Deacylation Transition States of a Bacterial DD-Peptidase†

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ABSTRACT: β-Lactam antibiotics restrict bacterial growth by inhibiting DD-peptidases. These enzymes catalyze the final transpeptidation step in bacterial cell wall biosynthesis. Although much structural information is now available for these enzymes, the mechanism of the actual transpeptidation reaction has not been studied in detail. The reaction is known to involve a double-displacement mechanism with an acyl−enzyme intermediate, which can be attacked by water, specific amino acids, peptides, and other acyl acceptors. We describe in this paper an investigation of acyl acceptor specificity and assess the need for general base catalysis in the deacylation transition state of the *Streptomyces* R61 DD-peptidase. We show, by the criterion of solvent deuterium kinetic isotope effect measurements and proton inventories, that the transition states of specific and nonspecific substrates are very similar, at least with respect to proton motion. The transition states for attack (tetrahedral intermediate formation) by D-amino acids and Gly-L-Xaa dipeptides do not include a general base catalyst, while such catalysis is essential for reaction with water and D-α-hydroxy acids. D-α-Hydroxy acids act as acyl acceptors for glycyl substrates but not for more specific D-alanyl substrates; hydroxy acids actually behave, more generally, as mixed inhibitors of the DD-peptidase. The structural and mechanistic bases of these observations are discussed; they should inform transition state analogue design.

The bacterial DD-peptidases [also known as transpeptidases or penicillin-binding proteins (PBPs)][1] catalyze the final step in cell wall biosynthesis, where the monomer unit is incorporated into the peptidoglycan polymer (*1*). These enzymes are essential to bacterial survival and, because of this, have been extensively targeted by inhibitors (antibiotics) produced by a variety of species (including *Homo sapiens*) in attempts to restrict bacterial growth. The β-lactams represent an important group of such antibiotics. Bacterial resistance to these antibiotics has risen with usage and currently presents a significant problem to their therapeutic application (*2*). Consequently, the DD-peptidases have been intensively studied in recent years in an effort to provide an informational base for novel antibiotic design.

Bacteria, in general, produce several variants of DD-peptidase enzymes, each having a specific role in the cell cycle (overlap of function between some of them also occurs) (*3*). They are all, however, evolutionarily related and have common elements of tertiary fold and active site structure. They have been classified into subgroups based on amino acid sequence homology (*4*). A general reaction scheme for these enzymes is depicted in Scheme 1. During turnover of a peptide substrate, RCONHR′, an acyl−enzyme intermediate, E−OCOR, is formed, which partitions between reaction with water (the carboxypeptidase reaction) and aminolysis (the transpeptidase reaction; R and R″ would represent adjacent peptidoglycan strands). Much structural information from X-ray crystallography is now available for these enzymes, but rather less is known about the details of their substrate specificity, kinetics, and reaction mechanism, particularly for the transpeptidation reaction. We have recently studied some elements of substrate specificity (both acyl donor and acceptor) of one particular DD-peptidase, that of *Streptomyces* R61, which has been investigated as a model DD-peptidase for many years (*5*, *6*). We have more closely established the structural features of both the enzyme and the substrate required for optimal activity (*7*−*10*). In this paper, we extend this work with a closer look at the mechanism of acyl transfer from the acyl−enzyme intermediate, generated from both specific (**1**−**3**) and nonspecific (**4**−**7**) substrates, to amine and hydroxyl acceptors. We have focused, particularly, on the requirement for general acid−base catalysis in these reactions. The results provide a more accurate definition of the important transition states involved in the transpeptidation reaction.

EXPERIMENTAL PROCEDURES

Materials. The DD-peptidase of *Streptomyces* R61 was generously provided by J.-M. Frère of the University of Liège (Liège, Belgium). Concentrations of enzyme stock solutions were determined spectrophotometrically by means of the published extinction coefficient of 3.9×10^4 cm^{-1} M^{-1} (*11*). Substrates **1**−**6** and the amine acceptor aminomalon-*N*-ethylamide were available from previous studies in this laboratory (*8*, *9*, *12*−*14*). *N*,*N*′-Diacetyl-L-lysyl-D-alanyl-D-alanine (**7**) was purchased from Sigma-Aldrich Corp.

*N-(Glycolyl)-L-phenylalanine (**13**).* First, *N*-(*tert*-butoxyacetyl)-L-phenylalanine *tert*-butyl ester was prepared from the condensation of L-phenylalanine *tert*-butyl ester (Chem-

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[1] Abbreviations: EDC, *N*-[(dimethylamino)propyl]-*N*′-ethylcarbodiimide; HOBT, 1-hydroxybenzotriazole; MOPS, 3-morpholinopropanesulfonic acid; NMR, nuclear magnetic resonance; PBP, penicillin-binding protein.

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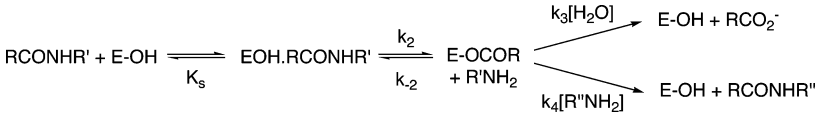
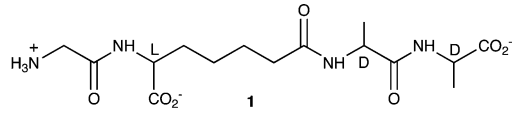
Scheme 1

