Streptomyces K15 active-site serine DD-transpeptidase: specificity profile for peptide, thiol ester and ester carbonyl donors and pathways of the transfer reactions

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The *Streptomyces* K15 transferase is a penicillin-binding protein presumed to be involved in bacterial wall peptidoglycan cross-linking. It catalyses cleavage of the peptide, thiol ester or ester bond of carbonyl donors Z-R^1-CONH-CHR^2-COX-CHR^3-COO^- (where X is NH, S or O) and transfers the electrophilic group Z-R^1-CONH-CHR^2-CO to amino acceptors via an acyl-enzyme intermediate. Kinetic data suggest that the amino acceptor behaves as a simple alternative nucleophile at the level of the acyl-enzyme in the case of thiol ester and ester donors, and that it binds to the enzyme·carbonyl donor Michaelis complex and influences the rate of enzyme acylation by the carbonyl donor in the case of amide donors. Depending on the nature of the scissile bond, the enzyme has different requirements for substituents at positions R^1, R^2 and R^3.

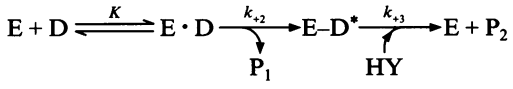
INTRODUCTION

All bacteria possess at least one, most often several, monofunctional serine DD-peptidases that catalyse transfer of the R-L-αα-D-alanyl moeity of R-L-αα-D-alanyl-D-alanine carbonyl donors to the γ-OH of their active-site serine and from this to a final acceptor. The general reaction is shown in Scheme 1. $k_{cat.}/K_m = k_{+2}/K$ is the second-order rate constant of enzyme acylation [1].

Depending on whether HY is water, D-alanine or another amino compound, the carbonyl donor is hydrolysed (DD-carboxypeptidase activity), regenerated or aminolysed (DD-transpeptidase activity). Penicillin is also a carbonyl donor of the DD-peptidases. As the scissile β-lactam amide bond is endocyclic, P_1 remains part of a long-lived serine-ester-linked acyl (penicilloyl)-enzyme, k_{+3} has a very small value and the DD-peptidases behave as penicillin-binding proteins (PBPs). The serine DD-peptidases/PBPs possess the active-site-defining motifs characteristic of the penicilloyl serine transferase family (reviewed in [2]). Usually, they are bound to the outer face of the plasma membrane by a C-terminal amphiphilic α-helix [3] and they function mainly as DD-carboxypeptidases.

The *Streptomyces* K15 DD-peptidase/PBP is unusual. After translocation through the membrane, the signal peptide-free 262-amino acid mature protein (conserved motifs S35TTK, where S35 is the essential serine residue, S96GC and K213TG) remains attached to the membrane although it lacks C-terminal anchors [4]. In addition, the *Streptomyces* K15 enzyme catalyses transfer of the R-L-αα-D-alanyl moiety of carbonyl donor peptides to exogenous amino acceptors related to the wall peptidoglycan (such as glycylglycine) with much greater efficacy than to water. In consequence, it behaves as a strict DD-transpeptidase under certain conditions [5].

Interpretation of the catalytic property of an enzyme at the atomic level requires elucidation of the three-dimensional structure of the protein. Overexpression of the *Streptomyces* K15 enzyme in *Streptomyces lividans* harbouring plasmid pDML225 allows the enzyme to be exported in the culture medium in substantial amounts [6]. The water-soluble enzyme still functions as a transpeptidase and binds benzylpenicillin, piperacillin, ticarcillin and temocillin with the same k_{+2}/K and (low) k_{+3} values as the membrane-bound enzyme purified in the presence of cetyltrimethylammonium bromide (CTAB) ([7] and unpublished work). Recent advances in X-ray diffraction of the water-soluble protein have been reported [8]. The transfer reactions that the enzyme catalyses on peptide, thiol ester and ester carbonyl donors have now been studied. The results are presented below.



Scheme 1 General reaction of bacterial serine DD-peptidase

E, enzyme; D, carbonyl donor; E·D, Michaelis complex; E–D*, serine ester-linked acyl (R-L-αα-D-alanyl)-enzyme; HY, final acceptor; P_1, leaving group of the enzyme acylation step (i.e. D-alanine); P_2, transfer product; K, dissociation constant; k_{+2}, k_{+3}, first-order rate constants.

