrations of ligands against the inactive, fluorescent enzymes were carried out by monitoring the perturbation of dansyl fluorescence on ligand binding by means of a Perkin-Elmer MP44A spectrofluorimeter. Routinely, 1 μL aliquots of a stock ligand solution were added to 100 μL of a 0.5 μM enzyme solution and the fluorescence emission intensity changes at the wavelength of greatest change, generally 520 or 530 nm, recorded. The excitation wavelength was 350 nm. Ligand dissociation constants, K_L, were determined from the measured intensities, F, by means of eq 1 and a nonlinear least-squares computer program.

$$F = \left(\frac{\nu_0}{\nu + \nu_0}\right)[F_0 K_L + F_\infty L_0 \nu_0/(\nu + \nu_0)]/ [K_L + L_0 \nu_0/(\nu + \nu_0)] \quad \text{(i)}$$

In this equation, v_0 is the initial volume containing the protein, v is the volume of ligand stock solution, of concentration L_0, added, F_0 is the initial fluorescence intensity prior to addition of ligand, and F_∞ the fluorescence intensity at saturating ligand concentration in the same volume as the original solution, v_0.

Determination of the Effect of Ligand on the Rate of Acylation of the Enzyme by β-Lactams. Acylation of the active site of the R61 DD-peptidase by β-lactams is accompanied by a decrease in protein fluorescence (*27*), largely that arising from Trp 233 which is adjacent to the active site (*28*). In the present experiment, this fluorescence change was monitored during acylation of the enzyme by dansylpenicillin. The protein fluorescence decreased much more on acylation by this penicillin than by benzylpenicillin, probably because of radiationless energy transfer from Trp 233 to the dansyl group. Thus, a small aliquot of a stock solution of the penicillin was added to 100 μL of a 0.77 μM DD-peptidase solution to give a final concentration of the penicillin of 0.54 mM and the ensuing reaction monitored. This experiment was carried out in the absence of, and in the presence of, various concentrations of ligand. The pseudo-first-order rate constants, k_{obs}, obtained from the fluorescence intensity vs time traces (assuming the dansylpenicillin concentration < K_s) were fitted to eq ii derived from Scheme 3. In this scheme, S represents dansylpenicillin, ES′ the stable acyl enzyme derived from reaction of E and S, L the ligand, EL, its noncovalent complex with E, and ELS′ the acyl enzyme derived from reaction of EL with S.

Scheme 3



$$k_{obs}/S_0 = \frac{k_s + (k_{1s}/K_{L'})L_0}{1 + L_0/K_{L'}} \quad \text{(ii)}$$

In eq ii, S_0 and L_0 are the total substrate and ligand concentrations, respectively, both much higher than enzyme concentration; the dissociation constant of L from the free enzyme is $K_{L'}$ (cf. K_L of eq. (i), which refers to dissociation from the active site-labeled enzyme).

Kinetics of Peptide Substrate Turnover. V_{max} and thus k_{cat} for turnover of **8** by the R61 DD-peptidase was obtained from spectrophotometric steady-state rate measurements. The decrease in absorbance at 220−230 nm accompanying the reaction was monitored by means of a Hewlett-Packard 8453 spectrophotometer. Reaction mixtures (100 μL) included the enzyme (0.02 μM) and substrate (0.4−1.0 mM). Since the enzyme was saturated at these substrate concentrations, V_{max} was obtained from an average of the initial rates measured in this concentration range. The value of K_m could not be obtained directly in this fashion because absorption changes were too small ($\Delta\epsilon$ = 64 M^{-1} cm^{-1} at 222 nm) to be accurately measured at concentrations below 0.4 mM. Determination of the K_m value of **7** was accomplished by a competition experiment where the inhibition of turnover of **6** by **7** was measured. The hydrolysis of **6** to phenylacetyl-D-alanine and m-hydroxybenzoate, catalyzed by the DD-peptidase, was monitored spectrophotometrically at 292 nm (*18*). In the presence of **7**, a lag in formation of m-hydroxybenzoate was observed due to competition by **7** for the active site. A typical reaction mixture (100 μL) contained the enzyme (5 nM), **6** (2 mM), and **7** (varied from zero to 0.6 mM). The initial portion of the total progress curve thus obtained, until essentially all of **7** was consumed by the enzyme and normal steady-state turnover of **6** obtained, was analyzed by means of the FITSIM program (*29*) and Scheme 4 to obtain K_{m1}. In this scheme, S_1 and S_2 represent **7** and **6** respectively, and P_1 and P_2 the respective products of their hydrolysis. The value of k_{cat2} and K_{m2} were 7.5 s^{-1} and 0.98 mM, respectively (*18*), and the value of k_{cat1} was taken to be that obtained from the experiment described above. The value of K_{m1} obtained was not changed significantly by addition of product inhibition by P1 (see below) to Scheme 4.

Scheme 4

$$E + S_1 \underset{K_{m1}}{\rightleftharpoons} ES_1 \xrightarrow{k_{cat1}} E + P_1$$

$$E + S_2 \underset{K_{m2}}{\rightleftharpoons} ES_2 \xrightarrow{k_{cat2}} E + P_2$$

Scheme 5

$$E + S_1 \underset{K_m}{\rightleftharpoons} ES_1 \xrightarrow{k_{cat}} EP_1 \xrightleftharpoons{K_{p1}} E + P_1$$

To test for product inhibition by glycyl-L-α-amino-ϵ-pimelyl-D-alanine, the hydrolysis of **7** (1 mM) in the presence of the DD-peptidase (20 or 40 nM) was monitored at 224 nm ($\Delta\epsilon = 50\ M^{-1}cm^{-1}$). After the reaction was complete, a further aliquot of **7** was added to bring its concentration back to 1 mM in the reaction mixture and the subsequent absorption change monitored. The total progress curve thus achieved was analyzed to obtain K_{p1} using the FITSIM program (*29*) and Scheme 5. Values of k_{cat1} and K_{m1} were taken from the experiments described above.