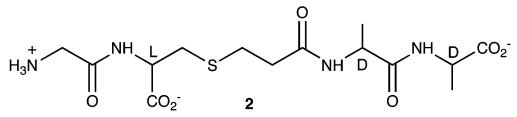


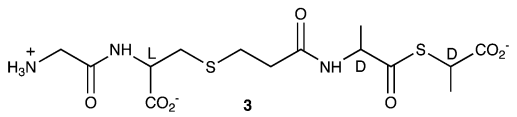
Impex) with *tert*-butoxyacetic acid (Oakwood Products, Inc.) (*15*). Thus, *tert*-butoxyacetic acid (200 mg, 1.51 mmol) was dissolved in ethyl acetate (7.5 mL), and sequentially, water (15 mL), L-phenylalanine *tert*-butyl ester hydrochloride (389 mg, 1.51 mmol), potassium carbonate (123 mg, 0.89 mmol), and HOBT (204 mg, 1.52 mmol) were added. The mixture was chilled in an ice bath with stirring and EDC (322 mg, 1.6 mmol) added. The resulting mixture was stirred overnight at 4 °C. An aqueous solution of 2 M citric acid (20 mL) was then added to the reaction mixture, and the layers were separated. The organic phase was further washed sequentially with citric acid (0.5 M, 2 × 20 mL), brine (10 mL), aqueous sodium bicarbonate (1 M, 2 × 20 mL), and brine (2 × 10 mL). It was dried over anhydrous sodium sulfate and evaporated to dryness to afford the solid product (371 mg, 73%): ^{1}H NMR (300 MHz, d_6-DMSO) δ 1.11 (9H, s, t-Bu), 1.36 (9H, s, t-Bu), 3.03 (2H, d, J = 6.6 Hz, CH_2), 3.75 (2H, s, CH_2O), 4.47 (1H, q, J = 7.5 Hz, CH), 7.24 (5H, m, ArH), 7.41 (1H, d, J = 7.8 Hz, NH).

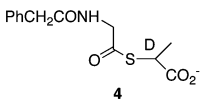
This material (169 mg, 0.54 mmol) was dissolved in 400 μL of dry, ice-cooled, dichloromethane and trifluoroacetic acid (8.4 mL) added (*16*). The mixture was stirred at 4 °C for 8−9 h and then evaporated to dryness under vacuum. The residue was suspended in water (1 mL) and brought to pH 7.0 by the addition of solid $NaHCO_3$ (1−2 equiv). The solution was freeze-dried and the residue purified by elution with water from a Sephadex G10 column: yield 80 mg (67%); ^{1}H NMR (300 MHz, D_2O) δ 2.99 (1H, dd, J = 7.5, 13.8 Hz, CHO), 3.21 (1H, dd, J = 4.8, 14.4 Hz, CHO), 3.97 (2H, ABq, J = 16.8 Hz, CH_2), 4.49 (1H, dd, J = 7.5, 5.3 Hz, CH), 7.3 (m, 5H, ArH).

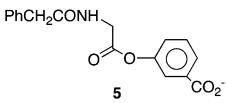
*N-(Glycolyl)-L-alanine (**12**).* This was prepared in 50% overall yield from L-alanine *tert*-butyl ester and *tert*-butoxyacetic acid as described above for the phenylalanine analogue: ^{1}H NMR (300 MHz, D_2O) δ 1.36 (3H, d, J = 7.2 Hz, CH_3), 4.08 (2H, s, CH_2), 4.21 (1H, q, J = 7.2 Hz, CH).

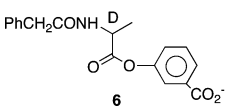


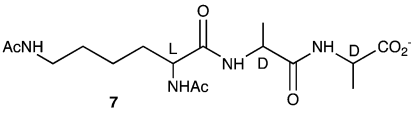










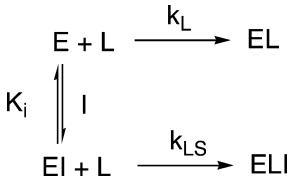


Kinetics. Kinetics measurements were carried out in either 10 mM phosphate buffer (**1** and **2**) or 20 mM MOPS buffer (**3**−**6**) at pH 7.5 and 25 °C, unless specified otherwise. The reactions of **1**−**6** were monitored spectrophotometrically at appropriate wavelengths: 224 nm ($\Delta\epsilon$ = 50 cm^{-1} M^{-1}) for **1**, 228 nm ($\Delta\epsilon$ = 50 cm^{-1} M^{-1}) for **2**, and 290 nm ($\Delta\epsilon$ = 1760 cm^{-1} M^{-1}) or 300 nm ($\Delta\epsilon$ = 950 cm^{-1} M^{-1}) for **5** and **6**. Reactions of **3** and **4** were generally carried out in the presence of 4,4′-dipyridyl disulfide (1.0 mM), and the release of 4-thiopyridone was monitored at 350 nm ($\Delta\epsilon$ = 2640 cm^{-1} M^{-1}) (*12*). With aliphatic acyl acceptors, the reactions of **3** and **4** could be monitored directly at 240 nm ($\Delta\epsilon$ = 2230 cm^{-1} M^{-1}) and 270 nm ($\Delta\epsilon$ = 100 cm^{-1} M^{-1}), respectively. Steady state kinetics parameters for **3**, **4**, and **6** were obtained from initial rate measurements; the data were fitted to the Henri−Michaelis−Menten equation by means of a nonlinear least-squares computer program. The stopped-flow measurements were obtained from a Durrum D110 instrument. Equal volumes of solutions of the enzyme (final concentration of 0.6 μM) and **1** (50, 100, or 200 μM) were mixed. The protein fluorescence beyond 300 nm was monitored as a function of time, following excitation at 282 nm. The data were fitted to Scheme 6 (see below) using Dynafit (*17*).

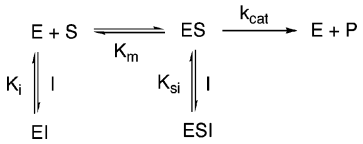
Solvent deuterium kinetic isotope effects on V for the R61 DD-peptidase-catalyzed reactions of **1**−**5**, in the absence and the presence of acyl acceptors, were obtained from spectrophotometric initial rates, essentially as previously described (*18*, *19*). The concentrations of the substrate that was used were at least 5K_m for ^{D_2O}V (the convention is used throughout whereby ^{D_2O}X represents the solvent kinetic isotope effect on X, i.e., X^{H_2O}/X^{D_2O}). Small corrections for the lack of complete saturation were made where appropriate (*18*). Reactions were initiated by the addition of a small volume of a solution of the enzyme in 1/1 (v/v) H_2O/D_2O MOPS buffer. For measurements of isotope effects at pH 8.9, a 10 mM Tris buffer was employed. At the pH values that were employed, k_{cat} is essentially invariant (*10*, *20*). Proton inventories on V for the hydrolyses of **1**, **3**, and **4** were determined at pH(D) 7.5, as described by Stein et al. (*21*). Initial rates were determined (in at least triplicate) in solutions containing D_2O mole fractions of 0, 0.5, and 1.0. This procedure has been shown to be as effective in determining the general shape of an inventory as that in which several mole fractions of D_2O are employed (*22*). As described above, the addition of aliquots of a common enzyme stock solution was used to initiate the reactions. Fits of the experimental proton inventories to an appropriate Gross−Butler equation (eq 2) were made by means of a nonlinear least-squares computer program.