MATERIALS AND METHODS

The carbonyl donors used are listed in Table 1 (Results section). Peptide 1 was a gift from Reanol (Budapest, Hungary), peptides 2–4 were used previously [5], peptide 5 was from UCB Bioproducts (Braine-l'Alleud, Belgium), peptides 6 and 7 were from Sigma (St. Louis, MO, U.S.A.), peptide 8 was synthesized as described [9]. The peptides and the soluble enzyme were incubated in 5.7 mM Tris/HCl, pH 7.2, containing 0.1 M NaCl and 0.045 mM dithiothreitol at 37 °C. The released D-alanine (for peptides 1 and 5–8) was measured by the D-amino acid oxidase/peroxidase procedure [10].

Thiol esters 9–12 were synthesized as described [11,12]. Thiol-esters 13–16 were gifts from UCB Bioproducts. The thiol esters and the soluble enzyme were incubated in 25 mM sodium phosphate, pH 7.2, containing 0.4 M NaCl and 0.004 mM dithiothreitol at 37 °C. The time-dependent decrease in absorbance

Abbreviations used: PBP, penicillin-binding protein; CTAB, cetyltrimethylammonium bromide.

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Table 1 Specificity profile and kinetic parameters of the transfer reactions catalysed by the *Streptomyces* K15 peptidase

The arrow denotes the scissile bond. *ms*A_2pm, *meso*-2,6-diaminopimelic acid.

$$Z\text{-}R^1\text{-}CO\text{-}NH\text{-}CH(R^2)\text{-}CO\text{-}\downarrow\text{-}X\text{-}CH(R^3)\text{-}COO^-$$

Carbonyl donor	Z	R^1	R^2	X	R^3	Amino acceptor	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
1	N^{α}-Ac	L-Lys |ϵ NH-Ac	CH_3	NH	CH_3	None	–	–	10*
						Gly-Gly (2 mM)	0.31	6.2	50
						Gly-Gly (10 mM)	0.46	9.4	49
2			H	NH	H	Gly-Gly (2 mM)	–	No activity†	–
3			H	NH	CH_3	Gly-Gly (2 mM)	–	No activity†	–
4			CH_3	NH	H	Gly-Gly (2 mM)	–	Very low activity†	–
5	N^{α}-Ac	L-Lys	CH_3	NH	CH_3	Gly-L-Ala (10 mM)	0.45	9.35	48
6	N^{α}-(L-Ala-γ-D-Glu)	L-Lys	CH_3	NH	CH_3	Gly-Gly (3 mM)	0.24	8.5	28
7	N^{α}-(L-Ala-γ-D-Glu)	*ms*A_2pm	CH_3	NH	CH_3	Gly-Gly (3 mM)	–	No activity	–
8		C_6H_5	CH_3	NH	CH_3	None	–	No activity	–
						Gly-Gly (5 mM)	–	No activity	–
9		C_6H_5	CH_3	S	H	None	0.11	1.5	75
						Gly-Gly (saturation)	0.53	7	75
						D-Ala (saturation)	0.80	11	73
10		C_6H_5	H	S	CH_3‡	None	0.017	0.22	75
11		C_6H_5	H	S	H	None	–	High	8
12		C_6H_5	CH_3	S	CH_3	None	–	High	3
13		C_6H_5-CH_2	CH_3	S	H	None	–	–	160
14		C_6H_5-CH_2	CH_3	S	CH_3‡	None	–	–	450
15	N^{α}-Ac	L-Lys |ϵ NH-Ac	CH_3	S	H	None	–	–	45
16			CH_3	S	CH_3‡	None	–	–	145
17			CH_3	O	CH_3	None	0.13	0.7	186
						Gly-Gly (10 mM)	1.78	9.8	184

* The $k_{cat.}/K_m$ value was determined at [S] < K_m (from 0.75 to 1.2 mM) under which conditions less than 10% of peptide 1 was consumed and the concentration of released D-alanine was < 0.03 mM.

† As determined on the membrane-bound detergent-purified enzyme [5].

‡ Racemic mixture.

was monitored at 250 nm using a UVIKON 800 spectrophotometer linked to a microcomputer via an RS232 interface [9,13].

