A New Kinetic Mechanism for the Concomitant Hydrolysis and Transfer Reactions Catalyzed by Bacterial DD-Peptidases†

Marc Jamin, Jean Marc Wilkin, and Jean-Marie Frère*

Laboratoire d'Enzymologie, Institut de Chimie, Université de Liège, B6, B-4000, Sart Tilman (Liège 1), Belgium

Received February 1, 1993; Revised Manuscript Received April 16, 1993

ABSTRACT: In the presence of an adequate nucleophilic acceptor substrate (A) and ester and thiolester donor substrates (S), the *Streptomyces* R61 soluble DD-peptidase catalyzes both hydrolysis and acyl group transfer reactions. Simple bisubstrate models do not explain the variations of the transfer to hydrolysis ratios with the donor and acceptor concentrations. A new kinetic mechanism for the concomitant hydrolysis and transfer reactions is proposed which involves an acceptor and a second, nonproductive donor substrate binding site. In this model, the acceptor essentially binds to the acyl–enzyme, and the second donor molecule only binds to the ternary ES*A complex. Hydrolysis can then proceed from the quaternary ES*AS complex. The values of all of the parameters involved in the reaction of a thiolester substrate with D-alanine as the acceptor substrate were determined at 15 and 37 °C. The results obtained with a protein modified by site-directed mutagenesis, and with which the transpeptidation reaction appeared to be specifically impeded, are discussed on the basis of the new kinetic mechanism. The data obtained with the soluble form of the high molecular weight penicillin binding protein 2× from *Streptococcus pneumoniae* are also in agreement with this model.

The transpeptidation reaction responsible for the cross-linking of the peptidoglycan peptide units is the essential reaction inhibited by β-lactam antibiotics in bacteria. The penicillin binding proteins (PBPs), the membrane-bound enzymes which catalyze these reactions, are active-site serine DD-peptidases [for a review, see Frère *et al.* (1992)]. They are members of a large family of active-site serine penicillin recognizing enzymes, which also contains β-lactamases of classes A, C, and D (Joris *et al.*, 1988, 1991). All of these enzymes have been studied extensively, and several conserved residues involved in the mechanism have been tentatively identified (Frère *et al.*, 1992; Waley, 1992), but few studies have been devoted to the transpeptidation reaction itself (Frère *et al.*, 1973; Perkins *et al.*, 1973). In addition, except for the active-site serine, which forms a transient ester bond with the acyl moiety of the donor substrate (Scheme I), the role played by the other residues in the transpeptidation reaction is not clearly understood.

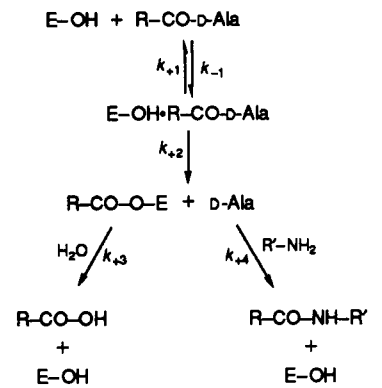
Some soluble bacterial DD-peptidases have been utilized as models for the study of the membrane-bound enzymes (Frère & Joris, 1985). *In vitro*, the extracellular DD-peptidase from *Streptomyces* R61 (in short, the R61 DD-peptidase) catalyzes the transfer of both the acyl group of D-alanyl-D-alanine-terminated peptides and the analogous esters and thiolesters on water (hydrolysis or carboxypeptidation), D-amino acids, or small peptides (transpeptidation) (Pollock *et al.*, 1972; Adam *et al.*, 1990; Jamin *et al.*, 1991).

Various enzyme-catalyzed reactions involving an acyl–enzyme intermediate are channeled into concomitant hydrolysis and acyl-transfer pathways when an exogenous nucleophile is added. In some cases, a simple partitioning mechanism is involved, but numerous studies demonstrated the binding of the exogenous nucleophile to the enzyme prior to its participation in the deacylation (Scheme II). This mechanism, first proposed for cysteine and serine proteases [for a review, see Fruton (1982)], also applies to other reactions involving an acyl–enzyme or glycosyl–enzyme intermediate, as observed with penicillin amidase (Kasche *et al.*, 1984) and β-galactosidase (van der Groen *et al.*, 1973).

However, with the R61 DD-peptidase, the simple bisubstrate mecanisms where the donor substrate binds first failed to explain all of the kinetic data (Frère *et al.*, 1973), and on this basis, an ordered mechanism where the acceptor binds first was initially proposed. However, attempts to visualize the binary acceptor–enzyme complex remained unsuccessful (Nieto *et al.*, 1973; Fuad *et al.*, 1976).

A related class C β-lactamase which also catalyzes concomitant hydrolysis and acyl-transfer reactions with dep-

Scheme I: Mechanism of the Hydrolysis and Transpeptidation Reactions Catalyzed by the Active-Site Serine Bacterial DD-Peptidases[a]



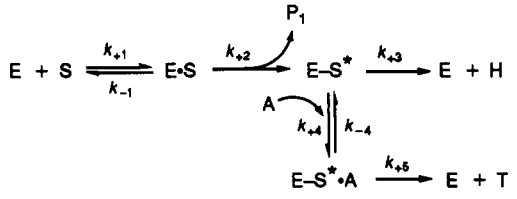
[a] The acyl group is generally R-CO-D-Ala. E-OH represents the enzyme where the OH group is that of the active-site serine side chain. R-CO-D-Ala and R'-NH2 are, respectively, the donor (S) and the acceptor (A) substrates.

† This work was supported in part by the Belgian government in the frame of the Pôles d'attraction interuniversitaires (PAI No. 19) and Actions concertées (conventions 86/91-90 and 89/94-130) and by the Fonds de la recherche scientifique médicale (Contract No. 3.4537.88). The stopped-flow equipment was acquired thanks to grants of the Fonds de la Recherche Fondamentale Collective (FRFC, Brussels) and the Fonds de la Recherche of the University of Lieège. M.J. and J.M.W. are Research Fellows of the Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture (I.R.S.I.A.).

* Author to whom correspondence should be addressed. FAX 32-41-56-33-64.

0006-2960/93/0432-7278$04.00/0

Scheme II: Mechanism of Hydrolysis and Transpeptidation Reactions Involving an Acceptor Site[a]



[a] E is the enzyme, S is the donor substrate, A is the acceptor substrate, ES is the Henri–Michaelis complex, ES* is the acyl–enzyme, ES*A is the acyl–enzyme–acceptor complex, P_1 is the donor leaving group, and H and T are the hydrolysis and transfer products, respectively. $K' = (k_{-1} + k_{+2})/k_{+1}$ and $\alpha = (k_{-4} + k_{+5})/k_{+4}$.