The effects of added acyl acceptors, D-amino acids, dipeptides, and D-α-hydroxy acids on V were determined spectrophotometrically, as described above; concentrations

Scheme 2



Scheme 3



of the acceptors were in the 0−50 mM range. Values of k_4/k_3 (Scheme 1) for the acceptors were determined from the slopes of plots of the initial rate versus acceptor concentration at low acceptor concentrations where no specific binding of acceptor to enzyme need be considered (*9*). Values of $^{D_2O}k_4$ could be determined from the measured values of $^{D_2O}(k_4/k_3)$ and $^{D_2O}k_3$. The solvent isotope effect on the amine pK_a of glycine was determined by measurements of the pH(D) of solutions containing 25 mM glycine and 25 mM sodium glycinate in H_2O and D_2O.

Inhibition of substrate hydrolysis by certain amino acid analogues was found to be of the mixed type. In a number of cases, the competitive contribution to this inhibition (K_i) was determined from measurements of the rate constants for inhibition of the reaction between the R61 DD-peptidase and the fluorescent dansylpenicillin according to Scheme 2 (*7*), where L represents dansylpenicillin and I the inhibitor. The concentrations of E, L, and I were typically 0.6 μM, 200 μM, and 0−50 mM, respectively. The decrease in fluorescence intensity at 320 nm was monitored as a function of time after excitation at 280 nm, and the data were fitted to an exponential function to obtain pseudo-first-order rate constants (k_{obs}) of inactivation (*7*). These constants, measured at several inhibitor concentrations, were fitted to eq 1 by a nonlinear least-squares procedure

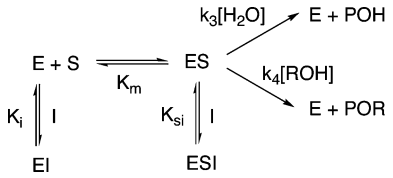
$$k_{obs} = \frac{k_L + (k_{LS}/K_i)I_0}{1 + I_0/K_i} \quad (1)$$

where I_0 represents the initial concentration of the inhibitor. The uncompetitive contribution could then be obtained from experiments where initial rates of reaction of a substrate were determined at a series of inhibitor concentrations. These results were fitted to Scheme 3 by Dynafit (*17*), where K_i was fixed to the value derived from the fluorescence experiment described above. The results were plotted as described by Cornish-Bowden (*23*).

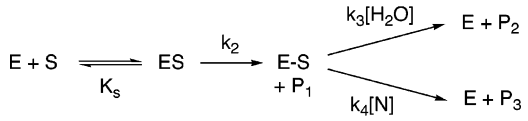
In cases where D-α-hydroxy acids acted as both acyl acceptors and mixed inhibitors, initial rate measurements at a series of hydroxy acid concentrations were fitted to Scheme 4 with Dynafit (*17*); here also, K_i was fixed at the value obtained from the relevant fluorescence experiment.

The presence or absence of acyl transfer from **5** and **6** to D-mandelate was determined from ^{1}H NMR spectra of reaction mixtures containing 2 mM depsipeptide (ester), 10 mM D-mandelate, and 2 mg of $NaHCO_3$ in 0.6 mL of D_2O. The reaction was initiated by addition of the R61 DD-peptidase (4 μM), and NMR spectra were then collected at appropriate times.

Scheme 4



Scheme 5



RESULTS AND DISCUSSION

As described in the introductory section, bacterial DD-peptidases catalyze the hydrolysis and aminolysis of C-terminal D-Ala-D-Ala peptides and analogues of these substrates by the kinetic mechanism of Scheme 5. In this scheme, ES represents the noncovalent complex of the enzyme and substrate, E-S represents the covalent acyl−enzyme intermediate formed on acylation of the active site serine residue by the substrate, with release of the leaving group as P_1, N represents an alternative nucleophile, amine or hydroxy acid, and P_2 and P_3 represent the hydrolysis (acid) and aminolysis/alcoholysis (amide/peptide/ester) products, respectively.

The nature of the rate-determining step, i.e., the relative size of k_2 and k_3, has been determined already for a variety of substrates for the *Streptomyces* R61 DD-peptidase (*14*, *18*, *20*, *24*, *25*). For example, deacylation is usually rate-determining for (thio) ester substrates; those employed in this study include thioglycolate, thiolactate, and *m*-hydroxybenzoate depsipeptides (*14*, *18*, *24*, *25*). This conclusion is readily obtained from a simple experiment: amine acyl acceptors, N in Scheme 5, accelerate the steady state reaction rate under saturating substrate (S) conditions if deacylation is rate-determining. Recently, this generality was also shown to be true for the thiodepsipeptide **3** with a side chain specific for the R61 DD-peptidase (*9*).

The situation with peptide substrates has been more problematic. It was demonstrated some years ago that acylation was rate-determining for the nonspecific peptide *N*,*N*′-diacetyl-L-lysyl-D-alanyl-D-alanine (**7**) (*20*). Initial experiments with the specific peptide **1** were also interpreted in terms of rate-determining acylation (*7*). These were, first, the absence of an effect of D-lactate on steady state turnover of **1** at saturating concentrations; Jamin et al. (*24*) had shown that D-lactate accelerated turnover of a nonspecific depsipeptide, a result that, at the time, suggested that D-α-hydroxy acids, like D-amino acids, were effective acyl group acceptors for this enzyme. Second, a manual mixing experiment suggested that saturating **1** had no effect on the intrinsic fluorescence of the R61 DD-peptidase. It was well-known that the fluorescence of this enzyme is quenched by active site acylation (*11*, *24*).