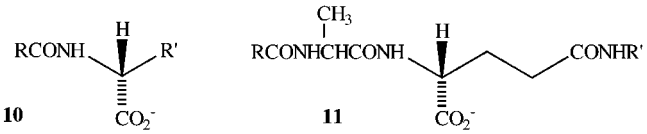
The steady-state kinetic parameters for hydrolysis of the glutamyl peptide **8** were determined from spectrophotometric (220 nm, $\Delta\epsilon = 60\ M^{-1}\ cm^{-1}$) initial rates by means of the Wilkinson method (*30*).

Kinetics of the R61 DD*-Peptidase-Catalyzed Reaction of **7** in the Presence of an Alternative Nucleophile*. The reaction of **7**, catalyzed by the R61 DD-peptidase, was studied in the presence of the alternative nucleophile D-lactate. Thus, the initial rates of absorption change at 224 nm of a reaction mixture (100 μL) containing the peptidase (20 nM), **8** (1 mM), and D-lactate (0−0.1 M) were measured.

Computational Methods. The computations were set up essentially as previously described (*31*) and run on an IBM 3CT computer with INSIGHT II 97.0 (MSI, San Diego, CA). The starting point for the structural simulations was the crystal structure of the R61 DD-peptidase [PBD file 3pte (*32*)] including the crystallographic water molecules. A tetrahedral intermediate, as in **9**, was built onto this structure by means of the Builder module of INSIGHT II. MNDO charges were employed for the ligand, while charges on the protein were assigned by INSIGHT II. The ligand was initially oriented with the oxyanion in the oxyanion hole and with the side chain amido group and leaving group (D-Ala) roughly oriented as they were in previous simulations carried out in this laboratory (*18*) and as suggested by the crystal structure of a covalent complex of the enzyme with cephalothin (*33*). The position of the extended side chain of **7** was of course quite unknown. A series of MD and simulated annealing runs were made in order to evaluate the range of conformations available to the side chain. A CV force field within the Discover program (version 2.98) was employed for all simulations and energy calculations. MD runs were carried out for up to 20 ps at both 300 and at 1000 K; at the latter temperature, the protein backbone was fixed. Simulated annealing was begun at 1000 K and the temperature reduced in 50 K steps to 300 K; again the protein backbone was fixed. No solvent was included in these exploratory simulations. Subsequently 20 ps MD runs were made on selected conformations at 300 K where all of the protein was released from constraint and a 20 Å sphere of water molecules was added, centered at Ser 62 O_γ. Typical snapshots from these were taken and the energy of each minimized in the CV force field by means of 1000 steps by the method of steepest descents following by 2000 steps of conjugate gradients. The final derivative of energy with respect to structural perturbation was then in the range of 0.02−0.03 kcal/Å. Bond distances and interaction energies could then be obtained directly from the minimized structure by means of the Discover program. E_{int} values were calculated as described previously (*31*) and included all residues of the protein.

RESULTS AND DISCUSSION

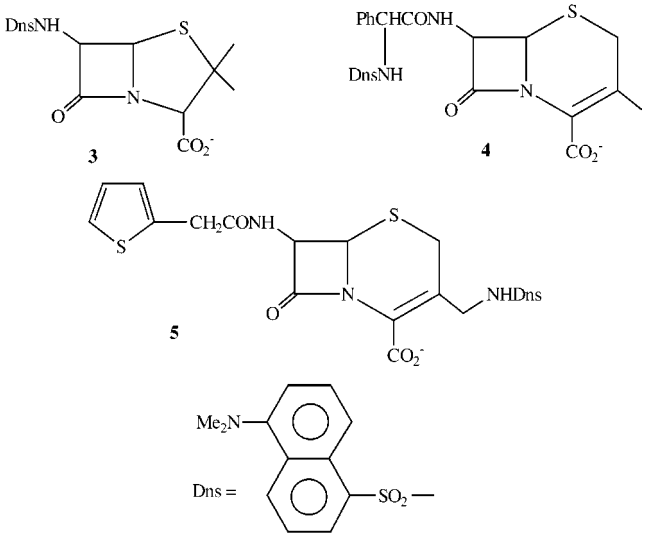
Peptide Binding. The technique of using a fluorescent probe to irreversibly occupy the active site of an enzyme in order to investigate the binding of potential ligands to an extended binding site has been previously employed in this laboratory to find such ligands of the P99 β-lactamase (*19*). In that study, it was found that ligands of general structure **10** bound to the enzyme where the active site serine had



10 **11**

been labeled by a fluorescent phosphonate. It was proposed that this fragment resembled the L-Ala-D-isoglutamyl dipeptide **11** of the gram negative bacterial cell wall. Since the R61 enzyme has a tertiary structure and active site structure very similar to that of the P99 β-lactamase (*6*, *32*), but also has the substrate specificity of a bacterial DD-peptidase, it would seem likely that an extended substrate binding site would be also found on it—indeed, more so than in the case of the β-lactamase where such sites would presumably only be evolutionary relics (*34*).