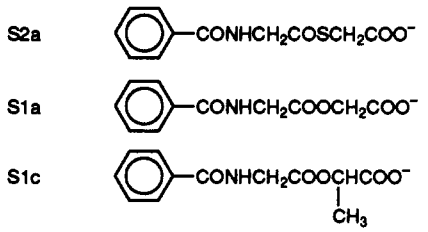
sipeptide substrates exhibited a similarly complex behavior (Pratt *et al.*, 1984; Pazhanisamy *et al.*, 1989). Pratt and co-workers proposed a model in which the acceptor substrate binds to the Henri–Michaelis complex formed with the donor and in which a second nonproductive binding site for the donor substrate is also involved (Pazhanisamy & Pratt, 1989a; Mazzella *et al.*, 1991). Arguing that β-lactamases and DD-peptidases are evolutionarily related, they proposed that this model could also account for the behavior of the DD-peptidases.

We have previously shown that deacylation was the rate-limiting step in the hydrolysis of several ester and thiolester substrates by the *Streptomyces* R61 DD-peptidase and that the quenching of the intrinsic fluorescence of the protein allowed a direct visualization of acyl–enzyme accumulation (Jamin *et al.*, 1991). In this article, we report a detailed kinetic investigation of the concomitant hydrolysis and transfer reactions catalyzed by this enzyme, and we propose a new kinetic mechanism. We also discuss the results obtained with one mutant which appeared to be specifically impaired in the transpeptidation branch of the mechanism (Wilkin *et al.*, 1993b).

MATERIALS AND METHODS

Enzymes and Substrates. The DD-peptidase from *Streptomyces* R61 was produced and purified according to Erpicum *et al.* (1990) and Fossati *et al.* (1978).

The ester and thiolester substrates were synthesized as described by Adam *et al.* (1990, 1991). Substrates S2a, S1a, and S1c are as follows:



The amino acids and peptides used as acceptor substrates were purchased from Sigma Chemical Co. (St. Louis, MO).

Kinetic Parameters. All measurements were performed in 10 mM sodium phosphate buffer, pH 7.0. The absorbance measurements were made with an UVIKON 860 spectrophotometer linked to a microcomputer *via* an RS232 interface. The k_{cat}, K_m, and k_{cat}/K_m parameters were determined either by analyzing complete time courses with the help of the integrated Henri–Michaelis equation (De Meester *et al.*, 1987) or by recording initial rates at different substrate concentrations and analyzing the data by direct fitting to the Henri–Michaelis equation or to its linearized form according to Hanes' transformation.

The fluorescence measurements were made with the help of a Perkin-Elmer LS 50 spectrofluorimeter equipped with a 1 × 0.4 cm thermostated cell. The apparent first-order rate constants for the decrease of the intrinsic fluorescence of the enzyme were computed with the help of the ENZFITTER software package (Leatherbarrow, 1987).

Simulation and Fitting. Two software packages were used for fitting and simulations on an AT286 microcomputer. The ENZFITTER software package allows data analysis by nonlinear rgression (Leatherbarrow, 1987). The SIMFIT software package, which was kindly given by Dr. H. Holzhütter (Institute of Biochemistry, Humbolt University, Berlin, Germany), allows simulations and data analysis using models based upon algebraic and/or differential equations (Holzhütter *et al.*, 1990). The fitting algorithm uses a modified Marquardt procedure, permitting the introduction of lower and upper bounds as constraints on the fitted parameters.

Pre-Steady-State Kinetics. The pre-steady-state kinetic measurements were performed with the help of a Bio-Logic SFM3 stopped-flow apparatus as described by Jamin *et al.* (1991).

(*a*) *Determination of k_2 and K' at Different Temperatures.* The reaction between substrate S2a and the R61 DD-peptidase was monitored by following the quenching of the intrinsic fluorescence of the enzyme, as previously described. The apparent first-order rate constants for the acylation of the enzyme (k_{app}) were obtained by analyzing the time courses of the fluorescence decrease. The k_2 and K' values were obtained by fitting the data to

$$k_{app} = \frac{k_2[S]}{K' + [S]} + k_3 \qquad (1)$$

The k_2/K' values were deduced from the linear part of the plots at low ($\ll K'$) substrate concentrations.

(*b*) *Influence of Acceptor and Donor Concentrations on the Formation of the Acyl–Enzyme.* In these experiments, which were performed at 15 °C in the stopped-flow apparatus, syringes 1, 2, and 3 contained the enzyme, the acceptor (D-alanine), and the donor substrates (S2a), respectively. The apparent first-order rate constants for the acylation of the enzyme were deduced from the protein fluorescence decrease. In the presence of the acceptor, the rate of the reaction increased and the amplitude of the recorded signal decreased. As a consequence, the concentration of the R61 DD-peptidase was adjusted in order to obtain an adequate signal.

(*c*) *Acyl–Enzyme Dilution Experiments.* These experiments were performed at 37 °C in slightly different conditions. The stopped-flow instrument contained two mixing chambers. The solutions from syringes 1 and 2 were mixed in the first chamber. This mixture was added to the solution from syringe 3 in the second mixing chamber. The delay between the two mixings could be modified by varying the length of an aging loop inserted between the two chambers.

Syringes 1, 2, and 3 contained 1 mM substrate S2a, 26.2 μM R61 DD-peptidase, and phosphate buffer or 200 mM D-alanine, respectively. Two hundred microliters of each of the first two solutions and 400 μL of the third were used in each experiment. The total flow rate was 12 mL/s. An aging loop of 190 μL was introduced between the two mixing chambers, which delayed the second mixing by 140 ms.

Transfer/Hydrolysis Ratio ([*T*]/[*H*]). The [T]/[H] ratios were measured by separating the products and substrate on a LiChrospher 100 (5 μM) C18 column (Merck, Darmstadt, Germany). Eluent A was 10 mM sodium acetate adjusted to pH 3.0 with HCl, and eluent B was acetonitrile. The flow

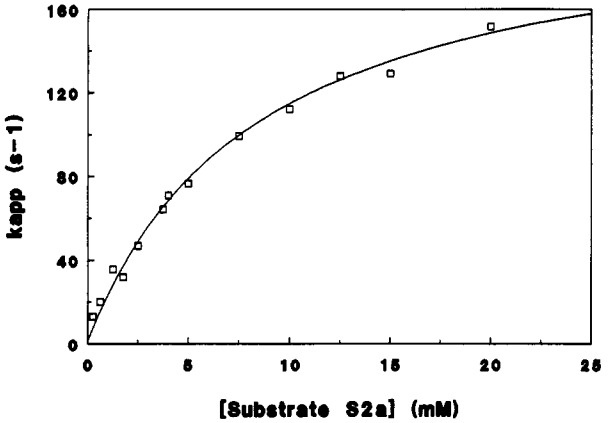


FIGURE 1: Variation of the pseudo-first-order rate constant for acyl–enzyme formation (k_{app}) with the concentration of substrate S2a at 15 °C. The final concentration of the *Streptomyces* R61 DD-peptidase was 6.6 μM. SD values were about 5%. The curve was fit to eq 1 by least-squares regression. The following values were obtained (with $k_3 = k_{cat} = 1.3 \pm 0.1$ s^{-1}): $k_2 = 210 \pm 10$ s^{-1} and $K' = 8.5 \pm 0.8$ mM.

rate was 1 mL/min. Elution was performed under isocratic conditions (90:10, v/v) for 15 min followed by a 3-min linear acetonitrile gradient (10–100%). Elution times for hydrolysis and transfer products (with D-alanine as acceptor substrate) were 12.4 and 13.9 min, respectively. The substrate was eluted in the gradient. Quantification was achieved by integrating the areas under the curves using hippuric acid (H) and the authentic aminolysis product (T, C_6H_5CO-Gly-D-Ala) as standards (Jamin *et al.*, 1991).