More recent studies (*10*) of **1** cast doubt on the major conclusion described above. First, the value of k_{cat} for thiodepsipeptide **3** was the same, within experimental

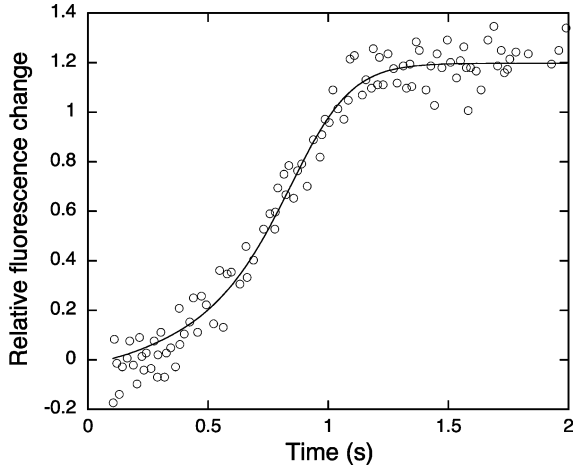


FIGURE 1: Fluorescence intensity as a function of time after R61 DD-peptidase (0.6 μM) is mixed with **1** (100 μM). The line represents the fit of the data to Scheme 6 (see the text).

Scheme 6

$$E + S \underset{K_m}{\rightleftharpoons} ES \xrightarrow{k_{cat}} EP \overset{K_p}{\rightleftharpoons} E + P$$

uncertainty, as that of peptide **2**, suggesting a common rate-determining step, which must be hydrolysis of the acyl−enzyme intermediate. Second, another manual mixing fluorescence experiment did indicate an initial fluorescence quenching, which then disappeared as the substrate was hydrolyzed. Finally, the relevance of the D-α-hydroxy acid acceptor experiment was called into question when it was shown that neither D-lactate nor D-phenyllactate accelerated the hydrolysis of **3**; this unexpected result is examined in detail later in this paper.

To finally convince ourselves of the validity of the more recent conclusion, we have now conducted a stopped-flow fluorescence experiment in which we reacted **1** with an enzyme concentration higher than that which was possible with the manual mixing experiment described above where artifacts from mixing were difficult to eliminate. The result of a typical experiment of this type (several were carried out at concentrations of **1** of 50, 100, and 200 μM) is shown in Figure 1. This shows the presence of a step involving partial quenching of the enzyme fluorescence, already complete within the mixing time of the instrument (4 ms; the pseudo-first-order rate constant of acylation would be expected to be determined from the relation $k_2[S]/K_s = k_{cat}[S]/K_m = 860\ s^{-1}$), followed by a slower regeneration of the enzyme as the substrate was turned over. These data were fitted to Scheme 6 [values of K_m and K_p of 7.9 and 160 μM, respectively, were assumed (*7*)], yielding a k_{cat} value of 97 $\pm$ 10 s^{-1}. This value agrees well with the steady state value of 69 $\pm$ 14 s^{-1} from initial rate measurements (*7*). Thus, we assumed for all subsequent experiments that deacylation is rate-determining for peptide **1**, as it is for depsipeptide **3**. Apparently, the presence of the peptidoglycan-specific side chain in **1** promotes acylation to such an extent that, unlike with nonspecific peptides such as **7**, deacylation becomes the slowest step under conditions of substrate saturation.

To learn more about the deacylation mechanism, and specifically about the deacylation transition state, we chose substrates **1**−**6** for further study. These include molecules with both peptidoglycan-mimetic side chains (**1**−**3**) and the nonspecific benzyl side chain (**4**−**6**), and with a variety of leaving groups. Steady state parameters for hydrolysis of these substrates are listed in Table 1. As noted previously (*7*−*10*), the peptidoglycan-mimetic substrates generally have higher k_{cat} values and lower K_m values than the nonspecific examples. It is interesting to note, however, that the deacylation rate constants, k_3, of nonspecific substrates **6** and **7** (*18*) are comparable to those of **1**−**3**. This indicates that the peptidoglycan-mimetic nature of the latter compounds is expressed in the acylation step [$k_{cat}/K_m = k_2/K_s$ (Scheme 5)], where the leaving group is present, rather than in the deacylation step. The transition state for acyl−enzyme aminolysis (k_4) would, of course, be identical to that of acylation of the enzyme by a peptide. Specific binding sites for the acyl group and the leaving group have been observed crystallographically (*9*, *10*, *26*); they presumably interact to bring about the kinetic effect noted above (*27*).

Table 1: Steady State Kinetic Parameters for Hydrolyses of **1**−**7**, Catalyzed by the R61 DD-Peptidase

substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}\ M^{-1}$)
1[a]	69	7.9×10^{-3}	8.6×10^6
2[b]	48	0.26	2.0×10^5
3	81 $\pm$ 4	(4.1 $\pm$ 0.9) $\times 10^{-3}$	2.1×10^7
4	5.7 $\pm$ 0.6	1.0 $\pm$ 0.4	5.5×10^3
5[c]	1.51	0.76	2.0×10^3
6	31 $\pm$ 1	1.37 $\pm$ 0.15	2.3×10^4
7[c]	34.5	9.8	3.5×10^3

[a] Data from ref *7*. [b] Data from ref *8*. [c] Data from ref *18*.

Table 2: Solvent Deuterium Kinetic Isotope Effects on V for Substrate Hydrolysis by the R61 DD-Peptidase

substrate	^{D_2O}V	substrate	^{D_2O}V
1	2.04 $\pm$ 0.14	**4**	3.08 $\pm$ 0.26
2	2.04 $\pm$ 0.14	**5**	2.56 $\pm$ 0.19[a]
3	1.79 $\pm$ 0.14		

[a] Data from ref *18*.

The solvent deuterium kinetic isotope effects on V (representing, as discussed above, the deacylation transition state in all cases **1**−**6**) for hydrolysis of the substrates at pH 7.5 by the R61 DD-peptidase are given in Table 2. These solidly normal values suggest general base catalysis for the deacylation reaction, as might be expected. The values for the specific substrates appear to be somewhat lower than for the nonspecific substrates, suggesting less proton motion in the transition state, but the difference is not large. Compounds **2** and **3**, which contain the same acyl group, as do **4** and **5**, should give the same isotope effects within each pair. In fact, they appear slightly different in each case, although not significantly beyond experimental uncertainty. A similar solvent isotope effect also suggested general base catalysis in the acylation of the R61 DD-peptidase by nonspecific peptide **7** (*18*).

