BIOCHEMISTRY

 Volume 44, Number 30 August 2, 2005

Articles

Transpeptidation Reactions of a Specific Substrate Catalyzed by the *Streptomyces* R61 DD-Peptidase: The Structural Basis of Acyl Acceptor Specificity†

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Received March 24, 2005; Revised Manuscript Received June 1, 2005

ABSTRACT: Bacterial DD-peptidases, the targets of β-lactam antibiotics, are believed to catalyze D-alanyl-D-alanine carboxypeptidase and transpeptidase reactions in vivo. To date, however, there have been few concerted attempts to explore the kinetic and thermodynamic specificities of the active sites of these enzymes. We have shown that the peptidoglycan-mimetic peptide, glycyl-L-α-amino-ε-pimelyl-D-alanyl-D-alanine, **1**, is a very specific and reactive carboxypeptidase substrate of the *Streptomyces* R61 DD-peptidase [Anderson, J. W., and Pratt, R. F. (2000) *Biochemistry 39*, 12200−12209]. In the present paper, we explore the transpeptidation reactions of this substrate, where the enzyme catalyzes transfer of the glycyl-L-α-amino-ε-pimelyl-D-alanyl moiety to amines. These reactions are believed to occur through capture of an acyl-enzyme intermediate by amines rather than water. Experiments show that effective acyl acceptors require a carboxylate group and thus are amino acids and peptides. D(but not L)-amino acids, analogues of the leaving group of **1**, are good acceptors. The effectiveness of D-alanine as an acceptor increases with pH, suggesting that the bound and reactive form of an amino acid acceptor is the free amine. Certain glycyl-L(but not D)-amino acids, such as glycyl-L-alanine and glycyl-L-phenylalanine, are also good acceptors. These molecules may resemble the N-terminus of the *Streptomyces* stem peptides that, presumably, are the acceptors in vivo. The acyl acceptor binding site therefore demonstrates a dual specificity. That D-alanyl-L-alanine shows little activity as an acceptor suggested that, on binding of acceptors to the enzyme, the carboxylate of D-amino acids does not overlap with the peptide carbonyl group of glycyl-L-amino acids. Molecular modeling of transpeptidation tetrahedral intermediates and products demonstrated the likely structural bases for the stereospecificity of the acceptors and the nature of the dual function acceptor binding site. For both groups of acceptors, the terminal carboxylate appeared to be anchored at the active site by interaction with Arg 285 and Thr 299.

Over the last 50 years, β-lactams have proven to be remarkably effective and versatile antibiotics (*1*). The antibiotic activity of these molecules derives from their inhibition of bacterial DD-peptidases (otherwise known as transpeptidases or penicillin-binding proteins), the enzymes that catalyze the final transpeptidation cross-linking reaction (Scheme 1) in bacterial cell wall (peptidoglycan) biosynthesis (*2*). Over the same 50 years since the introduction of β-lactams into clinical practice, bacterial resistance to them has increased dramatically by several mechanisms including by production of the β-lactamases, enzymes that catalyze β-lactam hydrolysis (*3*). Despite this problem of resistance

† This research was supported by the National Institutes of Health, Grant AI-17986.

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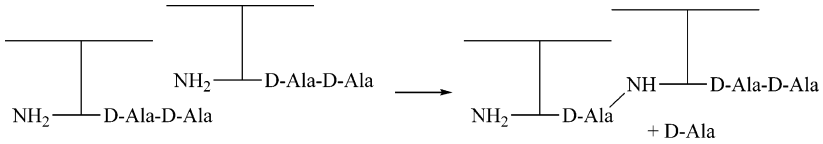
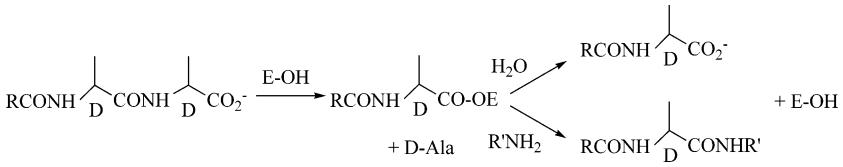
10.1021/bi0505417 CCC: $30.25
Published on Web 07/06/2005

Scheme 1



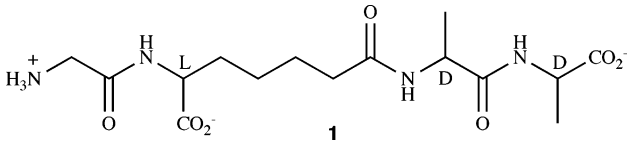
Scheme 2



and despite the allure of new targets (*4*), the DD-peptidases remain a very specific and accessible target for antibiotic research and development.

The DD-peptidases have been usefully classified by Ghuysen, on the basis of their size, in vivo function, and amino acid sequence homologies (*5*). A high molecular weight (60−90 K) group is believed to catalyze the transpeptidation reactions of Scheme 1 in vivo. Bacterial growth usually ceases on inhibition of these enzymes. A low molecular weight (20−50 K) group that also appears to catalyze transpeptidation in vitro, is believed, in many cases, to generally catalyze carboxypeptidation and endopeptidation in vivo. The latter enzymes are generally not essential for bacterial growth and survival. The carboxypeptidation and transpeptidation reactions appear to branch from a common acyl-enzyme, as shown in the mechanism of Scheme 2 (in this scheme, R and R′ represent the two peptidoglycan strands). In recent years, a number of crystal structures of DD-peptidases have been reported, of both the high (*6*, *7*) and low (*8*−*10*) molecular weight groups.

Despite much progress in the identification, purification, and structural studies of these enzymes, there is surprisingly little in the way of systematic studies of their turnover kinetics and substrate specificity. To a considerable extent, this is because many of these enzymes do not seem to catalyze substrate turnover at any significant rate in vitro (*11*). One notable exception to this situation is represented by the extensive studies of certain *Streptomyces*-derived DD-peptidases by the Belgian group led by Ghuysen and Frère. These low molecular weight enzymes, especially one from *Streptomyces* R61, have been examined in considerable detail (*12*, *13*). Kinetics and specificity studies to date, however, have been limited to some extent by the use of nonspecific small molecule substrates that display complicated reaction schemes and thus present some ambiguity of interpretation (*14*, *15*). Recently, we have introduced a very specific substrate, **1**, for the R61 DD-peptidase (*16*). This peptide



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contains the glycyl-L-α-amino-ϵ-pimelyl moiety of the *Streptomyces* peptidoglycan, and presumably because of this, reacts rapidly with the enzyme [$k_{cat}/K_m = 8.7 \times 10^6\ s^{-1}\ M^{-1}$ (*16*)], and interacts very strongly with the active site (*17*). The specificity elements of this molecule as an acyl donor in the carboxypeptidase reaction have been dissected by means of a structure−activity study (*18*). Henceforeward, in this and the accompanying paper (*19*), the glycyl-L-α-amino-ϵ-pimelyl moiety will be referred to as the side chain of peptides such as **1**. The present paper describes a study of the transpeptidation reaction using this substrate as the acyl donor, and thereby, in particular, an assessment of the structural specificity of the acyl acceptor.