RESULTS

Hydrolysis Reaction. The k_{cat}, K_m, and k_{cat}/K_m parameters for the hydrolysis of substrate S2a by the R61 DD-peptidase were measured at different temperatures ranging from 4 to 40 °C. Within this range, deacylation was always the rate-limiting step, as previously observed at 37 °C, and the Arrhenius plots for the the kinetic parameters were linear (Figures A and B of the supplementary material).

The temperature dependence of k_2, K', and k_2/K' was determined by plotting the apparent first-order rate constants for the formation of the acyl–enzyme (k_{app}) *versus* the concentration of substrate S2a (Figure 1). In the fitting procedure, the k_{cat} value determined above at the corresponding temperature was used as the k_3 value. Unfortunately, at 30 and 37 °C, k_{app} remained proportional to the S2a concentration up to k_{app} values of 140 and 160 s^{-1}, respectively, and it was not possible to further increase the substrate concentration. Under these conditions, only k_2/K' could be computed. The Arrhenius plots for the these parameters were linear within the accessible temperature range (Figures B and C of the supplementary material). The Arrhenius plots of the second-order rate constant of acyl–enzyme formation as determined by measuring k_2/K' or k_{cat}/K_m were superimposable within the limits of experimental error (Figure B of the supplementary material). Thus, the values of k_2 (700 ± 100 s^{-1}) and K' (7 ± 1 mM) at 37 °C could be estimated by extrapolation of the corresponding Arrhenius plots.

Concomitant Hydrolysis and Transfer Reactions. (*a*) *Initial Rate Measurements.* In the absence of acceptor substrate, the k_{cat} for the hydrolysis of substrate S2a was concentration independent up to 3 mM, indicating that the hydrolysis reaction obeyed the simple Henri–Michaelis equation. When a suitable acceptor substrate was added, an increase in the global reaction rate was observed when the

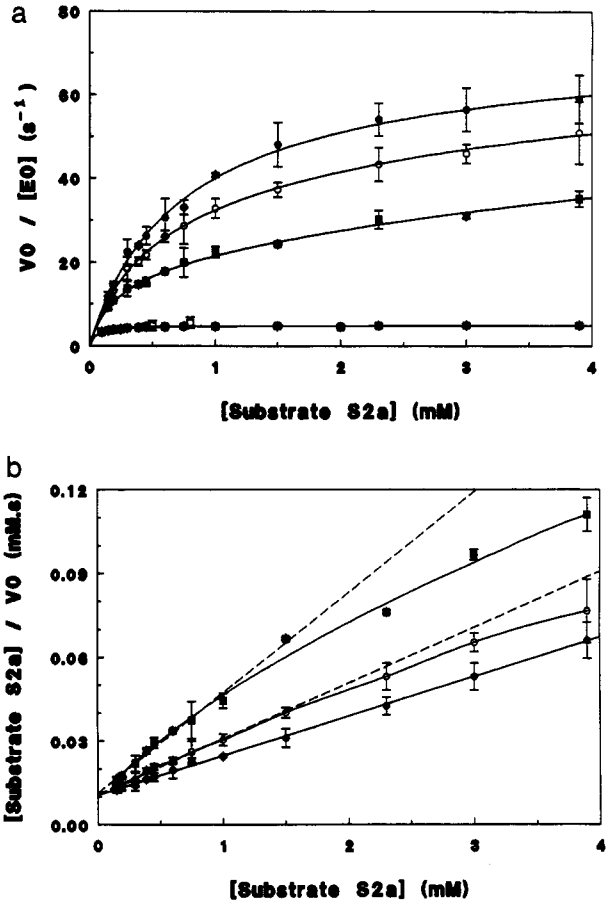


FIGURE 2: Dependence of the initial rates of the global donor utilization on the concentration of substrate S2a at different D-alanine concentrations. The final concentrations of D-alanine were as follows: 0 (□), 10 (■), 25 (○), and 50 mM (●). (a) Direct plots. The points represent the experimental results, and the curves were calculated on the basis of eq 5 using the values shown in Table I as described in the text. The temperature was 37 °C. (b) Hanes plots ($[S]/v_0$ *versus* [S]). The points represent the experimental results. The concentrations of D-alanine and the symbols are the same as in a. The dotted lines were obtained by a linear regression for [S] < 1.0 mM. The curves were calculated on the basis of eq 5 with the values shown in Table I.

donor concentration was sufficient. Note that no acceleration must be expected when the donor concentration is lower than the hydrolysis K_m, even when k_3 is smaller than k_2.

The variation of the initial rate with the concentration of substrate S2a was recorded at different D-alanine concentrations (Figure 2a). As previously observed with the peptide substrates, the Hanes plots were not linear in the presence of the acceptor (Figure 2b). Note that these deviations from linearity occurred at S2a concentrations higher than 1.0 mM, and as a consequence, the values of k_{cat} and K_m previously determined from complete time course analyses remained reliable (see below). Indeed, the k_{cat} and K_m values computed from the linear portion of the curves at [S2a] < 1.0 mM (Figure 2b) were in excellent agreement with those determined from complete time courses (Table A of the supplementary material).

(*b*) *Parameters for the Acylation Step.* The variations of the apparent first-order rate constant for the formation of the acyl–enzyme with the acceptor and donor substrate concentrations were recorded with the help of the stopped-flow apparatus. The k_{app} increased with both the D-alanine and donor substrate (S2a) concentrations (Figure 3).

(*c*) *[T]/[H] Ratios.* The [T]/[H] ratios varied during the progress of the reaction (Figure 4). Consequently, they were

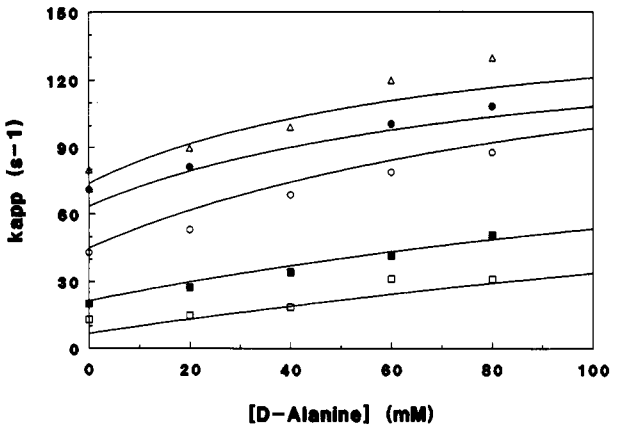


FIGURE 3: Variation of the apparent first-order rate constant for acyl–enzyme formation with acceptor and donor substrate concentrations. The final concentrations of donor substrate were as follows: 0.25 (□), 1 (■), 2.5 (○), 4 (●) and 5 mM (△). The temperature was 15 °C. The curves were calculated on the basis of eq 7 with the values shown in Table I. SD values were ±10%.