For further insight, proton inventories were also taken for **1**, **3**, and **4**. These all showed concave down, "dome" shapes (Figure 2). Although there are several possible interpretations of such curves (*28*, *29*), generally difficult to distinguish, one model generating self-consistent fits to the present data, and consistent with previous work on this enzyme, involves a combination of offsetting reactant state and transition state

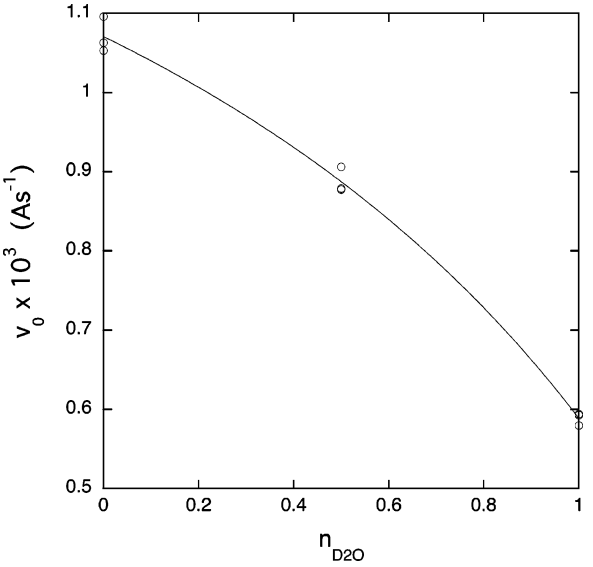


FIGURE 2: Proton inventory (initial rate, measured in absorbance units at 240 nm/s, vs mole fraction of D_2O in the solvent) for the hydrolysis of **3** (95 μM), catalyzed by the R61 DD-peptidase (5.4 nM).

Table 3: Fractionation Factors of Protons Involved in Hydrolysis of the Acyl−Enzyme Intermediate

substrate	ϕ^T	ϕ^R
1	0.40 ± 0.06	0.84 ± 0.11
3	0.36 ± 0.07	0.66 ± 0.12
4	0.37 ± 0.05	0.64 ± 0.09

fractionation factors, as indicated by the Gross−Butler equation (eq 2),

$$v_n = v_0(1 - n - n\phi^T)/(1 - n - n\phi^R) \qquad (2)$$

where v_0 represents the initial velocity in H_2O, v_n is the initial velocity in a solution containing D_2O at mole fraction n, and ϕ^R and ϕ^T are the fractionation factors of a proton (not necessarily the same one) in the reactant and transition state, respectively. It might be noted here that the likelihood ratio test (*30*) confirmed that eq 2 fitted the data better (99% confidence) than a linear (single-proton) equation. The fit of eq 2 to the proton inventories of **1**, **3**, and **4** generated the fractionation factors listed in Table 3. The qualitatively important conclusion is that these values for the three compounds are essentially the same within experimental uncertainty. This shows that the deacylation transition states for these compounds, two of them (one a peptide and the other a thiodepsipeptide) representing specific substrates and one nonspecific, are virtually the same, at least with respect to proton motion. The transition state fractionation factor of ca. 0.4 is consistent with the motion of a solvent exchangeable proton in the transition state, as would be expected for general base-assisted attack of water on the acyl−enzyme intermediate. The reactant state fractionation factor of ca. 0.7 is unusually low for an exchangeable proton but evokes the finding of a similar proton in the free enzyme, which was suggested to derive from a tight Lys65−Tyr159 hydrogen bond (*18*). Such an arrangement of these residues is certainly conceivable in the acyl−enzyme intermediate as well, although some difference must be present since the fractionation factor of the reactant proton is considerably smaller in the free enzyme than in the acyl−enzyme intermediate; the issue of a neutral acid−base pair versus a zwitterion has been discussed (*18*, *31*).

Table 4: Deuterium Solvent Kinetic Isotope Effects on Aminolysis of Depsipeptide **3**[a]

acyl acceptor	$^{D_2O}k_4$
D-alanine	2.5 ± 0.4
Gly-L-Ala	2.1 ± 0.4 (1.07 ± 0.21[b])
Gly-L-Phe	2.2 ± 0.6 (0.90 ± 0.15[b])
aminomalon-*N*-ethylamide	2.8 ± 0.4

[a] All measurements at pH 7.5 except where noted. [b] At pH 8.9.

It might be noted in passing that the solvent deuterium kinetic isotope effects on *V*/*K* for **2**−**4** were also measured as 0.96 ± 0.18, 0.93 ± 0.14, and 0.94 ± 0.05, respectively. Those are similar to these previously determined (*18*) for **5** (0.85 ± 0.06) and **7** (0.89 ± 0.06) and suggest that the acylation transition states of specific substrates are similar to those of nonspecific substrates; the (most likely) inverse values reflect the effect of the free enzyme fractionation factor, discussed above and in the previous paper (*18*).

More important than the carboxypeptidase reaction, to bacteria at least, is the transpeptidation reaction, also catalyzed by DD-peptidases (Scheme 1), including the R61 enzyme (*9*, *10*, *24*, *25*). To learn more about the transpeptidation reaction, we took measurements of $^{D_2O}k_4$, as described in the Experimental Procedures, for several effective small molecule acceptors. As expected from the relevant peptidoglycan structure, both D-amino acids and glycyl-L-amino acid dipeptides act as acceptors (*9*, *10*, *32*). In the work presented here, we employed D-alanine, Gly-L-Ala, Gly-L-Phe, and aminomalon-*N*-ethylamide; the last two of these were particularly effective as acceptors, and the question of different transition states between these and the poorer acceptors arose (*9*). Solvent deuterium kinetic isotope effects obtained for the transpeptidation reaction of specific depsipeptide **3** are reported in Table 4.