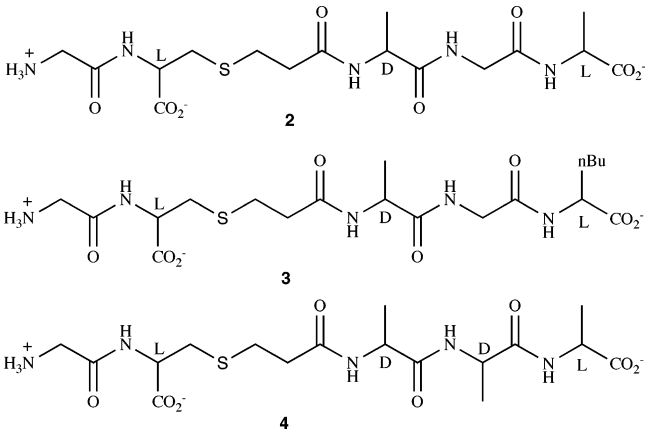
The transpeptidation reaction catalyzed by bacterial DD-peptidases is also of some broader interest because the leaving group (D-alanine, Scheme 1) is, in general, structurally different from the nucleophile that displaces it (the amine terminus of the stem peptide). Other examples of this type of acyl transferase are γ-glutaminyl transpeptidase, where both amino acids and dipeptides are acyl acceptors (*20*, *21*), and bacterial membrane sortases where the peptide nucleophile must differ in structure from the peptide leaving group (*22*, *23*). This situation contrasts with the perhaps better known cases of transpeptidation catalyzed by the common proteases. In the latter case, the same binding site (S_1', S_2', etc.) is used by the incoming nucleophile after departure of the leaving group, and thus the two have similar structural specificity (*24*−*26*). On the other hand, enzymes such as acetyl transferases which, unlike those mentioned above, do not employ an acyl-enzyme intermediate and thus catalyze acyl transfer by means of an obligate ternary complex, must have separate binding sites for acyl acceptor and leaving group (*27*). In this paper we also offer a structural interpretation of the dual acyl acceptor specificity of the *Streptomyces* R61 DD-peptidase that is in accord with the kinetics results.

MATERIALS AND METHODS

Materials. The DD-peptidase of *Streptomyces* R61 was generously supplied by Dr. J.-M. Frère of the University of Liège (Liège, Belgium). Glycyl-L-α-amino-ϵ-pimelyl-D-alanyl-D-alanine, **1**, was prepared by Dr. John W. Anderson in this laboratory (*16*). The highest quality commercially available amino acids were employed without further purification; D-alanine, in particular, was purchased from Fluka (puriss.). Dipeptides were purchased from Bachem and used as supplied. Gly-L-Lys(Ac)[1] and glycyl-DL-α-aminocaprylic acid were prepared as described (*16*). The synthesis of peptides **2**−**4** is described in the Supporting Information;

[1] Abbreviations: standard three-letter abbreviations for α-amino acids; Xaa, any α-amino acid.

thia analogues were employed because of ease of synthesis (*18*).



Kinetics. All kinetics studies were performed at 25 °C. The buffers used were 10 mM phosphate (pH 7.6) and 10 mM Tris (pH 8.9). Initial rates and total progress curves of reaction between **1** and amino acids were measured spectrophotometrically at 226 nm by means of a Hewlett-Packard 8453 diode array spectrophotometer. Reaction mixtures (100 μL) generally contained **1** (0.2−1.0 mM), DD-peptidase (20 nM), and amino acid acceptor (0−200 mM).

A hplc method was also employed, particularly for the dipeptide acceptors whose absorption at 226 nm precluded application of the direct spectrophotometric method described above. Reaction mixtures (200 μL) were prepared as described above and incubated at 25 °C. At appropriate times, samples (15 μL) were withdrawn and the reaction quenched by addition to 15 μL portions of an aqueous solution containing 3% acetonitrile, 0.2% trifluoroacetic acid, and 0.2 mM ϵ-*N*-acetyllysine, the latter as a concentration reference.

The samples were then analyzed by injection into a Varian Prostar 210 hplc system equipped with a Varian 340 UV/vis detector. Separation was achieved on a Macherey-Nagel Nucleosil 5 C-18 column with a mobile phase of 3% v/v acetonitrile/water mixture, also containing 0.05% trifluoroacetic acid. A flow rate of 0.9 mL/min was employed and the effluent monitored at 215 nm. Under these conditions, retention times of ϵ-*N*-acetyllysine (used as a reference), **1**, and its hydrolysis product were 4.17, 12.62, and 10.96 min, respectively. New peaks appeared from transpeptidation reactions; for example, retention times for glycyl-L-α-amino-ϵ-pimelyl-D-alanylglycine and glycyl-L-α-amino-ϵ-pimelyl-D-alanyl-glycyl-L-alanine were 8.19 and 14.20 min, respectively. Product fractions were collected and dried and their solutes' identity confirmed by mass spectrometry (Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois). Relative concentrations of products could be obtained from peak areas with the aid of directly determined extinction coefficients or by difference (aminolysis product = original reactant − hydrolysis product). Kinetics data from the acyl acceptors other than D-alanine were fitted to appropriate reaction schemes by means of the Dynafit program (*28*).

Steady state parameters for the hydrolysis of peptides **2**−**4** were generally obtained from spectrophotometric initial rate measurements at 220 nm. Reaction mixtures (100 μL) contained the peptide substrate (0−1 mM) and enzyme (0.1 μM). Where necessary, better estimates of K_m values were obtained from spectrophotometric (290 nm) competition experiments using *m*-[[(phenylacetyl)-D-alanyl]oxy]benzoic acid (1.0 mM; K_m = 1.0 mM) as substrate (*15*); enzyme and peptide (inhibitor) concentrations were 0−1.3 mM.

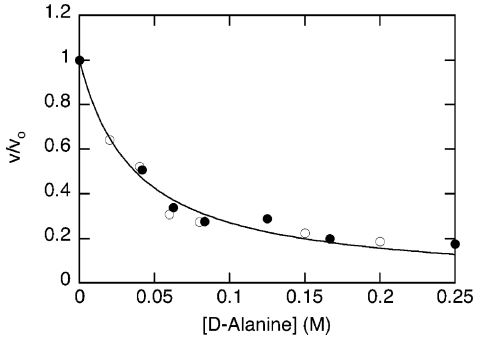


FIGURE 1: Initial rates of turnover of **1** (●, 0.5 mM; ○, 1.0 mM) by the R61 DD-peptidase (20 nM) as a function of D-alanine concentration at pH 7.6. The points are experimental, and the line was obtained from fitting the data to eq 1.