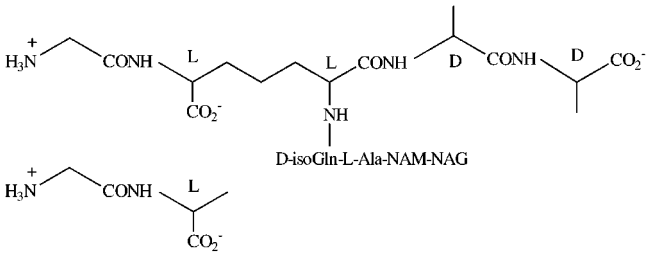
To test this idea, the active site of the R61 DD-peptidase was covalently labeled with three dansyl β-lactams, each with the fluorescent dansyl group differently positioned, in two cases, **3** and **4**, on the acyl donor side, and in one, **5**, on the



3 **4** **5**

acyl acceptor side. The dansylpenicillin(**3**)/enzyme complex was then titrated with the following ligands, all from the P99 β-lactamase study: 4-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-*N*-acetylmuramyl-L-alanyl-D-isoglutamine, *N*-

Scheme 6



(phenylacetyl)-glycyl-D-phenylalanine, *N*-(phenylacetyl)glycine, *N*-(phenylacetyl)glycyl-D-alanine, *N*-(phenylacetyl)-glycyl-D-glutamine, disodium benzylpenicilloate, D-phenylalanine, D-homophenylalanine, D-lysine, D-norleucine, and meso-diaminopimelic acid. None of these had measurable effect on the intensity of dansyl fluorescence emission except D-homophenylalanine which produced an enhancement of fluorescence. The dissociation constant of the D-homophenylalanine complex exceeded 20 mM, however. A number of these compounds were also titrated against the dansylcephalexin (**4**) and dansylcephalothin (**5**) complexes, but in these cases no perturbation of the dansyl fluorescence was observed.

Rather unexpectedly therefore, no evidence was found to suggest that significant binding of compounds of structure **10** occurred on the R61 DD-peptidase. If it is assumed that these ligands in fact do not bind to the enzyme (as opposed to binding without effect on the fluorescent probe or being precluded from binding by the presence of the β-lactam), it must be concluded that the DD-peptidase differs strikingly from the β-lactamase in this regard. The absence of such binding to this DD-peptidase would certainly call into question its role as an enzyme of cell wall biosynthesis and perhaps supports the suggestion (*35*) that the non-membrane bound R61 DD-peptidase may be a defensive stoichiometric β-lactam trap.

A number of years ago, Frére et al. reported that glycyl-L-alanine was a noncompetitive inhibitor of the R61 DD-peptidase (*36*) and proposed an additional binding site for this molecule. Such a site certainly made sense in terms of the structure of the *Streptomyces* cell wall, where one terminal L amino center of L,L-diaminopimelic acid is acylated by glycine (*13*); the amino group of glycine is thus the acyl group acceptor in cell wall cross-linking in these bacteria (Scheme 1). The structural analogy between glycyl-L-alanine and the acyl acceptor is shown in Scheme 6. We were pleased to find therefore that Gly-L-Ala did cause a fluorescence enhancement of dansylpenicillin bound at the R61 active site. A fluorescence titration indicated that the resulting complex had a dissociation constant of 10.3 ± 1.2 mM. Other Gly-L-Xaa peptides had similar effects. For example, Figure 1 shows the effect of Gly-L-Trp on the fluorescence of the bound dansylpenicillin; a titration in this case (Figure 1) yielded a dissociation constant of 0.86 mM.

These data and a comparison of the structures in Scheme 6 suggested that glycyl dipeptides with longer aliphatic side chains might bind more tightly. The results of structural variation in the ligand on the strength of binding to the dansylpenicillin-labeled DD-peptidase are presented in Table 1. It is noticeable, first, that although larger aliphatic side chains do not, in general, appear to lead to stronger binding

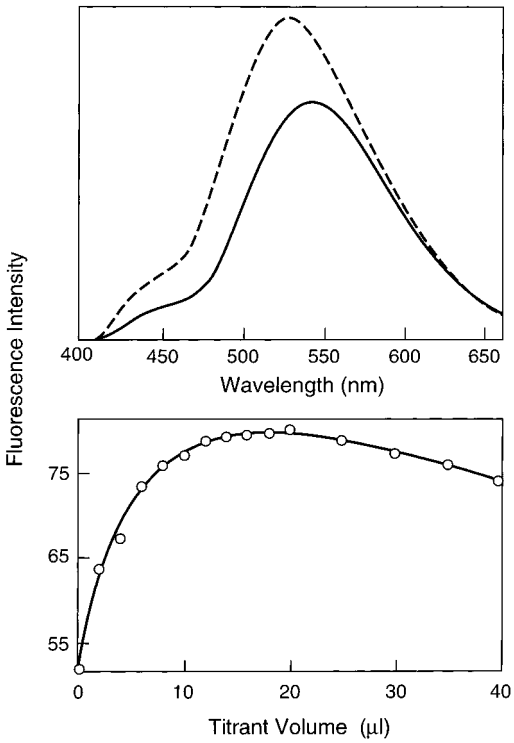


FIGURE 1: Upper panel: Emission spectra (λ_{ex} 350 nm) of the dansylpenicillin-labeled *Streptomyces* R61 DD-peptidase (0.5 μM) in the absence (—) and presence (- - -) of Gly-L-Trp (1.1 mM). Lower panel: Titration of the labeled enzyme (100 μL, 0.5 μM) with Gly-L-Trp (0−10 mM), as monitored by fluorescence emission at 520 nm on excitation at 340 nm.