At first glance, the $^{D_2O}k_4$ values, averaging around 2.6, suggest a proton or protons in motion and the likelihood of general base catalysis. However, it must be taken into account that, at pH 7.5, the acceptor amines are largely nitrogen protonated. Studies of the effect of pH on the aminolysis reaction showed that k_4 increases with pH (*10*), which suggests, reasonably, that the reactive forms of the acceptors are the free amines. It is interesting to note in this regard that a crystal structure of a complex of the R61 DD-peptidase with **1** at pH 6.8 shows bound products, including D-alanine (*26*). The amino nitrogen of this D-alanine does not appear to be hydrogen-bonded to either the protein or water molecules. This would suggest a neutral amine bound rather than one that was protonated, where strong hydrogen bonding to the ammonium ion would be expected. The situation at pH 7.5 for the experiments of Table 4 should therefore best be represented as in Scheme 7, and thus, eqs 3 and 4 would follow if pH < pK_a.

$$k_4^{obs} = k_4 K_a \qquad (3)$$

$$^{D_2O}k_4^{obs} = {}^{D_2O}k_4\,{}^{D_2O}K_a \qquad (4)$$

For acids in general, $^{D_2O}pK_a = 0.54 \pm 0.07$ or $^{D_2O}K_a = 3.47$ (*26*). To confirm this for an amino acid, we measured

Scheme 7

$$RNH_3^+ \overset{K_a}{\rightleftharpoons} RNH_2 + E\text{-}S \xrightarrow{k_4} E + PNHR$$

$^{D_2O}K_a$ for glycine and obtained a value of 3.5. Thus, from eq 4, $^{D_2O}k_4 = 0.74$. Finally, if $\phi^{ES} = 0.7$ (from above), ϕ^T for the aminolysis transition state would be given by $\phi^{ES}/^{D_2O}k_4$ ($= 0.95$). Thus, contrary to initial impressions, there appear to be no protons in motion in the aminolysis transition state. This conclusion is supported by the measurements of $^{D_2O}k_4$ for Gly-L-Ala and Gly-L-Phe at pH 8.9 where both would contain neutral amines; the $^{D_2O}k_4$ values, under these conditions, were 1.07 and 0.90, respectively (Table 4). The $^{D_2O}k_4$ values for Gly-L-Phe and aminomalon-*N*-ethylamide are essentially the same as those for D-alanine and Gly-L-Ala. The transition states for the better acceptors (the former pair) are thus similar, with respect to proton transfer at least, to those for the poorer acceptors (the latter pair).

The results of the previous paragraph suggest the aminolysis mechanism of Scheme 8, which describes formation and breakdown of a tetrahedral intermediate, where the proton transfers, presumably catalyzed by active site acids and bases E-BH^+ and E-B, respectively, accompany the breakdown step. If the proton transfers are efficiently catalyzed such that $k_2 > k_{-1}$, then the formation of the tetrahedral intermediate (k_1 step) will be rate-determining. Since the pK_a of the amine will not be significantly decreased in the formation of the tetrahedral intermediate, proton transfer to a base need not accompany this step (*33*). A direct analogy from the literature is found, for example, in the study by Bruice et al. of the aminolysis of phenyl acetates (*34*). They observed solvent deuterium kinetic isotope effects of 1.0 ± 0.1 and concluded that nucleophilic attack by amine was rate-determining. The transition state for the reverse reaction, acylation of the enzyme by a peptide, will, of course, be the same; i.e., the rate-determining step will be breakdown of the zwitterionic tetrahedral intermediate after rapid nucleophilic attack and proton rearrangement. This situation seems less common for the aminolysis of other acyl−enzyme intermediates since kinetic isotope effects suggest proton motion in the deacylation transition states of chymotrypsin (*35*), liver transglutaminase (*36*), and kidney γ-glutamyltranspeptidase (*37*).

Since water and D-amino acids are good acceptors for the acyl groups of acyl−enzyme intermediates derived from the R61 DD-peptidase, it might be expected that D-α-hydroxy acids would also have significant acceptor ability. Indeed, Jamin et al. (*24*) showed that this was true for D-lactate and D-mandelate when the nonspecific thiolesters **9** and **10** were employed as substrates. We were surprised, therefore, to find that neither D-lactate nor D-phenyllactate accelerated turnover of **3** or produced the expected acyl transfer products (*9*). Figure 3 shows the effect of D-mandelate on the turnover rate of **3** and **6** under $[S] > K_m$ conditions when deacylation is rate-determining.

D-Mandelate is clearly an inhibitor. Such inhibition by D-lactate, D-phenyllactate, and D-hexahydromandelate was also observed; L-phenyllactate also inhibited the reaction, but more weakly that the D-enantiomer. A more detailed investigation with D-hexahydromandelate revealed that the inhibition was of the mixed variety (Scheme 3); the intersecting lines of Figure 4 clearly exclude competitive inhibition (*23*).

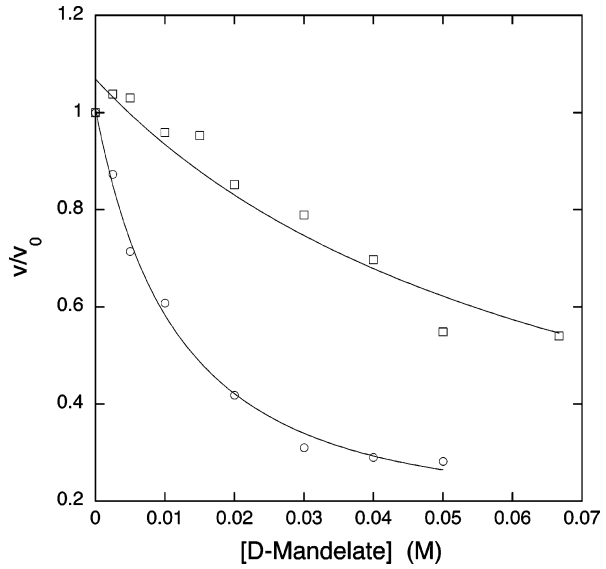


FIGURE 3: Inhibition of the R61 DD-peptidase-catalyzed hydrolysis of **3** [100 μM (○)] and **6** [5.0 mM (□)] by D-mandelate; the enzyme concentrations were 5.0 nM and 0.18 μM, respectively. The lines represent fits of the data to Scheme 3 (see the text).

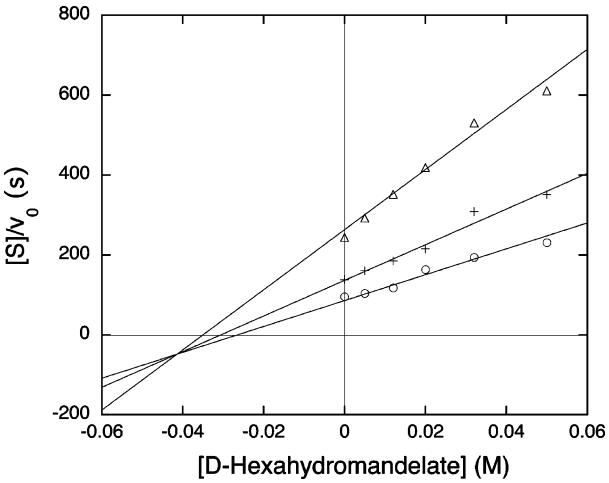


FIGURE 4: Inhibition of the R61 DD-peptidase-catalyzed hydrolysis of **3** [30 (○), 50 (+), and 100 μM (△)] by D-hexahydromandelate. The intersecting pattern of lines represents a simultaneous fit of all of the data to Scheme 3 (see the text) and indicates mixed inhibition.