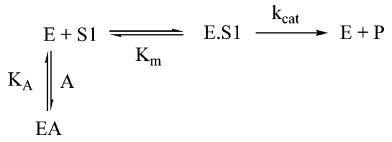
Molecular Modeling. The computations were performed by means of an sgi octane2 workstation with the InsightII 2000 suite of molecular modeling programs (Accelrys, San Diego, CA). The starting points for the models constructed were previously assembled models of **1** bound to the R61 DD-peptidase, either in a noncovalent (prereaction) complex or as a tetrahedral intermediate. These models, in turn, were constructed from the relevant crystal structures (*17*, *18*). In each case, the Lys 65 and Tyr 159 side chains were cationic and anionic, respectively, in the noncovalent complexes, and cationic and neutral, respectively, in the tetrahedral intermediates. Molecular dynamics simulations (200 ps), where a number of different ligand starting conformations were often separately tested, were run on each complex and typical snapshots selected and subjected to energy minimization. The relative strengths of interactions between the enzyme active site and the enantiomers or conformers of particular acyl acceptors were calculated as E_{int} values from the energy-minimized structures (*29*). These E_{int} values include interactions between the ligand and active site residues, interactions between the active site residues themselves, and internal energies of the ligand and active site residues. Active site residues included in these calculations were Ser 62, Lys 65, Phe 120, Thr 123, Tyr 159, Asn 161, Trp 233, Arg 285, His 298, Thr 299, Gly 300, Thr 301, Ser 326, and Asn 327.

RESULTS AND DISCUSSION

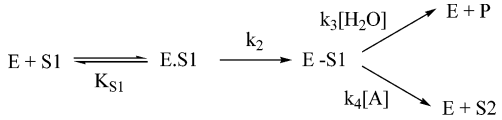
Early studies of the transpeptidation reaction catalyzed by the *Streptomyces* R61 DD-peptidase, using *N*,*N*′-diacetyl-L-lysyl-D-alanyl-D-alanine as the acyl donor, indicated that D-amino acids and Gly-Xaa dipeptides were effective acceptors (*14*, *30*, *31*). This result is in accord with the transpeptidation reaction that may be catalyzed by this enzyme in vivo (Scheme 2), where the leaving group would be D-alanine and the nucleophile a glycyl-L-α-amino-ϵ-pimelyl amine terminus. One would therefore anticipate that D-alanine (and perhaps other D-amino acids) and Gly-L-Xaa peptides would be acyl acceptors during the reaction of **1** with the enzyme.

Figure 1 shows the variation of the rate of reaction of **1** at saturating concentrations with the concentration of D-alanine,

Scheme 3



Scheme 4



catalyzed by the R61 DD-peptidase at pH 7.6 and followed spectrophotometrically at 226 nm. The observed rate obviously decreases with D-alanine concentration. The two most likely interpretations of this behavior are those of Schemes 3 and 4 [where S1 is the peptide substrate, A is the acyl acceptor, E.S1 is a noncovalent complex of E and S, E-S1 is the acyl-enzyme intermediate, P is the hydrolysis product glycyl-L-α-amino-ϵ-pimelyl-D-alanine, and S2 is the transpeptidation (aminolysis) product], where, in Scheme 3, D-alanine behaves as a classical competitive inhibitor while, in Scheme 4, it acts as an acyl acceptor, essentially a product inhibitor, intercepting the acyl-enzyme and, in effect in this case, regenerating the substrate (S2 = S1 if A = D-alanine). From Schemes 3 and 4, eq 1 can be derived, showing, in each case, how the observed steady state initial rate (as a fraction of the rate v_0, in the absence of the amino acid) should vary with [A] (D-alanine concentration). Under [S1] $\gg K_m$ conditions, according to Scheme 3, the effect of [A] on v, if any, will decrease as [S1] increases, i.e., K will decrease.

$$v/v_0 = (1 + K[\mathrm{A}])^{-1} \qquad (1)$$

Scheme 3: $K = K_m/\{(K_m + [\mathrm{S1}])K_A\}$

Scheme 4: $K = k_4/(k_3[\mathrm{H_2O}])$

In the case of Scheme 4, v/v_0 will decrease with [A] to the same extent at all [S1], i.e., K should be independent of [S]. The data of Figure 1, showing results of experiments at concentrations of **1** of 0.5 mM and 1.0 mM, yields values of K of 25.0 ± 2.5 M^{-1} and 28.0 ± 3.5 M^{-1}, respectively, which suggest that Scheme 4 is more likely to be correct. Under this interpretation, the data can be combined, as shown in Figure 1, to give a K value of (26.8 ± 1.6) M^{-1}, which yields a partition ratio k_4/k_3 value of 1490 if [H_2O] = 55.5 M (note that both k_3 and k_4 in Scheme 4 are second-order rate constants); at this pH, at least, D-alanine is thus a much more effective acyl acceptor than water.

The situation with other amino acid acyl acceptors was more complicated since the products of transpeptidation, S2, were themselves substrates of the enzyme and hydrolyzed after their formation—i.e., thermodynamically, in all cases the final product should be the hydrolysis product P. In order to obtain kinetics data for such a system it was necessary to distinguish reactants, intermediate products, S2, and final (hydrolysis) products, a distinction that could not be made from UV absorbance data alone. Thus, spectrophotometric data was supplemented with hplc data where the concentrations of S1, S2, and P could be monitored directly as a function of time. Figure 2 shows data for glycine. The

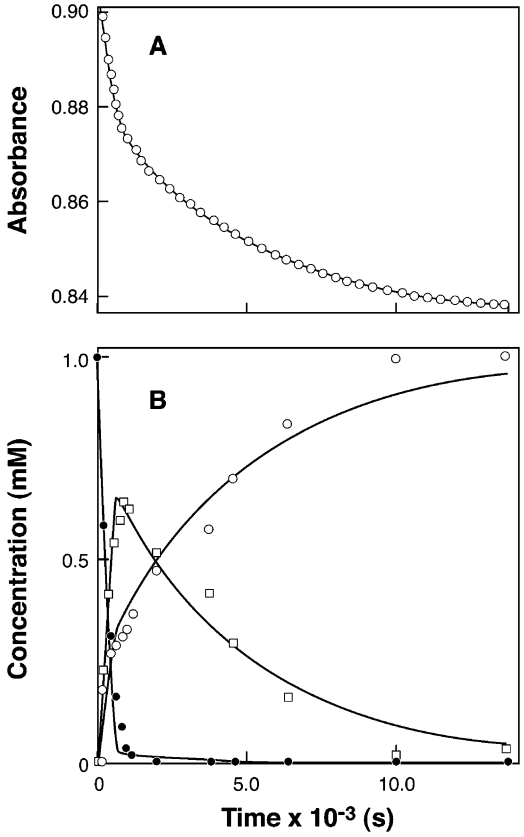
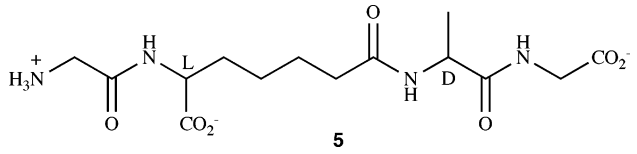