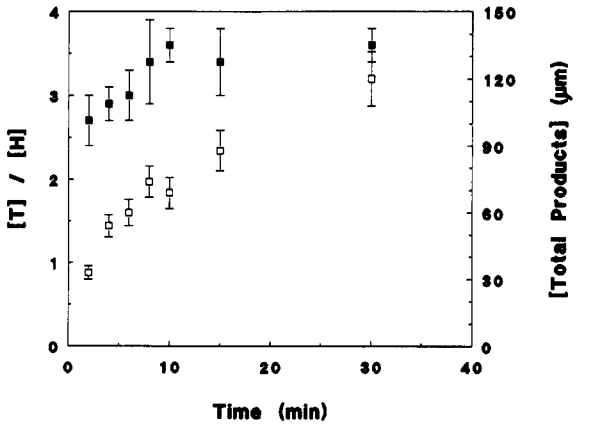


FIGURE 4: Variation of the [T]/[H] ratio (■) with the progress of the reaction. Initial concentrations: substrate S2a, 150 μM; D-alanine, 10 mM; and R61 DD-peptidase, 13 nM. The total concentration of products is also shown (□).

determined under initial rate conditions, *i.e.*, before less than 10% of the donor substrate had disappeared. At the same donor concentration, the [T]/[H] ratios exhibited superimposable hyperbolic dependencies *versus* the D-alanine concentration for substrates S1c and S2a (Figure 5a). As observed before with the peptide donor, the [H]/[T] *versus* 1/[acceptor] plots were linear. Since a model where the acceptor binds first had been rejected before (see above), these results indicated that hydrolysis could occur *via* an intermediate containing the acceptor molecule.

As with the peptide donor, the [T]/[H] ratios decreased with increasing donor (S2a) concentrations (Figure 5b). When three different donors yielding the same acyl–enzyme were utilized at a fixed acceptor concentration, the [H]/[T] ratio either increased linearly with the donor concentration or remained unchanged (Figure 6). The lines exhibited different slopes but extrapolated to the same [H]/[T] value at zero donor concentration, indicating that under conditions of low donor substrate concentration the [H]/[T] ratio was always independent of the donor's leaving group structure.

(*d*) *Acyl–Enzyme Dilution Experiments.* The absence of influence of the donor's leaving group on the [T]/[H] ratio at zero donor concentration suggested a direct binding of the acceptor substrate to the acyl–enzyme. This was confirmed by showing that D-alanine could actually bind to the preformed acyl–enzyme and directly channel the reaction into the transfer

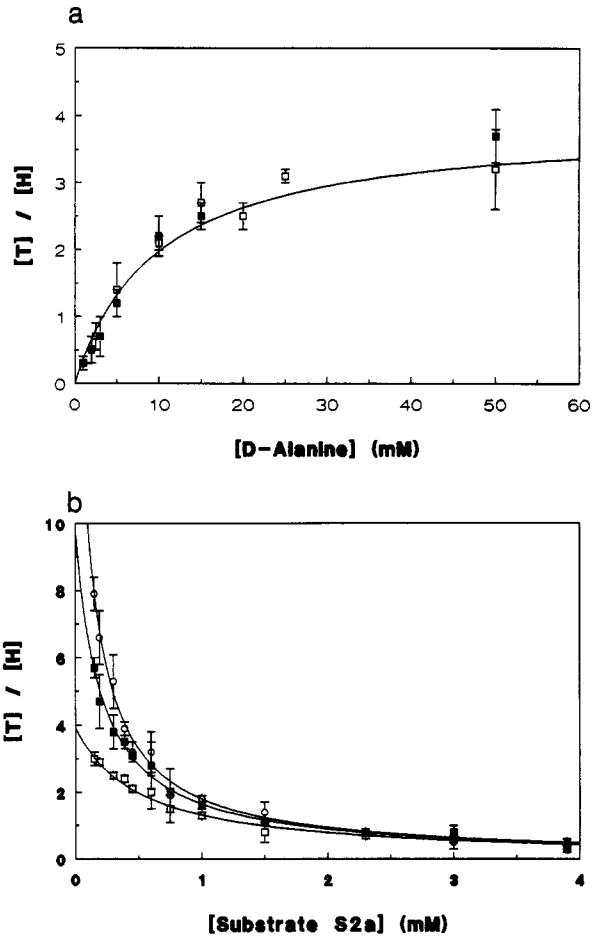


FIGURE 5: Variations of the [T]/[H] ratio with the donor and acceptor substrate concentrations. (a) Influence of the acceptor substrate (D-alanine). The final concentrations of substrates S2a (□) and S1c (■) were both 500 μM. The temperature was 37 °C. The curve was calculated (for substrate S2a) on the basis of eq 6 using the values shown in Table I. (b) Influence of the donor substrate (S2a) concentration observed at three D-alanine concentrations. The final concentrations of D-alanine and the symbols are the same as in Figure 2a. The error bars represent SD values (three determinations). The temperature was 37 °C. The curves were calculated on the basis of eq 6 using the values shown in Table I.

pathway without the need for a preliminary turnover of the acyl–enzyme.

Substrate S2a and the R61 DD-peptidase were mixed (in the first mixing chamber of the stopped-flow apparatus), and the conditions were such that the acyl–enzyme concentration reached its steady-state level in less than 150 ms. As previously shown, the intrinsic fluorescence of this protein decreases when the acyl–enzyme is formed. The fluorescence signal could thus be used to estimate the acyl–enzyme concentration.

After 190 ms, the preformed acyl–enzyme reached the second chamber, where it was mixed either with the sodium phosphate buffer or with 200 mM D-alanine in the same buffer. When acyl–enzyme was diluted with buffer, a two-phase phenomenon was recorded (Figure 7a). The first phase ($t_{1/2}$ = 28 ms) was a fast monoexponential increase of fluorescence corresponding to the adjustment of the acyl–enzyme concentration due to the dilution of the donor substrate according to eq 2, which is valid if $[S] < K'$ and $k_3 \ll k_2$:

$$\frac{[ES^*]_{SS}}{[E]_0} = \frac{[S]}{[S] + K_m} \qquad (2)$$

In the second phase, corresponding to the decrease of the acyl–enzyme concentration due to the disappearance of the substrate, the fluorescence returned to its initial value.