Table 1: Dissociation Constants of Ligands Bound to Dansylpenicillin-Labeled R61 DD-Peptidase

entry	ligand	K_L (mM)[a]	$\Delta F/F_0$[b]
1	Gly-L-Ala	10.3 ± 1.2	0.89
2	Gly-L-Val	2.2 ± 0.6	0.90
3	Gly-L-Leu	18.8 ± 3.7	0.66
4	Gly-L-Ile	11.8 ± 1.4	1.7
5.	Gly-L-Met	14.9 ± 3.8	1.2
6	Gly-L-Phe	2.2 ± 1.2	1.1
7	Gly-L-Phenylgly		n[c]
8	Gly-L-Trp	0.86 ± 0.10	1.3
9	Gly-L-His	9.3 ± 3.6	1.3
10	Gly-L-2-naphthylalanine	0.40 ± 0.07	0.34
11	Gly-L-1-naphthylalanine		n
12	Gly-L-glu		n
13	Gly-L-Lys	52 ± 16	1.3
14	Gly-ϵ-*N*-Ac-L-Lys	19.8 ± 1.1	1.2
15	Gly-DL-aminocaprylic acid		n
16	Glycinamide		n
17	Gly-D-Ala		n
18	Gly-D-Phe		n
19	L-Ala-L-Ala	18.2 ± 4.3	0.43
20	NAc-L-Ala		n
21	NAc-L-Phe		n
22	Gly-L-PheNH_2		n
23	Gly-L-Ala-Gly		n
24	Gly-Gly-L-Phe	7.5 ± 1.0	0.48
25	Gly-L-Phe-Gly		n
26	Gly-L-Phe-L-Phe		n
27	*m*-aminophenylacetic acid		n
28	*N*-Ac-D-alanyl-D-alanine		n
29	*N*-*Z*-D-alanyl-D-alanine		n

[a] Defined in eq i. [b] Fluorescence enhancement on peptide binding. [c] No fluorescence change observed.

(entries 2−5, 12−15), Gly-L-Val is one exception, immediately indicating specificity. Polar side chains (entries

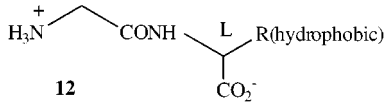
Table 2: Ligand Dissociation Constants from Acylation Kinetics

ligand	$K_{L'}$ (mM)[a]	k_{ls}/k_s[a]
Gly-L-Ala	22 ± 3	0.26
Gly-L-Phe	1.6 ± 0.3	0.12
Gly-L-Met	0.19 ± 0.07	0.07
Gly-L-Val	40 ± 8	0[b]
Gly-ϵ-*N*-Ac-L-Lys	1.09 ± 0.13	0.03
Gly-DL-aminocaprylic acid	0.19 ± 0.04[c]	0.02
GlyGly-L-Phe	10.3 ± 1.5	0

[a] Defined in Scheme 3. [b] $k_{ls} = 0$ assumed. [c] Only the L-enantiomer is assumed to bind.

12−14), however, did not help. Aromatic side chains often appeared advantageous (entries 6, 8, 9, 10), although the lack of binding by two members of this group (entries 7 and 11) again point to significant specificity. The terminal L-carboxylate is clearly important, as seen by the absence of binding in entries 16, 22, 23, and 25 (although the affinity of GlyGly-L-Phe, entry 24, is striking). A positive charge may well be required at the N-terminus in view of the results from entries 20, 21, and 27. Replacements for the N-terminal glycine were not pursued although its replacement by L-Ala (entry 19) was not encouraging. The L stereochemistry of the C-terminal amino acid was clearly important as indicated by the negative results with entries 17 and 18. Finally, D-alanyl-D-alanine peptides (entries 28 and 29) showed no sign of interaction, as would be expected if the major D-Ala-D-Ala binding site was occupied by dansylpenicillin.

The results of Table 1 therefore suggest that optimal binding is achieved by **12** where a hydrophobic side chain

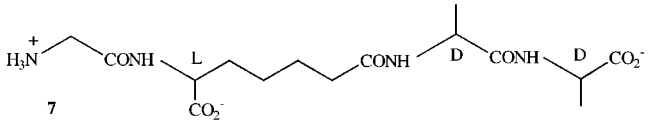


12

at the C-terminus, of rather specific size and shape, can indeed improve the interaction between Gly-L-Ala and the labeled protein. A caveat which should be taken into account before any further interpretation of these data is attempted is that if the model of Scheme 6 does in fact apply, i.e., if the Gly-L-Ala binding site does represent part of the binding site of the acyl donor substrate, it is likely that an extended side chain on the dipeptide would sterically interact with the dansyl group of the covalently bound penicillin. Thus the binding specificity of the dipeptide may also include this interaction with the β-lactam. It is therefore possible that the strength of binding of ligands with extended side chains, which might well overlap with the penicillin side chain, might be greater in the free enzyme than in the test complex.