Ester 17 was from Serva Feinbiochemica (Heidelberg, Germany). The ester and the soluble enzyme were incubated under the same conditions as those described above for the peptides. The released D-lactate was measured by the D-lactate dehydrogenase procedure [10].

$k_{cat.}$ and K_m were calculated from initial-rate measurements by fitting the data to the Henri–Michaelis equation or to its linearized form according to Hanes' transformation using the ENZFITTER program. $k_{cat.}/K_m$ values for thiol esters 13–16 were computed by analysing the complete time courses [13].

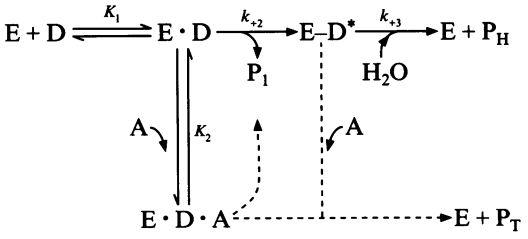
In some cases, the carbonyl donor D, the hydrolysed product H and the aminolysis product T of the transfer reactions were separated by HPLC on an ET250/8/4 Nucleosil-5 C18 column (Macherey-Nagel). The flow rate was 1 ml/min. With the cosubstrates, peptide 1/Gly-Gly, the retention times for D, H and T were 11.6, 9.9 and 8.2 min respectively (solvent 0.1% trifluoroacetic acid/6% acetonitrile). With the cosubstrates, thiol ester 9/D-alanine, the retention times of D, H and T were 14.7, 7.3 and 6.6 min respectively (solvent 80% 10 mM sodium acetate, pH 3.0/20% acetonitrile). With the cosubstrates, ester 17/Gly-Gly, the retention times of D, H and T were 18.5, 6.5 and 5.2 min respectively (solvent 0.1% trifluoroacetic acid/8% acetonitrile). Detection was performed at 215 nm (peptide 1 and ester 17) or at 235 nm (thiol ester 9).

RESULTS

Table 1 gives the values of $k_{cat.}$, K_m and $k_{cat.}/K_m$ (i.e. k_{+2}/K; see reaction 1) for the consumption of the carbonyl donors.

Transfer reactions on peptide carbonyl donors 1–8

The reactions catalysed by the membrane-bound enzyme (purified in the presence of CTAB) on peptide 1 have been studied previously [5]. In water, the acyl (Ac_2-L-Lys-D-alanyl)-enzyme accumulates, the second-order rate constant of enzyme acylation k_{+2}/K is small (14 $M^{-1} \cdot s^{-1}$) and D-alanine, as it is released during enzyme acylation, is utilized as amino acceptor and regenerates peptide 1. In the presence of Gly-Gly at concentrations sufficient to overcome the acceptor activity of the released D-alanine, the enzyme activity undergoes partitioning between hydrolysis (reaction product Ac_2-L-Lys-D-Ala) and aminolysis (reaction product Ac_2-L-Lys-D-Ala-Gly-Gly). Under saturating concentrations of Gly-Gly, peptide 1 is quantitatively converted into the transpeptidation product. The effect of increasing concentrations



Scheme 2

of Gly-Gly is to increase the value of $k_{cat.}$ and to a lesser extent the value of K_m so that the value of $k_{cat.}/K_m$ (i.e k_{+2}/K) increases from 14 $M^{-1} \cdot s^{-1}$ to 90 $M^{-1} \cdot s^{-1}$, leading to the conclusion that the amino acceptor Gly-Gly enhances the rate of enzyme acylation by peptide 1. Attempts to detect a binary complex (enzyme·Gly-Gly) (by equilibrium dialysis with radioactive Gly-Gly) failed and the value of the second-order rate constant of acylation of the enzyme by benzylpenicillin was not modified by the presence of Gly-Gly (20 mM), suggesting that the amino acceptor binds to the Michaelis complex E·D.

The water-soluble enzyme behaved similarly. The value of $k_{cat.}/K_m$ increased from 10 $M^{-1} \cdot s^{-1}$ in water to 50 $M^{-1} \cdot s^{-1}$ in the presence of 2 mM (or more) Gly-Gly (Table 1). Under saturating concentrations of the amino acceptor, as shown by HPLC analysis of the reaction products, the enzyme functioned as a strict transpeptidase.