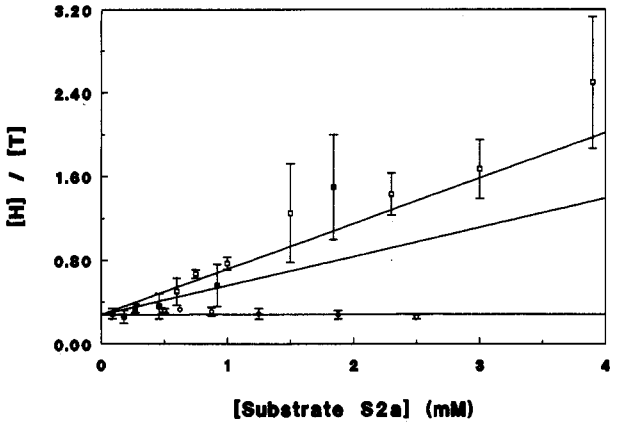


FIGURE 6: Variation of the [H]/[T] ratio with the concentration of various donors yielding the same acyl–enzyme. The acceptor substrate was 10 mM D-alanine and the donor substrates were S2a (□), S1c (■), and S1a (○). For the sake of clarity, the values obtained below 0.5 mM are not shown in the figure for substrate S2a (five values) and S1a (three values), but they were included in the linear regressions. When these were performed for each substrate individually, the results were as follows: S2a, 0.25 + 0.51[S]; S1a, 0.33 + 0.25[S]; S1c, 0.21 + 0.38[S]. The lines shown in the figure were thus drawn with a common intercept with the ordinate (0.28), and new linear regresssions were performed. The χ^2 values found with these new regressions were not significantly higher than those obtained after the first set of regressions.

When the acyl–enzyme was diluted with D-alanine (Figure 7b), the first phase became extremely rapid ($t_{1/2} < 6$ ms) and resulted in the disappearance of 85% of the acyl–enzyme, which was consistent with the very rapid establishment of a new steady state with a strongly increased rate of acyl–enzyme degradation. This decrease of the steady-state acyl–enzyme concentration was in agreement with an nondelayed attack of the acyl–enzyme by the nucleophilic acceptor. In a mechanism where the acceptor substrate only binds to the Henri–Michaelis complex, the nearly complete disappearance of the acyl–enzyme would essentially be k_3 dependent and would be slower than that observed. The second, slower phase of fluorescence increase was, as before, due to the disappearance of the substrate.

The Asn 161 Ser Mutant. With this modified enzyme, obtained by site-directed mutagenesis, the presence of the acceptor increased the total rate of donor utilization, but surprisingly, an important proportion of this increase could be attributed to the hydrolysis reaction itself (Wilkin *et al.*, 1993b). The initial rate of donor disappearance was measured at different donor and acceptor concentrations. The results are shown in Figure 8. At a fixed, 20 mM D-alanine concentration, the [T]/[H] ratio did not change significantly when the donor concentration was larger than 0.1 mM.

MODEL AND DISCUSSION

The R61 DD-peptidase is presently the most widely studied active-site serine penicillin binding protein. It is the only one for which 3D structural data (Kelly *et al.*, 1982, 1985, 1989) and detailed analysis of the transpeptidation reaction (Frère *et al.*, 1973) are available, in addition to some site-directed mutagenesis studies (Hadonou *et al.*, 1992a,b; Bourguignon-Bellefroid *et al.*, 1992a,b; Wilkin *et al.*, 1993a,b). Although it is a soluble, extracellular enzyme, it has been used successfully as a model for membrane-bound PBPs (Frère *et al.*, 1985). In the presence of suitable acceptor substrates, it catalyzes concomitant carboxypeptidation and transpeptidation reactions by transferring the acyl moiety of the donor substrate on either a water molecule or the nucleophilic group

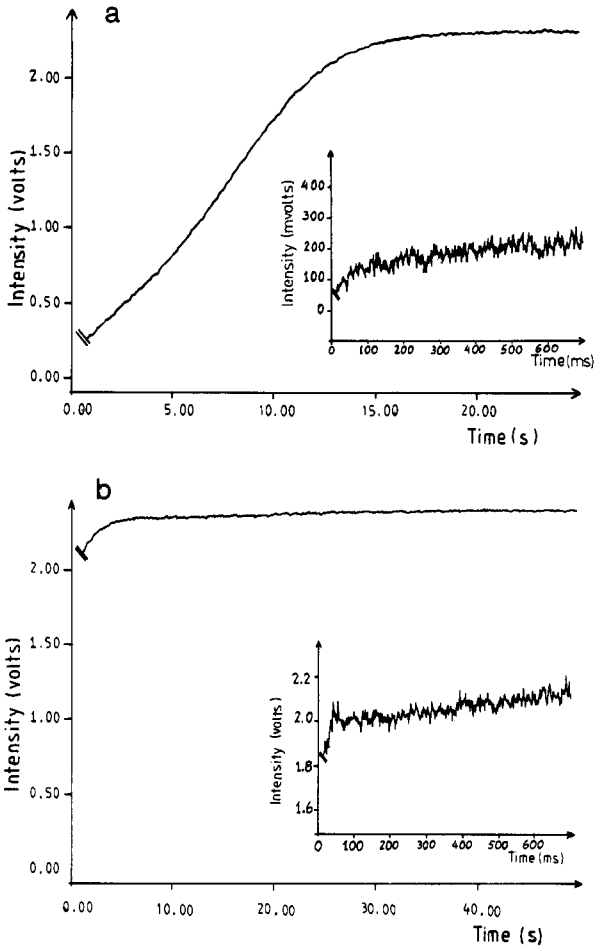


FIGURE 7: Variation of the R61 DD-peptidase intrinsic fluorescence with the preformed acyl–enzyme diluted in buffer (a) or mixed with an acceptor substrate (D-alanine) (b). The signal was recorded with the help of the stopped-flow apparatus. The excitation wavelength was 290 nm and the emission was recorded through a 310–490-nm band-pass filter. The final concentrations after the second mixing were [R61] = 6.6 μM and [S2a] = 250 μM, and in b [D-alanine] = 100 mM. The temperature was 37 °C.

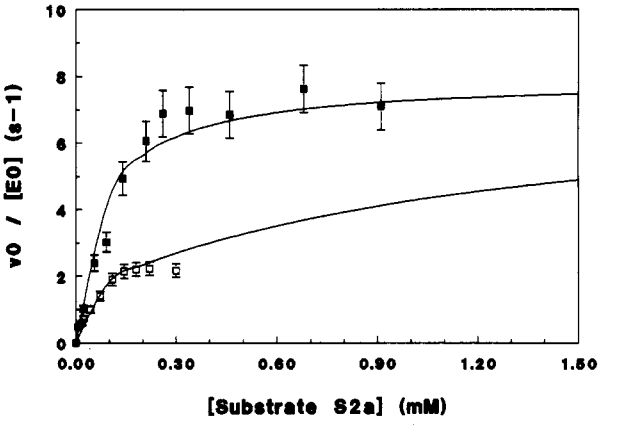


FIGURE 8: Concentration dependence of the initial rate of donor utilization at two different acceptor concentrations [10 (□) and 100 mM (■)] observed with the modified protein Asn 161 Ser. The curves were calculated on the basis of eq 5 with the values shown in Table I.

of the acceptor substrate. Frère (1973) has shown that the partitioning between the two pathways, characterized by the [T]/[H] ratio, was a sensitive indicator allowing a distinction between various mechanisms. Indeed, when an acyl–enzyme intermediate is channeled into simple hydrolytic and transfer pathways as depicted by Schemes I and II, the [T]/[H] ratios

should be independent of the donor concentration and strictly proportional to that of the acceptor.