An indication that the final proposition of the last paragraph is in fact true was obtained by studying several of the ligands, particularly of structure **12**, as inhibitors of acylation of the DD-peptidase by dansylpenicillin. The results of these experiments, interpreted in terms of Scheme 3 are presented in Table 2. The comparison of the $K_{L'}$ values of Table 2 with the K_L values of Table 1 is informative. The binding of Gly-L-Ala to the enzyme reduces its reactivity with dansylpenicillin ($k_{ls}/k_s < 1$) but not to zero. Apparently both Gly-L-Ala (with a short side chain) and dansylpenicillin can simultaneously bind to the enzyme. The binding of Gly-L-Ala is somewhat weaker to the free enzyme than to the acyl-enzyme, perhaps because of hydrophobic interaction between the methyl group and the dansyl group. The enzyme is also still reactive when the larger Gly-L-Phe is bound although not as reactive as the Gly-L-Ala complex, presumably because of interaction, possibly direct, between the side chain and the dansyl moiety. Peptides containing the longer aliphatic side chains, Gly-L-Met, Gly-ϵ-NAc-L-Lys, and glycyl-L-aminocaprylic acid, provide more interesting results.Their complexes with the enzyme are essentially unreactive with dansylpenicillin (almost as good a fit to the data is obtained in these cases if $k_{ls} = 0$), and the dissociation constants of their complexes with the free enzyme are much lower than with the acyl enzyme. This suggests a competitive interaction where, because of side-chain clashes, the dansylpenicillin is unable to react when the peptide is bound. The complex with GlyGly-L-Phe is also unreactive although the binding is not strengthened, possibly because of the less specific structure of this ligand. The case of Gly-L-Val is intriguing. Two interpretations seem possible. Either Gly-L-Val strongly interacts with the acyl enzyme, perhaps by way of direct hydrophobic interaction with the dansyl group, but not with the free enzyme, or Gly-L-Val can bind to the free enzyme without affecting its reactivity with dansylpenicillin. In the latter scenario, the observed inhibition could arise from weak binding at the D-Ala-D-Ala site. At any event, these results, in general, appear to provide evidence supporting the proposition that long aliphatic side chains in Gly-L-Xaa peptides directly and competitively interact with the side chains of β-lactams, both when the latter are covalently bound and in the process of reacting. Thus, these results support the concept that Gly-L-Xaa peptides, **12**, bind in the extended active site where the N-terminus of the donor peptide usually binds (Scheme 5).

Substrate Design, Synthesis, and Evaluation. As described in the introduction of the paper, there are very few molecules known that bind tightly to DD-peptidases in a noncovalent fashion or, with the exception of β-lactams, react rapidly and covalently. The experiments described above suggested that connecting Gly-L-Ala to D-Ala-D-Ala by means of a suitable linker might generate tight-binding substrates or inhibitors of the *Streptomyces* R61 DD-peptidase. The obvious linker, suggested by the *Streptomyces* cell wall structure (Scheme 6), would derive from replacement of the methyl side chain of L-Ala with a carboxybutyl side chain to generate the molecule **7**. The dipeptide side chain leading to NAG-



7

NAM was omitted for ease of synthesis (one less asymmetric center) and because there is no evidence that this moiety binds tightly to this enzyme (see below).

The molecule **7**, as the zwitterion, was prepared by classical peptide chemistry according to Scheme 2 and characterized by NMR and ES/MS. It was shown to be a substrate of the R61 DD-peptidase, first by an NMR experiment. Thus, approximately 100 μg of the enzyme was added to an NMR tube containing 2 mg of **7** in 20 mM sodium bicarbonate. Reaction was complete after ca. 10 min. The most noticeable spectral change was the disappearance of a methyl group doublet at 1.38 ppm and a methine quartet at

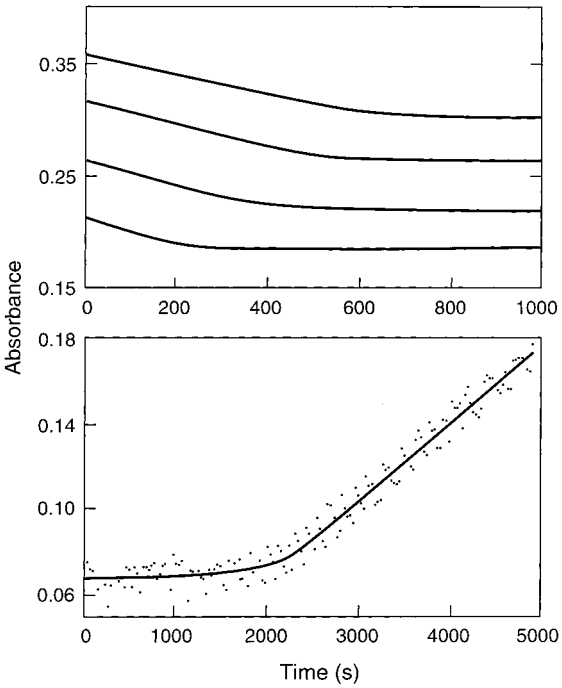


FIGURE 2: Upper panel: Reaction of **7** (0.4, 0.6, 0.8, and 1.0 mM, lower to upper trace) with the *Streptomyces* R61 DD-peptidase (20 nM), monitored spectrophotometrically at 228 nm. The data have been computer-smoothed. Lower panel: Reaction of the depsipeptide **6** (2 mM) in the presence of **7** (0.4 mM) and the DD-peptidase (5.0 nM), monitored spectrophotometrically at 292 nm. The points are experimental and the line calculated as described in the text. A small linear absorption change from the background hydrolysis of **6** has been subtracted.

4.32 ppm and the appearance of similar resonances at 1.48 and 4.16 ppm, respectively. The latter resonances correspond to the appearance of D-alanine in solution, presumably from hydrolysis of the C-terminal peptide bond, as shown by a spectrum taken after addition of authentic D-alanine to the sample.