FIGURE 2: Time course of reaction of **1** (1.0 mM) with glycine (200 mM) in the presence of the R61 DD-peptidase (20 nM). The upper panel shows the absorption at 226 nm as a function of time. The lower panel shows the concentrations of **1** (●), the transpeptidation product **5** (□), and the hydrolysis product, glycyl-L-α-amino-ϵ-pimelyl-D-alanine (○), all obtained from hplc analysis, as a function of time. The points are experimental, and the lines were obtained by fitting the data to Scheme 5.

absorbance at 226 nm shows a two-phased decrease with time. The hplc data shows that the faster phase corresponds to formation of S2 and the slow phase to its disappearance. S2 was identified in this case as glycyl-L-α-amino-ϵ-pimelyl-D-alanylglycine, **5**, by mass spectrometry (ES^+, *m/e* 361.5).



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Quantitative treatment of data such as presented in Figure 2 has previously been complicated, as mentioned in the introduction, by noncompetitive and nonproductive substrate binding modes (*14*, *15*). The same problem, also with small molecule substrates, has been observed with class C β-lactamases (*32*, *33*). It was important therefore to check whether these problems also obtained with **1**. The definitive experiment involves measurement of the ratio of the initial rates of aminolysis to hydrolysis (v_A/v_H) as a function of substrate concentration (*30*−*32*). If the simple Scheme 4 is a good representation of the situation, v_A/v_H will be independent of [S1] and determined only by the partitioning of E-S1, whereas if an additional binding site for S1 is present, v_A/v_H will generally be a function of [S1] because there will be two covalent acyl enzyme species, E-S1 and E-S1.S1, which

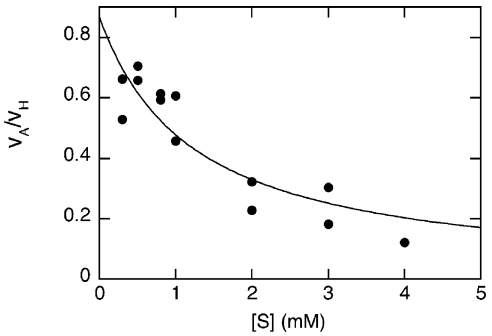


FIGURE 3: Ratios of rates of aminolysis to hydrolysis on reaction of **1** with glycine (100 mM) in the presence of the R61 DD-peptidase (20 nM) as a function of the concentration of **1**. The points are experimental, and the line was obtained by fitting the data to eq 2.

Table 1: Kinetic Parameters (Scheme 5) for Transpeptidation by the *Streptomyces* R61 DD-Peptidase

acyl acceptor	pH	$(k_4/k_3) \times 10^{-3}$	$k_{-4} \times 10^{-4}$ (s^{-1} M^{-1})
D-alanine	7.6	1.49 ± 0.09	870[a]
glycine	7.6	0.53 ± 0.01	3.0 ± 0.1
D-leucine	7.6	1.31 ± 0.09	7.2 ± 0.4
D-norleucine	7.6	2.0 ± 0.6	19 ± 6
D-alanine	8.9	6.3 ± 1.0	300 ± 10
D-norleucine	8.9	16.7 ± 0.9	29 ± 2
glycinamide	8.9	*b*	*b*
phenylethylamine	8.9	*b*	*b*
L-alanine	7.6	*b*	*b*

[a] Taken from ref 16. [b] No aminolysis observed.

will, in general, partition differently. Figure 3 shows the results of such an experiment for the reaction of **1** with glycine as A, also at pH 7.6. These data were fitted to eq 2 (*33*), where K_1 is the dissociation constant of S1 from the second binding site. The data of Figure 3 yielded values of

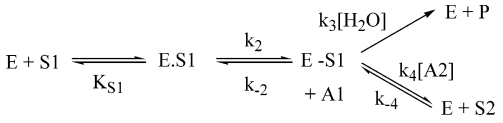
$$v_A/v_H = K_1(k_4/k_3)[A]/(K_1 + [S1]) \qquad (2)$$

k_4/k_3 and K_1 of 480 ± 60 [in good agreement with the value from the direct kinetics experiment (Table 1, see below)] and (1.2 ± 0.4) mM, respectively. The results show that, even for **1**, evidence of additional binding is observed at millimolar concentrations. The effect of the binding of a second molecule of **1** to the enzyme is to divert the acyl-enzyme from aminolysis to hydrolysis, an effect previously observed with this DD-peptidase (*15*) and also with a class C β-lactamase (*32*, *33*). The aminolysis reaction generally seems more sensitive to local structural perturbation than is hydrolysis (*13*). The presence of an additional binding site for **1** is an important issue. It should be noted, however, that the dissociation constant of **1** from the active site is 7.9 μM, whereas that from the nonproductive second site is some 150 times higher; the biological relevance of the latter is thus questionable.

In view of these results, where it seemed that the effects of second site binding of **1** would not be large at substrate concentrations of 1 mM and below, we quantitatively interpreted the data of Figure 2 according to Scheme 5 (where A1 represents D-alanine and A2 the alternative acyl acceptor, glycine in the case described above).

In fitting these data to the scheme [Dynafit (*28*)], K_{S1} was assumed to be the K_m of **1** (7.9 μM), k_2 to be k_{cat} for **1** (85 s^{-1} M^{-1}), and $k_{-2}/k_3 = 1490$ (see above); note that $k_{-2} = k_4$ when A1 = A2 and thus S1 = S2. Note also that it is

Scheme 5

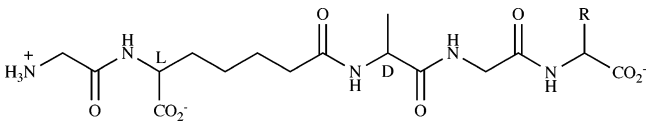


implicitly assumed in this treatment that $k_3 \gg k_2$, i.e., acylation of the enzyme by **1** is rate determining at saturation; evidence for this assumption has been previously obtained (*16*). The lines shown in Figure 2 then gave $k_4/k_3 = (530 \pm 10)$ and $k_{-4} = (3.0 \pm 0.1) \times 10^4$ s^{-1} M^{-1}. These results show that glycine is a somewhat poorer acyl acceptor than D-alanine and that S2 (**5**) is a considerably (300-fold) poorer substrate of the enzyme than is **1**. These conclusions are in qualitative accord with those reached from experiments with nonspecific substrates (*12*, *30*).