The previous studies performed with D-alanyl-D-alanine-terminated peptides as donor substrates indicated that the R61-catalyzed reactions did not obey these simple rules (Frère *et al.*, 1973), and a mechanism where the acceptor substrate binds to the free enzyme was proposed. However, the presence of an acceptor substrate failed to affect the rates of β-lactam binding and of the thermal denaturation of the protein (Nieto *et al.*, 1973), and attempts to directly detect such a complex by equilibrium dialysis remained unsuccessful (J. M. Frère, unpublished results).

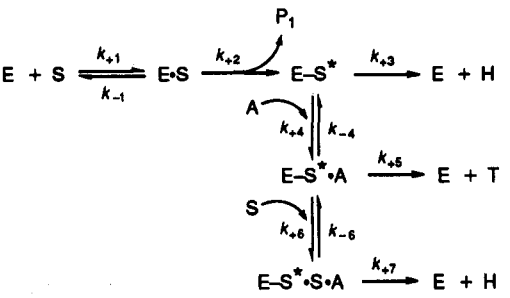
Pratt and co-workers obtained similar results when investigating the concomitant hydrolysis and aminolysis reactions catalyzed by the *Enterobacter cloacae* P99 β-lactamase (Pazhanisamy *et al.*, 1989). In particular, they proved that a kinetically competent acceptor–enzyme complex did not exist (Pazhanisamy & Pratt, 1989b) and observed that the [T]/[H] ratios depended upon the structure of the donor substrate's leaving group. Accordingly, they proposed a complex model, where binding of the acceptor occurred at the level of the Henri–Michaelis complex, thus prior to the formation of the acyl–enzyme and where a second molecule of donor substrate could bind to an additional, nonproductive site (Pazhanisamy & Pratt, 1989a; Mazzella *et al.*, 1991).

In the present study of the R61 DD-peptidase, we also observed that the [T]/[H] ratio was apparently modified when the structure of the donor leaving group was changed, but the [T]/[H] ratios extrapolated to the same value (within the limits of experimental errors) at zero donor concentration. The subtle influence of the donor concentration was also underlined by the variation of the [T]/[H] ratio with the progress of the reaction in an experiment where the acceptor concentration could be considered as constant (Figure 4). Moreover, the addition of a large concentration of D-alanine induced an immediate and nearly complete disappearance of the acyl–enzyme, indicating a nondelayed increase in the rate of utilization of this intermediate and, thus, a direct interaction with the acceptor substrate.

As a consequence, the model that is proposed here rests on the following observations: (1) In the absence of acceptor substrate, the hydrolysis reaction obeys the hyperbolic Henri–Michaelis equation up to a 3 mM concentration of substrate S2a (i.e., 60-fold the K_m value), and the rate of acyl–enzyme formation is also consistent with a simple linear pathway. (2) The acceptor binds to the acyl–enzyme to form a ternary complex (ES*A) which subsequently undergoes aminolysis (Scheme II). The presence of a specific acceptor binding site can be inferred from previous studies (Jamin *et al.*, 1991). (3) The nonlinearity of the variation of the [T]/[H] ratio with the acceptor concentration indicates that the hydrolysis reaction can occur *via* the pathway which involves the acceptor substrate. This might be accounted for by assuming that ES*A can give rise to E + A + H but also by a more profound modification of Scheme II. (4) The deviation from the simple Henri–Michaelis kinetics, illustrated by the nonlinear Hanes plots, which occurs only in the presence of the acceptor, and the dependence of the [T]/[H] ratio on the donor substrate concentration support the involvement of a second donor molecule after formation of the ES*A complex.

Scheme III represents the most simple model accounting for all experimental data. It could be further complicated by assuming for instance that ES*A and ES*AS could give rise to hydrolysis and transpeptidation products, but that seems difficult if not impossible to verify and it did not appear necessary to involve additional reactions unless compelled by experimental evidence. Similarly, a random sequential mechanism for the binding of the acceptor and the second donor substrate molecules to ES* could be proposed, but with the R61 DD-peptidase, the dissociation constant of the ES*S complex would be very large (≫3 mM). Equation 3 represents the steady-state equation derived from Scheme III:

Scheme III: New Kinetic Mechanism Proposed for the Bacterial DD-Peptidases[a]



[a] E is the enzyme, S is the donor substrate, A is the acceptor substrate, ES is the Henri–Michaelis complex, ES* is the acyl–enzyme, ES*A is the acyl–enzyme–acceptor complex, ES*AS is the quaternary acyl–enzyme–acceptor–donor, P_1 is the leaving group, and H and T are the hydrolysis and transfer products, respectively. $K' = (k_{-1} + k_{+2})/k_{+1}$, $\alpha = (k_{-4} + k_{+5})/k_{+4}$, and $\beta = (k_{-6} + k_7)/k_{+6}$.

$$\frac{v_0}{[E_0]} = \frac{a[S] + b[S]^2}{c + d[S] + e[S]^2} \tag{3}$$

where

$$a = k_2k_3k_4\alpha\beta + k_2k_4k_5\beta[A]$$

$$b = k_2k_3k_7 + k_2k_4k_7[A]$$

$$c = k_3k_4\alpha\beta K' + k_4k_5\beta K'[A]$$

$$d = k_3k_7K' + k_2k_4\alpha\beta + k_3k_4\alpha\beta + k_2k_4\beta[A] + k_4k_5\beta[A] + k_4k_7K'[A]$$

$$e = k_2k_7 + k_3k_7 + k_2k_4[A] + k_4k_7[A]$$

and eq 4 gives the [T]/[H] ratio under initial rate conditions.

$$\frac{[T]}{[H]} = \frac{k_4k_5\beta[A]}{k_3k_4\alpha\beta + k_3k_7[S] + k_7[A][S]} \tag{4}$$

The values of the various parameters, *i.e.*, k_{+4}, k_{-4}, k_5, k_7, and $\beta = (k_{-6} + k_7)/k_{+6}$, at 37 °C were computed with the help of the SIMFIT program in a fitting procedure based on two sets of data: the variations in the steady-state rate of the reaction and in the [T]/[H] ratios with the donor substrate concentrations at three different acceptor concentrations (Figures 2a and 5b). In this analysis, the k_2, k_3, and K' values were those derived from the data obtained in the absence of acceptor, *i.e.*, the k_3 value was the measured k_{cat} value for the hydrolysis reaction and the k_2 and K' values were those extrapolated to 37 °C on the basis of the Arrhenius plots (Figure C of the supplementary material). The fitting procedure yielded coherent results with different sets of k_{+4} and k_{-4} values as long as k_{-4} was larger than k_{+5}, and it thus appeared that the binding of the acceptor could be considered as a rapid equilibrium step and the steady-state equations simplified to

$$\frac{v_0}{[E_0]} = \frac{a'[S] + b'[S]^2}{c' + d'[S] + e'[S]^2} \tag{5}$$

Table I: Kinetics Parameters for the Concomitant Hydrolysis and Aminolysis (D-Alanine) of Substrate S2a Catalyzed by the R61 DD-Peptidase[a]

	parameters for the wild type		parameters for Asn 161 Ser
	37 °C	15 °C	37 °C
k_{cat} (s^{-1})	5 ± 0.5	1.3 ● 0.1	0.33 ± 0.05
K_m (μM)	50 ± 5	75 ± 5	9 ± 2
k_2 (s^{-1})	700 ± 100	210 ± 10	600 ± 150
K' (mM)	7 ± 1	8.5 ± 0.8	6 ± 2
k_3 (s^{-1})	5 ± 0.5	1.3 ± 0.1	0.5 ± 0.2
k_5 (s^{-1})	200 ± 50	170 ● 20	15 ± 5
α (mM)	100 ± 16	500 ± 100	100 ± 20
k_7 (s^{-1})	84 ± 2	70 ± 20	8 ± 2
β (mM)	0.82 ± 0.01	0.8 ± 0.2	0.12 ± 0.05
SD	0.0002	0.0380	0.3667