To assess the degree and nature of the competitive component of this inhibition (K_i in Scheme 3), the ability of several compounds to inhibit the reaction between the enzyme and a β-lactam was determined. β-Lactams react

Scheme 8

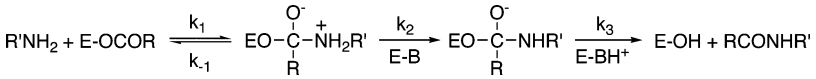


Table 5: Rate and Equilibrium Constants for Reactions of the R61 DD-Peptidase with Hydroxy Acids

substrate	acceptor/inhibitor	K_i (mM)	K_{si} (mM)	k_4 (s^{-1} M^{-1})
3	D-mandelate	11.6 ± 1.2	15.3 ± 1.3	≤10^3 [b]
3	D-phenyllactate	17.8 ± 4.7	16.0 ± 1.7	≤10^3 [b]
3	D-hexahydromandelate	7.0 ± 2.4	41 ± 7	≤10^3 [b]
3	glycolyl-L-Phe	19.3 ± 4.6	32.3 ± 3.1	≤10^3 [b]
3	D-α-phenylpropionate	15.5 ± 3.1	50 ± 10	
6	D-mandelate	11.6 ± 1.2	≤60	≤10^b
4[a]	D-mandelate	11.6 ± 1.2	9.5 ± 4.7	1500 ± 200
5[a]	D-mandelate	11.6 ± 1.2	9.5 ± 4.7	1500 ± 200

[a] Data fitted simultaneously (Figure 5). [b] Maximum values that would not substantially affect the fit to the data; for perspective, the value of k_4 for D-Phe, under the same conditions, is ca. 4×10^4 s^{-1} M^{-1} (*9*).

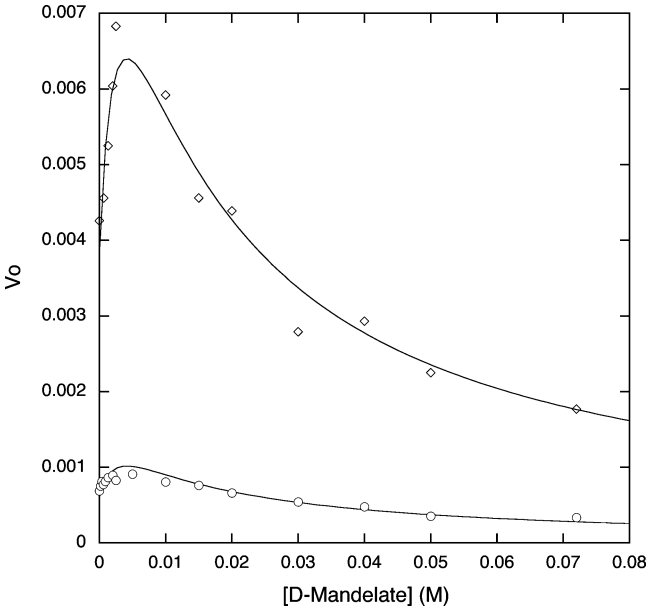


FIGURE 5: Effect of D-mandelate on the turnover of **4** [2.0 mM (◇)] and **5** [2.0 mM (○)], catalyzed by the R61 DD-peptidase (0.54 and 0.44 μM, respectively). The lines represent a simultaneous fit of all of the data to Scheme 4 (see the text).

essentially irreversibly with the R61 DD-peptidase [hydrolysis of the acyl−enzyme intermediate is very slow (*5*)], and inhibition of this process would most likely reflect direct competition for the active site. In fact, D-mandelate, for example, did inhibit reaction of the enzyme with dansylpenicillin, yielding, by the method described in Experimental Procedures, a K_i value of 11.6 ± 1.2 mM. When the data for inhibition of the hydrolysis of **3** and **6** by D-α-hydroxy acids and analogues were fit to Scheme 5 (see Figure 3, for example), where values of K_i were derived from the dansylpenicillin experiments described above, values of K_{si} for several inhibitors were obtained (Table 5).

The effect of D-mandelate on turnover of **4** and **5** by the R61 DD-peptidase was quite different from that on turnover of **3** and **6**, described above. Data obtained with the former substrates are shown in Figure 5. In these cases, the rate of turnover increased at low hydroxy acid concentrations but decreased, reflecting inhibition, at higher concentrations. These data were therefore fitted to Scheme 4 (where, as before, K_i was fixed at the values obtained from the dansylpenicillin experiments). The fitting parameters from this procedure are also found in Table 5. The increase in rate at low hydroxy acid concentrations was, according to Scheme 4, ascribed to the action of these compounds as acyl

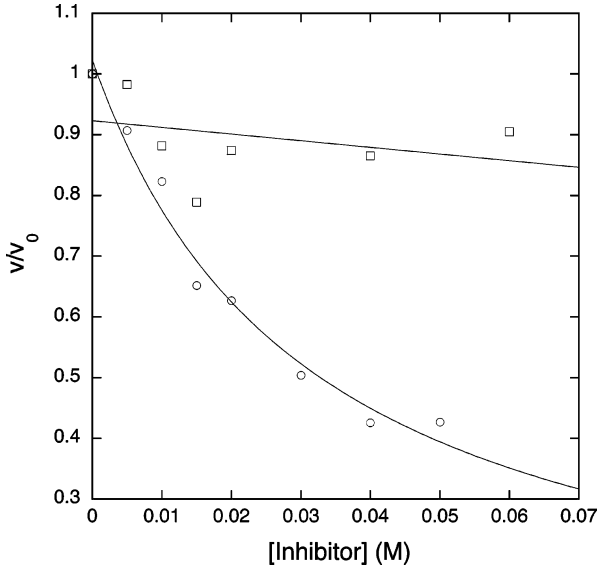
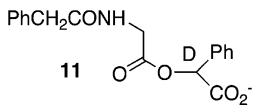
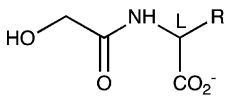