Similar experiments were performed with several other amino acids, with the results presented in Table 1. There are a number of points of interest here. First, the more hydrophobic amino acids, D-leucine and D-norleucine, are comparably effective as acyl-acceptors as D-alanine, with D-norleucine somewhat better than the latter. This appears to correlate with the k_{-4} value, effectively k_{cat}/K_m, for the S2 peptides as substrates: D-norleucine > D-leucine > glycine. In accord, also, with previous results with nonspecific substrates, L-alanine is an unobservably poor acceptor (*30*); *N*,*N*′-diacetyl-L-lysyl-D-alanyl-L-alanine is also not a substrate (*12*).

The ability of D-amino acids with respect to water as acyl acceptors increases with pH. For example, values of k_4/k_3 for D-alanine increased as follow: 650 (pH 6.5), 1490 (pH 7.6), 2740 (pH 8.3), 6290 (pH 8.9), and 13 700 (pH 9.5). This suggests, as might be expected, that the reactive forms of the acceptors are the free amines. Simple amines, however, do not act as acceptors—glycinamide and phenethylamine have no such activity, for example, indicating the importance of a D-amino acid carboxylate group.

A variety of dipeptide mimics of the putative in vivo acyl acceptor were then tested. The reactions of these with **1** were studied by means of the hplc method since direct spectrophotometric observation was not possible because of peptide absorption. These experiments were performed at pH 8.9, to take advantage of the more efficient aminolysis at high pH. At this pH, the steady state parameters for hydrolysis of **1** were $k_{cat} = (100 \pm 10)$ s^{-1}, $K_m = (45 \pm 3)$ μM, $k_{cat}/K_m = 2.3 \times 10^6$ s^{-1} M^{-1}. An example of the results obtained is shown in Figure 4, for glycyl-L-phenylalanine. Similar data were obtained with other dipeptides. The aminolysis product was identified by mass spectroscopy. For example, the product from glycyl-L-alanine, **6a**, had the expected *m*/*e* value



6a: R = L-Me
6b: R = D-Me
6c: R = L-CH_2Ph

(ES^+) of 432.60. These data were fitted to Scheme 5, as described above, yielding the results presented in Table 2. It is noticeable that, as with the products of aminolysis by amino acids, the products of the transpeptidation reaction

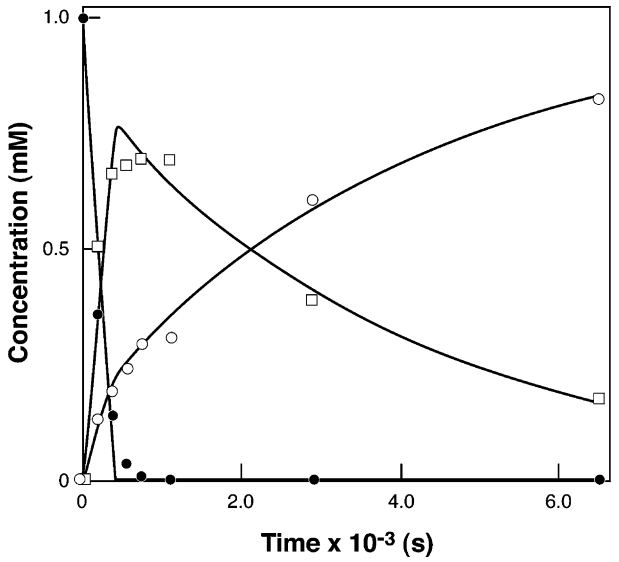


FIGURE 4: Time course of reaction of **1** (1.0 mM) with glycyl-L-phenylalanine (10 mM) in the presence of the R61 DD-peptidase (20 nM) showing the concentrations of **1** (●), the transpeptidation product **6c** (□), and the hydrolysis product, glycyl-L-α-amino-ϵ-pimelyl-D-alanine (○), as a function of time. The points are experimental, and the lines were obtained by fitting the data to Scheme 5.

Table 2: Kinetic Parameters (Scheme 5) for Transpeptidation by the *Streptomyces* R61 DD-Peptidase with Dipeptide Acyl Acceptors[a]

acyl acceptor	$(k_4/k_3) \times 10^{-3}$	$k_{-4} \times 10^{-4}$ (s^{-1} M^{-1})
Gly-L-Ala	5.5 ± 0.2	2.7 ± 0.2
Gly-D-Ala	*b*	*b*
Gly-L-Phe	20 ± 2	5.7 ± 0.6
Gly-L-Leu	0.59 ± 0.14	1.7 ± 1.0
Gly-L-Met	3.5 ± 0.3	≤0.2
Gly-L-Nle	*b*	*b*
Gly-L-aminocaprylic acid	*b*	*b*
Gly-L-Lys(Ac)	*b*	*b*
D-Ala-L-Ala	*b*	*b*

[a] All experiments at pH 8.9. [b] No aminolysis observed.

by dipeptides were, in general, also substrates of the DD-peptidase, i.e., the transpeptidation product disappeared with time (see Figure 4) to generate more of the hydrolysis products. Hplc experiments demonstrated that the products were glycyl-L-α-amino-ϵ-pimelyl-D-alanine and the original dipeptide. In order to unequivocally demonstrate this point, the peptide **2** was prepared and shown to be a substrate of the enzyme [$k_{cat} = (5.1 \pm 0.7)$ s^{-1}, $K_m = (0.75 \pm 0.18)$ mM, and $k_{cat}/K_m = 6870$ s^{-1} M^{-1} at pH 8.9]. This appears to be a much poorer substrate than **1**, but it should be noted in that regard that the presence of the sulfur in **2** may reduce its performance, perhaps by 30-fold in k_{cat}/K_m (*18*). These results show that, as one would expect, the enzyme can also catalyze the reverse reaction of peptide products and, thermodynamically, the final products are hydrolysis products. Thus the R61 DD-peptidase has formal endopeptidase activity. Such activity was previously noticed by Frère and Ghuysen (*14*, *34*).