[a] SD values are the sums of the squared deviations obtained at the end of the fitting procedure. At 37 °C, the k_3 value was experimentally determined in the absence of acceptor, the k_2 and K' values were obtained by extrapolation of the Arrhenius plots, and values of the other parameters were obtained by fitting initial rates and [T]/[H] ratios on eqs 5 and 6, respectively. At 15 °C, the values of k_2, K', and k_3 were determined experimentally in the absence of the acceptor substrate, and the values of the other parameters were obtained by fitting apparent first-order rate constants of acyl–enzyme formation on eq 7. With the modified enzyme, the values of k_2, K', and k_3 were determined experimentally at 37 °C in the absence of the acceptor substrate, and values of the other parameters were obtained by fitting initial rates and [T]/[H] ratios on eqs 5 and 6, respectively.

where

$$a' = k_2k_3\alpha\beta + k_2k_5\beta[A]$$

$$b' = k_2k_7[A]$$

$$c' = k_3\alpha\beta K' + k_5\beta K'[A]$$

$$d' = k_2\alpha\beta + k_3\alpha\beta + k_2\beta[A] + k_5\beta[A] + k_7K'[A]$$

$$e' = (k_2 + k_7)[A]$$

$$\frac{[T]}{[H]} = \frac{k_5\beta[A]}{k_3\alpha\beta + k_7[A][S]} \quad (6)$$

On this basis, the fitting procedure yielded the various values displayed in Table I.

Similarly, the value of the pseudo-first-order rate constant, k_{app}, characterizing the accumulation of the acyl–enzyme is given by eq 7,

$$k_{app} = \frac{k_2[S]}{K' + [S]} + \frac{k_3\alpha\beta + k_5\beta[A] + k_7[A][S]}{\alpha\beta + \beta[A] + [A][S]} \quad (7)$$

and the variations of the k_{app} parameter with the acceptor and donor substrate concentrations, obtained at 15 °C, could be fit to the model (Figure 3), yielding the values displayed in Table I, where the K', k_2, and k_3 values were again those measured in the hydrolysis experiments. To decrease the number of variable parameters in the fitting procedure, the values of α and β obtained at 37 °C were first considered as valid at the lower temperature. However, a much better fit was obtained when the value of α was somewhat increased.

In previous studies, complete time courses of the disappearance of substrate S2a had been recorded in the presence of various concentrations of D-alanine, and the k_{cat} and K_m values were derived with the help of the integrated Henri–Michaelis equation. The steady-state rate equation (eq 5) derived from Scheme III does not account for a hyperbolic dependence of v_0 upon [S], and this was confirmed by the nonlinear Hanes plots. However, the k_{cat} and K_m values derived from the linear part of these plots were in good agreement with those obtained from the complete time courses (Table A of the supplementary material). Furthermore, with the help of a Runge–Kutta integration routine, time courses of substrate disappearance were simulated using differential equations deduced from Scheme III and the values of the parameters presented in Table I. The simulated curves were then analyzed according to the simple integrated Henri–Michaelis equation and yielded k_{cat} and K_m values in good agreement with the experimentally determined values (up to a 1 mM concentration of donor substrate) (Table A of the supplementary material).

On the basis of the same differential equations, the variations of the acyl–enzyme concentration upon addition of either buffer or D-alanine to the preformed acyl–enzyme could also be simulated. The simulated curves agreed with the experimental data, whereas no agreement could be obtained on the basis of a model where the acceptor was assumed to bind only to the ES complex. The simultaneous binding of D-alanine to the Henri–Michaelis complex (ES) and to the acyl–enzyme could not be rejected, but there was no reason to introduce additional complications in a model which explained all of the observations.

With the Asn 161 Ser mutant, the values of the various parameters shown in Table I also yielded good agreement with the experimental data. The paradoxical effect of the presence of the acceptor, which resulted in a significant increase of the hydrolysis and a strongly decreased [T]/[H] ratio when compared to the wild-type enzyme, was easily explained by the very different effects of the mutation on the k_5/α and k_7/β ratios. Indeed, the former significantly decreased from 2 to 0.15 mM^{-1} s^{-1} while the second was barely affected (100 to 70 mM^{-1} s^{-1}). Thus, these differences nicely account for the preference of the modified protein for the second hydrolysis branch when compared to the transpeptidation pathway.

The proposed model also qualitatively explains the results obtained with the peptide donor substrate (Frère *et al.*, 1973), but those results are somewhat too incomplete to allow a detailed analysis similar to that presented here. Moreover, the high K_m values observed for this latter substrate, the fact that acylation is severely rate-limiting (Varetto *et al.*, 1987), and the technical difficulties due to the utilization of the peptide unfortunately preclude a more complete study of this substrate system.

The model presented here is quite similar to that proposed by Pazhanisamy and Pratt for the interaction between a class C β-lactamase and an ester substrate. The main difference is that the acceptor would essentially bind to the Henri–Michaelis complex (ES) with the β-lactamase and to the acyl–enzyme with the DD-peptidase. This might not be surprising since the kinetic parameters are such that, in the hydrolysis reaction with the substrates studied, different intermediates accumulate. With the DD-peptidases, binding of the acceptor to the ES complex cannot be rejected, but would only involve a small proportion of the reaction flux if present.

Finally, the number of binding sites for various substrates in or near the enzyme active site can appear to be surprisingly high. It should be noted that indications for such a complex situation had already been obtained (Frère *et al.*, 1973; Ghuysen *et al.*, 1973). Indeed, large concentrations of some acceptors have been shown to induce an inhibition of both the hydrolysis and transpeptidation reactions, which could only be interpreted by the existence of an additional binding site for these compounds. A "minimum" model could imply partial

or complete overlapping of this second acceptor binding site with the second donor binding site described here, but the binding of the acceptor would completely "freeze" the system while that of a donor would still allow hydrolysis to proceed.

Finally, one might wonder about the relevance of this complex model for other PBPs. In this respect, it is interesting to note that with the *Streptococcus pneumoniae* PBP 2×, which is the lethal target of cefotaxime in this bacterium, the [T]/[H] ratio appeared to depend upon the donor and acceptor concentrations in a way similar to that described here for the R61 DD-peptidase (Jamin *et al.*, 1993).