The hydrolysis of **7** in the presence of the enzyme could also be monitored spectrophotometrically at low wavelengths. Figure 2, upper panel, shows traces of absorption vs time for kinetic runs at concentrations of **7** between 0.4 and 1.0 mM. Noticeable here is the linearity of the traces until close to completion and the parallel nature of the lines, both indicating enzyme saturation. These data yielded a k_{cat} value of 69 ± 14 s^{-1}. Since this method was not sufficiently sensitive to be used at the substrate concentrations required to obtain K_m, the latter parameter was determined from the action of **7** as an inhibitor of turnover of a depsipeptide substrate, **6**, where release of m-hydroxybenzoate was monitored spectrophotometrically at 292 nm. A typical trace from such an experiment is shown in Figure 2 (lower panel). From these experiments, performed at concentrations of **7** between 100 and 600 μM, the value of K_m for **7** was determined (see Experimental Procedures) to be 7.9 ± 0.7 μM.

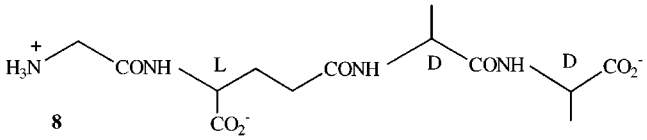
Product inhibition, presumably by glycyl-L-α-amino-ϵ-pimelyl-D-alanine since D-alanine had no effect at the concentrations involved, was also observed, and a K_{p1} value of 0.16 ± 0.04 mM determined (see Experimental Procedures). Product binding is therefore of comparable strength to that of the stronger-binding ligands of Tables 1 and 2. The peptide **7** (to 1 mM concentration) did not affect the fluorescence of dansylpenicillin (**3**)-labeled enzyme. It seems likely therefore that the tight binding of **7** is eliminated when the active site is occupied by a β-lactam. This result would of course be expected if **7** bound as a substrate.

Scheme 7

$$E + S \underset{K_s}{\rightleftharpoons} ES \xrightarrow{k_2} E\,S' \xrightarrow{k_3} EP \overset{K_p}{\rightleftharpoons} E + P$$

To understand the meaning of the K_m value of **7** it was necessary to determine whether it represented k_2 or k_3 in the usual turnover sequence (Scheme 7; ES′ represents the covalent acyl-enzyme intermediate). The alternative nucleophile D-lactate was employed for this purpose. Jamin et al. have shown that D-lactate is an effective nucleophile toward acyl-enzymes formed from depsipeptides and the R61 DD-peptidase (*37*). Thus, in cases of substrates where deacylation is rate-determining at saturation, e.g., depsipeptides, the initial rates of substrate turnover are increased by D-lactate. In the present case, with **7**, no such increase in initial rate was observed at a saturating **7** concentration (1 mM) and with D-lactate at concentrations up to 0.1 M. In fact, the observed rates decreased with D-lactate concentration, presumably due to binding of D-lactate somewhere in the extended substrate binding site. If competitive inhibition were assumed, the K_I of D-lactate would be 0.79 mM. These results suggest that enzyme acylation is rate determining during turnover of **7** at saturation. Also in support of this proposition was the observation that the intrinsic fluorescence emission intensity of the R61 DD-peptidase (λ_{ex} = 280 nm, λ_f = 320 nm) was not affected by **7** (1.5 mM). Acylation of the peptidase by either a β-lactam or a depsipeptide (deacylation is rate determining in both cases) has been shown to produce a significant decrease in fluorescence intensity (*27*, *37*). This is thought to represent quenching of the fluorescence of Trp 233 (*28*) which lies adjacent to the active site and may directly contact bound substrates (*32*, *18*). The absence of any such effect with **7** suggests that no acyl−enzyme accumulates and thus acylation is most likely rate determining. No quenching of fluorescence is produced by the peptide substrate *N*,*N*′-diacetyl-L-lysyl-D-alanyl-D-alanine (DALAA) (*27*), where acylation is known to be rate determining (*38*).

These experiments appear to demonstrate that during turnover of **7** by the R61 DD-peptidase, $k_{cat} = k_2$ and $K_m = K_s$. Thus, the K_m of 7.9 μM represents dissociation of the noncovalent Michaelis complex formed between **7** and the enzyme. To determine the degree to which the length of the pimelyl side chain contributed to this impressively tight binding, **8**, the glutamyl analogue of **7**, was prepared. Steady-



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state parameters for turnover of this substrate by the R61 DD-peptidase are included with those of **7** and DALAA, the latter hitherto the best amide substrate of this enzyme, in Table 3.

The data of Table 3 show that **7** is indeed an impressive substrate of the R61 DD-peptidase, more specific for the enzyme than DALAA by greater than 3 orders of magnitude.

Table 3: Steady-State Parameters for Turnover of Peptide Substrates by the R61 DD-Peptidase

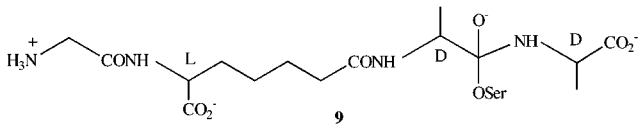
substrate	$k_{cat}(k_2)$ (s^{-1})	$K_m(K_s)$ (μM)	k_{cat}/K_m ($s^{-1}M^{-1}$)
7	69 ± 14	7.9 ± 0.7	8.7×10^6
8	12.8 ± 0.8	860 ± 190	1.5×10^4
DALAA[a]	34.5	9800	3.5×10^3

[a] Ref *43*.