The results show that Gly-L-Ala, Gly-L-Phe, Gly-L-Leu, and Gly-L-Met are acceptors but Gly-D-Ala is not. This result is in general accord with expectations that an in vivo acceptor for this enzyme would be a glycyl-L-α-aminopimelyl derivative. The apparent relative reactivity of these acceptors, however, may be modified by their accompanying inhibitory ability [see the accompanying paper (*19*)] and should not be overinterpreted at this point. It was rather striking, in view of these results, that Gly-L-Nle did not seem to be an acceptor; this was unexpected because its aliphatic chain might be expected to be a good mimic of that of pimelic acid. In order to exclude the possibility that the product peptide was an excellent substrate and thus would not have been detected as an intermediate, the peptide **3** was prepared and its reactivity with the enzyme investigated. The peptide **3**, however, was a much poorer substrate than **2** ($k_{cat}/K_m \leq 1000$ s^{-1} M^{-1} at pH 8.9), and thus the analogous peptide product from **1** should have been observed if it was in fact formed during a reaction between **1** and Gly-L-Nle. The (low) reactivity of Gly-L-Nle as an acceptor correlates well with that of **3** as an acyl donor. Other extended analogues of glycyl-L-alanine, viz., Gly-L-Lys(Ac) and glycyl-L-α-aminocaprylic acid, were also inactive as acceptors. This result is addressed further in the accompanying paper (*19*).

Another significant result is that D-Ala-L-Ala did not appear to be an acceptor in that no aminolysis product was observed in the hplc experiment. The absence of this product was also not due to its unexpectedly rapid hydrolysis by the enzyme since the analogue **4** was only a modest substrate of the DD-peptidase [$k_{cat} = (3.3 \pm 1.5)$ s^{-1}, $K_m = (0.92 \pm 0.08)$ mM, and $k_{cat}/K_m = 3450$ s^{-1} M^{-1} at pH 8.9], in accord with the observations with **2** and **3**, described above. D-Ala-L-Ala can be seen as a "first generation" dual specificity acceptor, combining the features of D-Ala and Gly-L-Ala. That it is, in fact, not an acceptor is rather striking because, if on binding of Gly-L-Ala to the enzyme as an acyl acceptor, the peptide carbonyl simply replaced the D-alanine carboxylate, then D-Ala-L-Ala would be expected to be an acceptor. The negative result suggests that the amide carbonyl of dipeptide acceptors does not occupy the same position when bound to the active site as the carboxylate of D-amino acid acceptors.

The results reported above are thus, to a first approximation, what one might expect of in vivo acceptors and also in general accord with previous results with smaller and less specific substrates. At the time of the early work, however, the crystal structure of the R61 DD-peptidase was not available. It is useful, therefore, at the current vantage point, to attempt a structural rationalization of the transpeptidation results. The issues to be considered are (i) the reasons for the stereospecificity of amino acid and Gly-Xaa acceptors, D in the former and L in the latter, and (ii) how amino acids and dipeptides with the observed stereospecifity occupy the dual function acceptor binding site. The latter issue, as discussed in the introduction, is a general one to transpeptidases.

Molecular Modeling. A common property of effective acceptor molecules is the presence of a carboxylate group. If the carboxylate is omitted or modified so as to remove the negative charge, the reactivity of the molecule as an acceptor decreases dramatically (Table 1); it is also shown in the accompanying paper (*19*) that Gly-L-PheNH_2 is not an acceptor. It seems likely that the requirement for a carboxylate reflects the importance of electrostatic interaction between the acceptor and Arg 285, the positively charged residue that, in crystal structures, interacts with the terminal carboxylate of **1** in both noncovalent prereaction and product complexes (*17*). The crystal structures also suggest that direct

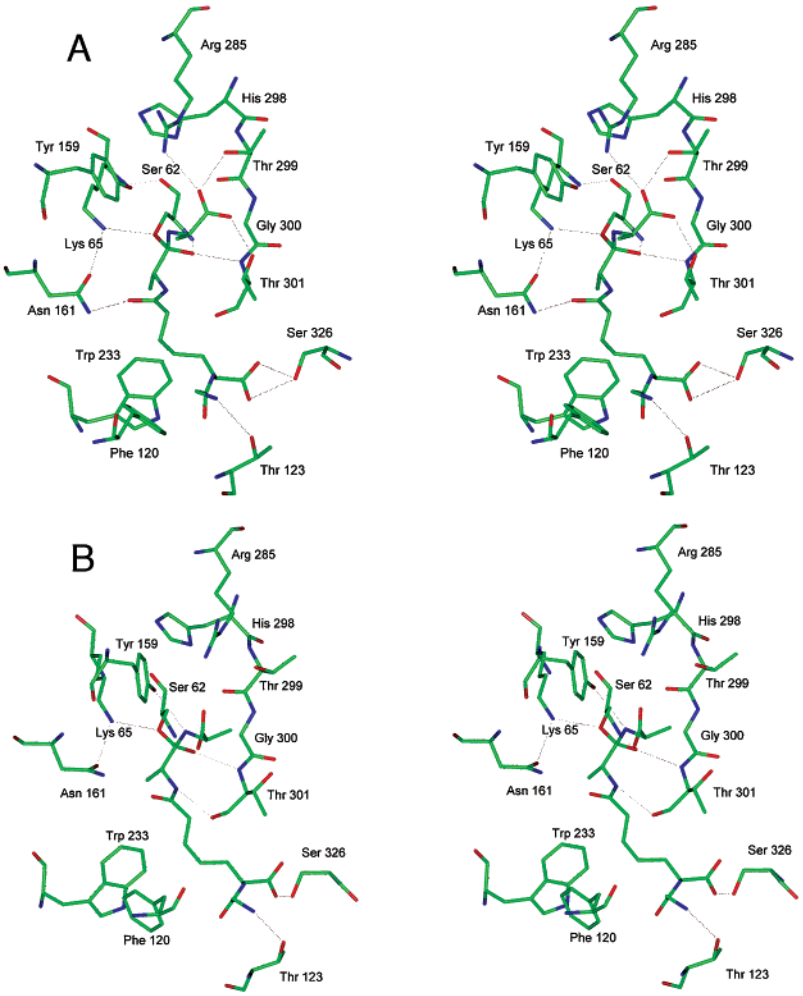
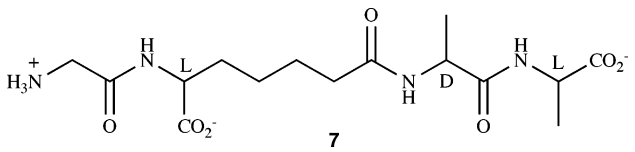


FIGURE 5: Stereoviews of energy-minimized tetrahedral intermediate structures formed on reaction of the R61 DD-peptidase with the peptides **1** (A) and **7** (B). Only heavy atoms are shown.

hydrogen bonding between the acceptor carboxylate and the side chain hydroxyl group of Thr 299 may occur. Previous modeling has shown that these interactions are also likely to be present in intermediate species (tetrahedral intermediates and transition states) (*18*). Thus, in the modeling of the peptide complexes described below, particular attention was paid to bound conformations where these interactions were possible. Noncoincidentally, presumably, such conformations did dominate the molecular dynamics simulations of effective acceptors and were, therefore, strongly reflected in the interaction energy calculations.