SUPPLEMENTARY MATERIAL AVAILABLE

Additional figures showing Arrhenius plots of k_{cat} (Figure A) and k_{cat}/K_m (Figure B) for the hydrolysis of substrate S2a and plots of k_2 (Figure C) and k_2/K (Figure B) for acyl–enzyme formation determined between 4 and 40 °C and a table (Table A) showing the comparison of k_{cat} values at different concentrations of D-alanine obtained from the complete time courses, initial rate measurements, and simulations (5 pages). Ordering information is given on any current masthead page.

REFERENCES

Adam, M., Damblon, C., Plaitin, B., Christiaens, L., & Frère, J. M. (1990) *Biochem. J. 270*, 525–529.

Adam, M., Damblon, C., Jamin, M., Zorzi, W., Dusart, V., Galleni, M., el Kharroubi, A., Piras, G., Spratt, B. G., Keck, W., Coyette, J., Ghuysen, J. M., Nguyen-Distèche, M., & Frère, J. M. (1991) *Biochem. J. 279*, 601–604.

Bourguignon-Bellefroid, C., Joris, B., Van Beeumen, J., Ghuysen, J. M., & Frère, J. M. (1992a) *Biochem. J. 283*, 123–128.

Bourguignon-Bellefroid, C., Wilkin, J. M., Joris, B., Aplin, R. T., Houssier, C., Prendergast, F. G., Van Beeumen, J., Ghuysen, J. M., & Frère, J. M. (1992b) *Biochem. J. 282*, 361–367.

De Meester, F., Joris, B., Reckinger, G., Bellefroid-Bourguignon, C., Frère, J. M., & Waley, S. G. (1987) *Biochem. Pharmacol. 36*, 2393–2403.

Erpicum, T., Granier, B., Delcour, M., Lenzini, M. V., Nguyen-Distèche, M., Dusart, J., & Frère, J. M. (1990) *Biotechnol. Bioeng. 35*, 719–726.

Fossati, P., Saint-Ghislain, M., Sicard, P. J., Frère, J. M., Dusart, J., Klein, D., & Ghuysen, J. M. (1978) *Biotechnol. Bioeng. 20*, 577–587.

Frère, J. M. (1973) *Biochem. J. 135*, 469–481.

Frère, J. M., & Joris, B. (1985) *CRC Crit. Rev. Microbiol. 11*, 299–396.

Frère, J. M., Ghuysen, J. M., Perkins, H. R., & Nieto, M. (1973) *Biochem. J. 135*, 483–492.

Frère, J. M., Nguyen-Distèche, M., Coyette, J., & Joris, B. (1992) in *The Chemistry of β-Lactams* (Page, M. I., Ed.) pp 148–195, Chapman and Hall, Glasgow.

Fruton, J. S. (1982) *Adv. Enzymol. 53*, 239–291.

Fuad, N., Frère, J. M., Ghuysen, J. M., Duez, C., & Iwatsubo, M. (1976) *Biochem. J. 155*, 623–629.

Ghuysen, J. M., Leyh-Bouille, M., Campbell, J. N., Moreno, R., Frère, J. M., Duez, C., Nieto, M., & Perkins, H. R. (1973) *Biochemistry 12*, 1243–1250.

Hadonou, A. M., Jamin, M., Adam, M., Joris, B., Dusart, J., Ghuysen, J. M., & Frère, J. M. (1992a) *Biochem. J. 282*, 495–500.

Hadonou, A. M., Wilkin, J. M., Varetto, L., Joris, B., Lamotte-Brasseur, J., Klein, D., Duez, C., Ghuysen, J. M., & Frère, J. M. (1992b) *Eur. J. Biochem. 207*, 97–102.

Holzhütter, H. G., & Colosimo, A. (1990) *CABIOS 6*, 23–28.

Jamin, M., Adam, M., Damblon, C., Christiaens, L., & Frère, J. M. (1991) *Biochem. J. 280*, 499–506.

Jamin, M., Damblon, C., Millier, S., Hakenbeck, R., & Frère, J. M. (1993) *Biochem. J. 292*, 735–741.

Joris, B., Ghuysen, J. M., Dive, G., Renard, A., Dideberg, O., Charlier, P., Frère, J. M., Kelly, J., Boyington, J., Moews, P., & Knox, J. (1988) *Biochem. J. 250*, 313–324.

Joris, B., Ledent, P., Dideberg, O., Fonzé, E., Lamotte-Brasseur, J., Kelly, J. A., Ghuysen, J. M., & Frère, J. M. (1991) *Antimicrob. Agents Chemother. 35*, 2294–2301.

Kasche, V., Riechmann, L., & Haufler, U. (1984) *Ann. N.Y. Acad. Sci.* 99–105.

Kelly, J. A., Moews, P. C., Knox, J. R., Frère, J. M., & Ghuysen, J. M. (1982) *Science 218*, 479–481.

Kelly, J. A., Knox, J. R., Moews, P. C., Hite, G. J., Bartolone, J. B., Zhao, H., Joris, B., Frère, J. M., & Ghuysen, J. M. (1985) *J. Biol. Chem. 260*, 6449–6458.

Kelly, J. A., Knox, J. R., Zhao, H., Frère, J. M., & Ghuysen, J. M. (1989) *J. Mol. Biol. 209*, 281–295.

Leatherbarrow, R. J. (1987) ENZFITTER, Elsevier Biosoft, Cambridge, UK.

Mazzella, L. J., Pazhanisamy, S., & Pratt, R. F. (1991) *Biochem. J. 274*, 855–859.

Nieto, M., Perkins, H. R., Frère, J. M., & Ghuysen, J. M. (1973) *Biochem. J. 135*, 493–505.

Pazhanisamy, S., & Pratt, R. F. (1989a) *Biochemistry 28*, 6875–6882.

Pazhanisamy, S., & Pratt, R. F. (1989b) *Biochemistry 28*, 6870–6875.

Pazhanisamy, S., Govardhan, C. P., & Pratt, R. F. (1989) *Biochemistry 28*, 6863–6870.

Perkins, H., Nieto, M., Frère, J. M., Leyh-Bouille, M., & Ghuysen, J. M. (1973) *Biochem. J. 131*, 707–718.

Pollock, J. J., Ghuysen, J. M., Linder, R., Salton, M. R. J., Perkins, H. R., Nieto, M., Leyh-Bouille, M., Frère, J. M., & Johnson, K. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 662–666.

Pratt, R. F., & Govardhan, C. P. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1302–1306.

van der Groen, G., Wouters-Leysen, J., Yde, M., & De Bruyne, C. K. (1973) *Eur. J. Biochem. 28*, 122–129.

Varetto, L., Frère, J. M., Nguyen-Distèche, M., Ghuysen, J. M., & Houssier, C. (1987) *Eur. J. Biochem. 162*, 525–531.

Waley, S. G. (1992) in *The Chemistry of β-Lactams* (Page, M. I., Ed.) pp 198–228, Chapman and Hall, Glasgow.

Wilkin, J. M., Jamin, M., Damblon, C., Zhao, G. H., Joris, B., Duez, C., & Frère, J. M. (1993a) *Biochem. J. 291*, 537–544.

Wilkin, J. M., Jamin, M., Joris, B., & Frère, J. M. (1993b) *Biochem. J. 293*, 195–201.