This difference arises almost exclusively from the much greater strength of noncovalent binding ($K_m = K_s$). Apparently the aliphatic side chain is insufficient in itself to produce tight binding, suggesting that the charged terminal functional groups on **7** are essential. It is clear, however, from the results with **8** that the length of the aliphatic linker is also important. The binding of **8**, although some 10-fold tighter than that of DALAA, is still 100-fold poorer than that of **7**. This suggests that specific binding of the glycylammonium group and/or the pimelyl α-carboxylate of **7** is required for tight binding. The shorter side chain of **8** is apparently unable to deliver the charged residues to their binding sites.

The other reaction that might be expected of an acyl−enzyme derived from **7** and the R61 DD-peptidase is of course aminolysis/transpeptidation (*7*, *9*). In principle, **7** itself should be an acyl acceptor (see Scheme 1). If there is a specific binding site for an acyl acceptor on this enzyme, then **7** would be expected, on the basis of its structure, to be a very good acceptor. When 1.6 mM **7** was reacted with the enzyme until the absorbance at 220 nm no longer changed, electrospray mass spectral analysis of the dried product showed the presence of only the hydrolysis product, glycyl-L-α-amino-ϵ-pimelyl-D-alanine (*m/e* 304, M + 1) and none of the aminolysis product of molecular weight 659. Apparently, **7** does not bind strongly as an acyl acceptor to this enzyme. This may reflect the fact that the enzyme's natural role is not that of a transpeptidase or that the high effective concentration of closely adjacent cell wall acceptors makes tight binding unnecessary. It should be noted that in early studies Ghuysen and co-workers did detect transpeptidation reactions of oligopeptides catalyzed by the R61 DD-peptidase (*39*, *40*). In those experiments, however, significant transpeptidation was only observed in solutions of low water content (e.g., 25% glycerol plus 58% ethylene glycol) and/or higher acyl acceptor concentrations (*36*, *41*).

Molecular Modeling. A model of the peptide substrate **7**, as a tetrahedral intermediate, **9**, during the enzyme acylation



9

step of hydrolysis, was constructed computationally as described in Experimental Procedures. MD and simulated annealing procedures led to several well-populated structures with **7** in different conformations. These differed largely in the conformation of the glycylpimelyl side chain beyond the amido group connecting it to D-Ala-D-Ala. To that point, the leaving group D-alanine, the penultimate D-alanine, and the amido group are oriented as expected based on the crystal structure of a cephalothin complex (*33*) and previous modeling of a smaller peptide, phenylacetyl-D-alanyl-D-alanine (*18*). In particular, the terminal carboxylate is hydrogen-bonded to Arg 285 and Thr 301, Lys 65 is hydrogen-bonded to Ser 62 O_γ, the oxyanion is in its hole, hydrogen-bonded to backbone NH groups of Ser 62 and Thr 301, the methyl group of the penultimate D-alanyl residue is in its hydrophobic pocket (*18*), and the side-chain amido group is hydrogen-bonded to Asn 161. Tyr 159 is hydrogen bonded, not to Ser 62 O_γ or to the leaving group nitrogen atom, but to the terminal carboxylate. This position of Tyr