Figure 5A shows the energy-minimized tetrahedral adduct presumably formed transiently on reaction of **1** with the R61 DD-peptidase, and, conversely, by the principle of microscopic reversibility, on reaction of D-alanine with the acyl-enzyme generated on reaction of **1**, or an analogue of **1**, with the enzyme. Essentially all of the interactions between the enzyme and ligand anticipated on the basis of crystal structures and previous modeling (*16*, *17*) are present. In particular, distances appropriate for hydrogen bonding are present between the Lys 65 ammonium ion and Ser 62 O_γ, the tetrahedral oxyanion of the ligand and the components of the oxyanion hole (backbone NH groups of Ser 62 and Thr 301), the side chain amide group of the ligand and the Asn 161 side chain and the backbone carbonyl oxygen atom of Thr 301, and the polar side chain termini with Thr 123 and Ser 326. The close, presumably hydrophobic (van der Waals) interactions between the ligand tetramethylene chain and the nonpolar side chains of Trp 233 and Phe 120 are also evident. In this structure, the terminal D-alanine carboxylate of the ligand appears to be tightly held by hydrogen bonds/electrostatic interactions with the side chains of Arg 285, Thr 299, and Thr 301. The D-alanine methyl group is directed out of the active site into solution, although it may be in hydrophobic contact with the side chain of Tyr 159 (these calculations do not, of course, directly include contributions from the classical hydrophobic effect).

In Figure 5B, the tetrahedral adduct of **7**, the terminal L-alanine analogue of **1**, is shown. Here, although many of the favorable interactions with the active site, noted above, are present, the terminal carboxylate is directed out of the active site into solution. Although rotation about the N−C_α bond of the terminal D-alanine would bring the carboxylate into contact with Arg 285, this would also bring the L-methyl group into unfavorable contact with the ligand oxyanion and Thr 301; hence the stability of the structure shown. E_{int} calculations suggest that the interactions between the ligand



7

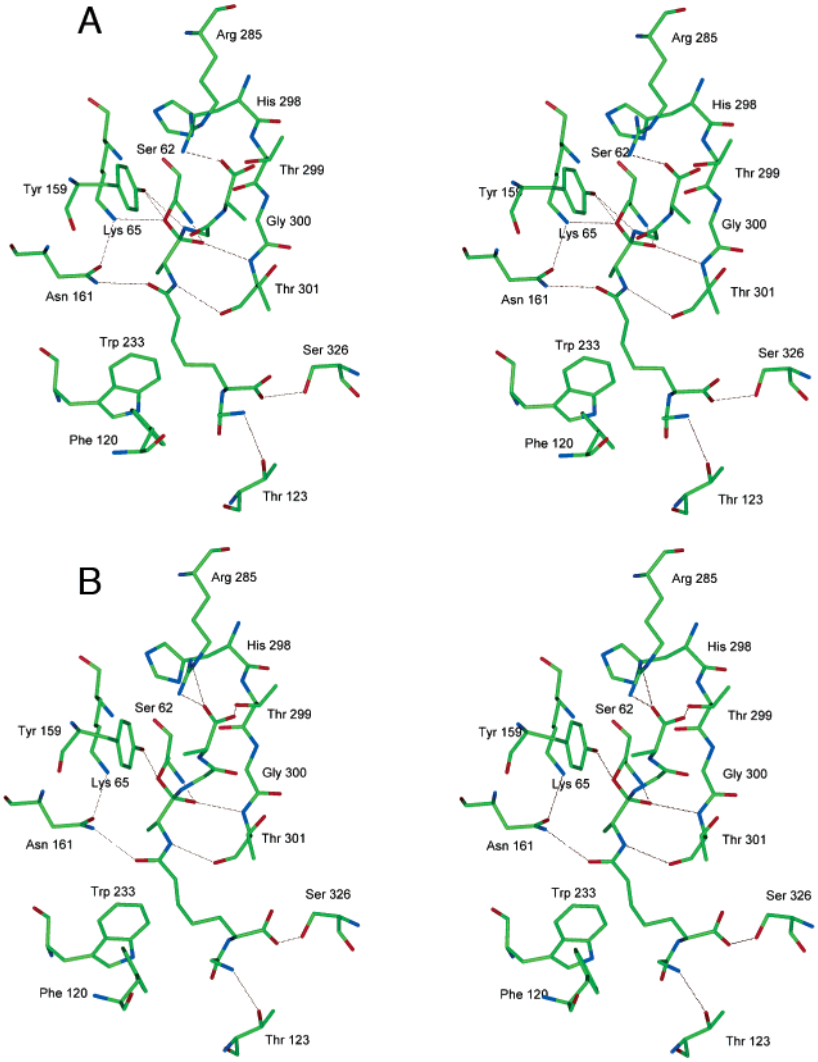


FIGURE 6: Stereoviews of energy-minimized tetrahedral intermediate structures formed on reaction of the R61 DD-peptidase with the peptides **6a** (A) and **6b** (B). Only heavy atoms are shown.

and the active site are more favorable in the D-alanine complex (Figure 5A) than in the L-alanine complex (Figure 5B) by 53.5 kcal/mol, the bulk of this difference arising from the electrostatic interactions between the carboxylate of D-alanine and the active site; this difference would probably be ameliorated to some considerable degree by the more extensive interactions between the carboxylate and water (not shown) in the structure of Figure 5B.

A similar picture emerged from analysis of the energy-minimized noncovalent complexes of **1** and **7** with the enzyme [Supporting Information; also shown in this location is an overlay of the modeled noncovalent complex of **1** with the most relevant crystal structure (*17*)]. Again, there is close interaction between the terminal carboxylate of **1** and Arg 285 and Thr 299 but not of the carboxylate of **7**, leading to E_{int} values in favor of the former complex by 64.6 kcal/mol. Both sets of models, therefore, lead to the conclusion, in accord with experiment (Table 1), that D-alanine should be a better acyl acceptor than L-alanine, and **1** a better substrate (faster acylation and therefore larger k_{cat}/K_m) than **7**.