FIGURE 6: Effects of glycolyl-L-Ala [**12** (□)] and glycolyl-L-Phe [**13** (○)] on the turnover of **3** (100 μM), catalyzed by the R61 DD-peptidase (5.0 nM). The lines represent fits of the data to Scheme 3 (see the text).

acceptors. This assignment was confirmed by ^{1}H NMR experiments, carried out as described in Experimental Procedures. NMR spectra of reaction mixtures consisting of **4** or **5** with D-mandelate showed the presence of acyl transfer product **11** [the mandelate ester distinguished by the methine singlet resonance at δ 5.7, cf. δ 4.8 for mandelate itself (*13*)]. This mandelate ester was itself a poor substrate of the enzyme and disappeared slowly after all of the **4** or **5** had been consumed. In contrast, D-mandelate appeared to be only an inhibitor of turnover of **6** by the DD-peptidase; no D-mandelate ester was observed in the NMR spectrum of mixtures of **6** with the enzyme.



Gly-L-Xaa dipeptides are also, as noted above (*9*, *10*, *32*), effective acyl acceptors for all of the substrates employed in these experiments. One might, therefore, expect that analogous hydroxy compounds, **12** and **13**, for example, would be acceptors. Reality, again, was different. The L-Phe derivative, **13**, inhibited the hydrolysis of **3**, **5**, and **6**, while **12** had very little effect on any substrate (e.g., see Figure 6).



12: R = CH_3
13: R = CH_2Ph

The interesting generalization from the experiments with hydroxy acceptors described above is that the D-α-hydroxy acids appeared to act as acyl acceptors for glycyl substrates **4** and **5** but not for the presumably more specific D-alanyl compounds **3** and **6**. Further, dipeptide analogues **12** and **13** did not act as acceptors for any of these substrates. The D-α-hydroxy acids and **13** did, however, act as mixed inhibitors

of hydrolysis of these substrates (Table 5), most likely competing with the substrate for the active site, but also binding elsewhere on the acyl−enzyme intermediate, affecting turnover. This second site could be the acyl acceptor site, although the presence of separate small molecule binding sites on this enzyme, with inhibitory activity, has been previously demonstrated (*10*, *14*, *25*). Structural characterization of the latter site(s) has not yet been achieved. It should also be noted at this stage that the hydroxyl group of the hydroxy acids is not required for inhibition on binding to either the competitive or uncompetitive site. Both D,L-α-fluorophenylacetate and D-α-phenylpropionate (Table 5) also inhibited turnover of **3** in a mixed fashion, although somewhat more weakly than D-mandelate.

Further insight into the issue of hydroxy acid acceptors was obtained through measurements of solvent deuterium kinetic isotope effects on k_4 values for D-mandelate as an acyl acceptor with substrate **5**. The value obtained at pH 7.5 was 2.5 ± 0.5. This shows that the hydroxy acid D-mandelate, like water and unlike amino acids, requires a general base catalyst in the transition state for deacylation of the enzyme. This is not unexpected since the hydroxyl proton would become very acidic as nucleophilic attack progressed, unlike the situation for amine attack (Scheme 8). It seems that this general base, although not needed in the formation of the aminolysis tetrahedral intermediate, although it probably would be needed in breakdown of the initially formed tetrahedral species, is required and available for D-α-hydroxy acid attack on glycyl substrates but is not available for D-alanyl substrates. This may reflect the greater mobility of the glycyl acyl enzyme versus that of the D-alanyl enzyme, where the D-methyl group is firmly anchored in a hydrophobic pocket (*14*, *26*). Thus, it may be that the greater mobility of the acyl−enzyme intermediate derived from the glycyl substrates enables it to access both the nucleophilic hydroxy acid and the general base catalyst earlier along the reaction coordinate to an extent not possible with those derived from D-alanyl substrates.

The lack of acceptor activity in **12** and **13** is also puzzling. Modeling, however, has suggested that D-amino acid and Gly-L-Xaa dipeptide acceptors probably bind in different orientations at the R61 DD-peptidase active site (*10*). Thus **12** and **13**, perhaps binding in a manner analogous to that of the dipeptide acceptors, may not be able to access the general base catalyst as readily as the D-hydroxy acids which may be bound in a manner analogous to that of the D-amino acids. The early requirement of the general base catalyst in hydrolysis and alcoholysis, but not in aminolysis, may be exploited by bacteria to limit the extent of acyl−enzyme hydrolysis in transpeptidation.

It is interesting to note in passing here that aminolysis of amino acid esters at the active site of the ribosome does not appear to require general base catalysis (*38*); a direct alcohol analogue of the amine nucleophile reacts several orders of magnitude more slowly than the amine, and the reaction is pH-independent. This may also be a device to limit the metabolic waste of hydrolysis.

We have shown in this paper, by the criterion of solvent kinetic isotope effects, that the transition states of the deacylation step of the R61 DD-peptidase-catalyzed hydrolysis of specific and nonspecific substrates are very similar. In spite of the strong accelerating effect of a specific peptidoglycan-mimetic side chain, the rate-determining step of the deacylation reaction probably remains the same. This suggests that the effect of the specific side chain is transmitted uniformly along the reaction coordinate; certainly, this is in accord with structural evidence which shows that the polar side chain terminus of **1** seems firmly bound in all of the reaction coordinate analogue structures obtained to date (*26*, *27*, *39*). The transition states for formation of the tetrahedral intermediate in hydrolysis and alcoholysis (by D-α-hydroxy acids) contain a general base catalyst, while those for aminolysis do not; in the latter case, rate-determining formation of the tetrahedral intermediate, without accompanying proton transfer, is probably followed by fast proton transfer, presumably catalyzed by the acid/base functional groups of the active site (Lys67 and Tyr159). Alcoholysis of acyl−enzyme intermediates derived from D-alanyl substrates by hydroxy acids does not occur at a measurable rate, although that of glycyl substrates does. This result may reflect the inability of a nucleophile approaching the D-alanyl reaction center to access the general base catalyst during nucleophilic attack, while one approaching the more flexible glycyl center does have this ability. We hope to apply this knowledge of mechanism to the further design of transition state analogue inhibitors.

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