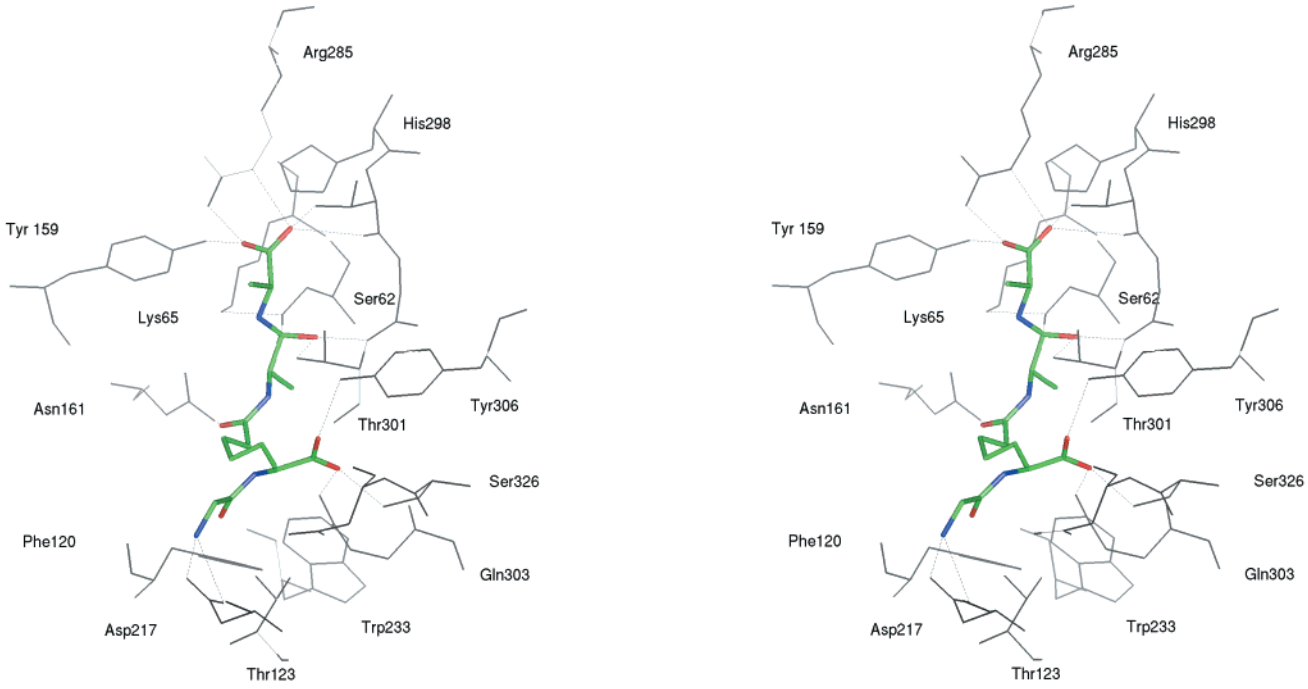


FIGURE 3: Optimized tetrahedral intermediate structure, in stereoview, formed on interaction of the *Streptomyces* R61 DD-peptidase with the substrate **7**. The substrate is shown in stick form, the protein as lines; only heavy atoms are shown. Dotted lines represent likely hydrogen bonds (heavy atom distance ≤ 3.0 Å).

159, although perhaps thermodynamically favored, is not the one that would be expected mechanistically since Tyr 159 is thought to act as a general acid/base catalyst of turnover. This model, therefore, may represent, to this extent at least, a nonproductive conformation.

The calculated interactions between the peptide ligand and protein [as indicated by the E_{int} parameter which takes into account direct enzyme−ligand interactions as well as enzyme−enzyme and ligand−ligand interactions (*31*)] suggested that the conformation shown in Figure 3 may be the most likely. Of particular interest here is the orientation of the glycyl-pimelyl side chain. The tetramethylene chain appears to be in close hydrophobic contact with Trp 233. As mentioned above, it is this residue whose fluorescence is quenched on acylation of the enzyme by β-lactams. Mutagenesis of Trp 233 also severely depresses DD-peptidase activity (*28*). The pimelyl α-carboxylate is hydrogen-bonded to the Tyr 306 hydroxyl, to Ser 326 O_γ, and to the amido group of the side chain of Gln 303. The N-terminal glycylammonium group is strongly hydrogen-bonded to the Asp 217 carboxylate. Thus both the aliphatic side chain and the terminal charged residues seem appropriately accommodated.

In a complete, natural substrate, a NAG-NAM-dipeptide substituent would be present at the L-ϵ-position of the L-α-aminopimelyl residue (Scheme 6). The tetramethylene bridge in **7** seems flexible enough, even when bound to the enzyme as shown in Figure 3, to allow such an extension. Incorporation of an ϵ-(*N*-acetamido) group into the model of Figure 3 followed by energy minimization led to a structure where the polar interactions with the enzyme were maintained but the conformation of the pimelyl side chain changed somewhat. Presumably the larger substituent of the presumed natural substrate could also fit into this structure.

GENERAL DISCUSSION AND CONCLUSIONS

This paper demonstrates that specific subsites of the extended substrate binding site of a bacterial DD-peptidase can be detected by small peptide binding experiments. In this particular case, that of the *Streptomyces* R61 DD-peptidase, we have demonstrated the affinity of Gly-L-Xaa dipeptides, **12**, for part of the acyl donor binding site. By analogy with the situation with proteases, it seems likely that such subsites contribute to the specificity of natural substrates and thus can be exploited in the design of specific substrates and inhibitors. Indeed, in this instance, we have shown that a combination of the subsite specificity with the essential C-terminal D-alanyl-D-alanine of a stem cell wall peptide (**1**) produced the most specific peptide substrate (**7**) yet achieved for a DD-peptidase. It is clear from the results yielded by the shorter analogues, DALAA and **8**, that the specificity of **7** derives in large measure from binding of the amino group extension (glycine) of the stem peptide into a specific subsite.

The question arises however whether the specificity of **7** could be further improved by incorporation of other elements of the stem peptide and its associated disaccharide (NAG-NAM-L-Ala-D-isoGln) or perhaps an even larger fragment of peptidoglycan structure. There is, however, it should be noted, not a lot of room for further improvement since anything approaching another order of magnitude increase in k_{cat}/K_m would almost certainly produce a "perfect" (diffusion-controlled) substrate. There is also experimental evidence suggesting that such further elaboration of the substrate structure in this case would not contribute a great deal to further specificity. There was no indication, for example, of binding of NAG-NAM-L-Ala-D-isoGln to the fluorescent β-lactam-labeled enzyme nor did this glycopeptide affect acylation of the enzyme by dansylpenicillin.

Early experiments by Ghuysen and co-workers do not indicate strong binding by muramyl pentapeptide species to the R61 DD-peptidase (*42*). Thus, for this particular enzyme, it seems that the majority of acyl donor specificity arises from the elements present in **7** and that further elaboration of the substrate would have little quantitative effect. It is possible, however, that a C-terminal (thio) depsipeptide rather than the D-alanine peptide of **7** would produce a substrate with diffusion-controlled acylation and rate-determining deacylation at saturation (*37*).

The tight binding evinced by **7** also suggests directions for the design of specific inhibitors of this enzyme. The model of Figure 3 may assist in this process. Such a venture would be aided of course by a high-resolution crystal structure of **7** or its hydrolysis product bound to the enzyme. It is hoped that experiments now in progress (M. McDonough, J. A. Kelly, and J. R. Knox, University of Connecticut) will soon achieve such a structure. The extension of these methods to other DD-peptidases (penicillin-binding proteins) may reveal tight-binding peptide motifs that will aid substrate/inhibitor design in these cases also.

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