The structures of Figure 6 show the lowest energy model structures for tetrahedral adducts of the enzyme with **6a** and **6b**. These represent the intermediates that would be achieved on reaction of the acceptors glycyl-L-alanine and glycyl-D-alanine, respectively, with the acyl-enzyme derived from **1**. All of the expected interactions are also present in these structures. In both cases, direct hydrogen bonding distances are present between the terminal carboxylate of the ligand and Arg 285 and Thr 299. The structures differ markedly in the orientations of the C═O and NH groups of the glycyl-alanine peptide bond; in neither case, however, does either of these moieties appear to directly hydrogen bond with a protein functional group. The methyl groups of **6a** and **6b** may be in hydrophobic contact with the side chains of Leu 332 and Tyr 159, respectively. Calculation of the interaction energies (E_{int}) leads to a result where the glycyl-L-alanine adduct (Figure 6A) is favored over the glycyl-D-alanine (Figure 6B) by 8.0 kcal/mol. Similar models of noncovalent complexes of **6a** and **6b** with the peptidase (Supporting Information) led to the same trend: the complex with **6a** was 21.4 kcal/mol more stable than that with **6b**. The greater difference between the noncovalent complexes may reflect the fewer degrees of freedom available to the ligand in this species, where the glycine and alanine residues are linked by a torsionally resistant amide bond. The models in this case, therefore, also agree with experiment (Table 2) in finding glycyl-L-alanine to be a better acceptor than glycyl-D-alanine.

Modeling of the noncovalent complexes of the glycyl-L-phenylalanine product **6c** with the enzyme indicated that a

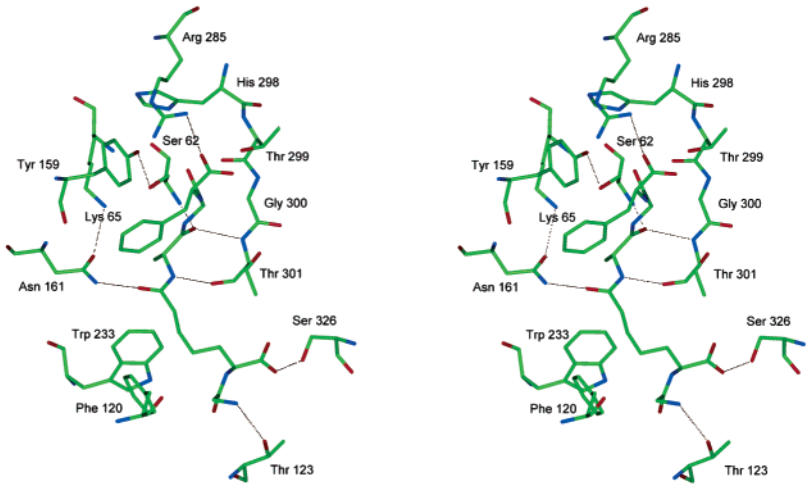


FIGURE 7: Stereoview of the energy-minimized noncovalent structure formed on interaction of the R61 DD-peptidase with the peptide **6c**. Only heavy atoms are shown.

FIGURE 8: Stereoview of the overlay of ligands **1** and **6a** in the complexes shown in Figures 5A and 6A.

conformation similar to that of **6a** in Figure 6A, where the L-phenylalanine side chain could be in van der Waals/hydrophobic contact with Leu 332, would be quite favorable. A somewhat more favorable conformation, however, in this case, by 24.4 kcal/mol, is shown in Figure 7. In this structure, the terminal carboxylate of **6c** also interacts with Arg 285 and Thr 299 but the L-phenylalanine side chain is oriented differently and may undergo hydrophobic interactions with the side chain of Tyr 159. This alternative conformation with **6c** might lead to the significantly higher activity of glycyl-L-phenylalanine than glycyl-L-alanine as an acceptor (Table 2), although it is not clear what effect the positioning of the phenyl group of L-Phe adjacent to Tyr 159 would have on the ability of the latter residue to participate as a general acid/base in catalysis. It could, however, influence the pK_a values of the active site functional groups.

Finally, Figure 8 shows the overlap of ligands from the energy-minimized tetrahedral intermediate structures for the good acceptors D-alanine and glycyl-L-alanine, i.e., from overlap of the structures of Figures 5A and 6A. It can be seen, as predicted from the experimental results (see above), that the peptide carbonyl group of the latter acceptor does not overlap in space with the carboxylate of the former and thus, as discussed in the introduction, the DD-peptidase does have, effectively, two distinct acceptor sites. They are situated, however, such that interaction with Arg 285 is possible with both of them, one from below, the other from above (Figure 8).

General Conclusions. The *Streptomyces* R61 DD-peptidase catalyzes not only the hydrolysis of the peptidoglycan-mimetic substrate **1** but also its aminolysis by specific amino acids and their derivatives (acyl acceptors): the transpeptidation reaction. This represents a good model for the transpeptidation reaction that DD-peptidases carry out in vivo to bring about the final step in bacterial cell wall synthesis. The effective acyl acceptors for the R61 enzyme in their reaction with **1**, all containing a free carboxylate group, were D-amino acids and glycyl L-amino acid dipeptides. This specificity does reflect the structure of the likely acyl donor and acceptor in vivo, and is generally similar to that observed earlier with less specific acyl donors (*14*, *15*). It was striking that glycyl-L-norleucine and Gly-L-Lys(Ac), the molecules apparently most similar to the putative in vivo acceptor, were, like **1** itself (*16*), completely inactive as acceptors. This issue is further addressed in the accompanying paper (*19*).

D-Alanine increased in effectiveness as an acceptor as the pH rose, suggesting that the bound and reactive form of the acceptor was the free amine. The transpeptidation products were also hydrolysis substrates since, in the absence of acceptors, the enzyme catalyzes a carboxypeptidation reaction. In general, the k_{cat}/K_m values of these peptides mirrored the effectiveness of the respective acceptor in the reverse (transpeptidation) reaction. The peptide **1**, which most resembles the natural substrate, was still, by far, the best substrate of those examined.

The ineffectiveness of D-Ala-L-Ala as an acceptor indicated that the carboxylate group of amino acid acceptors and the peptide carbonyl of dipeptide acceptors probably did not overlap in their binding to the enzyme and, therefore, conversely, the mode of binding of the terminal glycyl-L-alanine of **6a** must be different from that of the terminal D-alanine of **1**. As suggested in the introduction, this is not unexpected for an acyl transferase where the leaving group and acyl acceptor have different structures. This issue has not, until now, been examined in detail with bacterial DD-peptidases.

Molecular modeling produced structures that agreed with the experimental results with respect to the stereospecificity of the acyl acceptors and the different orientations of the bound D-amino acid and glycyl-L-amino acid acceptors. The

most likely binding sites for both classes of effective acceptors included Arg 285 as an anchor for the terminal carboxylate. Dual function acceptors could, in principle, be designed to take advantage of the two modes of acceptor binding. This matter is taken up in the accompanying paper (*19*).

SUPPORTING INFORMATION AVAILABLE

Details of the syntheses of peptides **2**−**4**. Stereoviews of noncovalent complexes of peptides **1**, **7**, **6a**, and **6b** with the R61 DD-peptidase. Also a figure showing overlap of the modeled noncovalent complex of **1** with a relevant crystal structure. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI0505417