The simplest scheme that best explains the above observations is that of a branched pathway (Scheme 2). Scheme 2 is similar to that proposed for the transfer reaction catalysed by the class-C β-lactamase of *Enterobacter cloacae* on the system *m*-[(phenylacetylglycyl)oxy]benzoic acid as carbonyl donor and D-phenylalanine as amino acceptor [14].

Using peptide 1 as reference, transpeptidase activity of the CTAB-purified membrane-bound enzyme [5] (i) strictly requires a D-alanine at position 2, (ii) is much decreased or abolished when a D-amino acid residue other than D-alanine or glycine is at position 3, (iii) is decreased when the neutral side chain at position 1 is shortened (as observed when Ac_2-L-Lys-D-Ala-D-Ala is replaced by Ac_2-L-A_2bu-D-Ala-D-Ala; Ac is acetyl; A^2bu is 2,4-diaminobutyric acid) and (iv) is maximal when Gly-Gly or Gly-L-Ala are used as amino acceptors of the transfer reaction [5].

The water-soluble enzyme behaved similarly. In addition (see peptides 1 and 5–8 in Table 1), transpeptidase activity appeared to be compatible with a wide range of substituents, Z, suggesting that the substituents on the amino side of R^1 did not interact, at least directly with the enzyme active site. Transpeptidase activity was also compatible with the presence of a free ε-amino group at position 1 but did not tolerate a carboxylate in the α-position to the ε-amino group. As expected, peptide 8 in which a benzoyl moiety at position 1 was fused to the amino end of D-Ala-D-Ala, had no carbonyl-donor activity.

Transfer reactions on thiol ester carbonyl donors 9–16

The use of thiol esters instead of peptides as carbonyl donors modified the features of the catalysed reactions in several respects.

In contrast with D-alanine (i.e. the leaving group of enzyme acylation by D-alanyl-D-alanine-terminated carbonyl donors), thioglycolate and D-thiolactate, the leaving groups of enzyme acylation by thiol esters 9, 11, 13, 15 and thiol esters 10, 12, 14, 16 respectively, had no acceptor activity with the corresponding

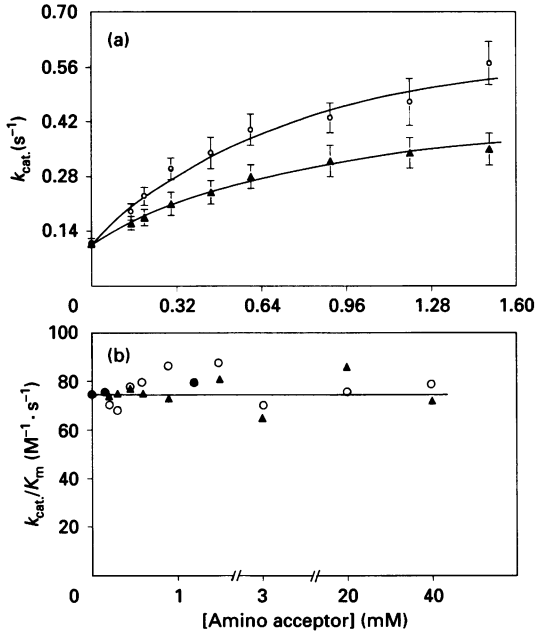


Figure 1 Effect of increasing concentrations of amino acceptors [D-alanine (○); glycylglycine (▲) on (a) the $k_{cat.}$ and (b) the $k_{cat.}/K_m$ values for consumption of the thiol ester carbonyl donor benzoyl-D-alanine-thioglycollate by the *Streptomyces* K15 acyltransferase

Enzyme concentration was 1 μM. For amino acceptor concentrations of 1.5 mM or less, the $k_{cat.}/K_m$ values were computed from individual $k_{cat.}$ and K_m values. For larger concentrations, the $k_{cat.}/K_m$ values were determined at [D] ≪ K_m; S.D. values are 17% or less.

acyl-enzymes. In consequence, hydrolysis reached completion (but at various rates; see Table 1).

The reaction catalysed by the enzyme on thiol ester 9 was studied in detail. In water, hydrolysis proceeded with $k_{cat.} = 0.11 \pm 0.026\ s^{-1}$, $K_m = 1.5 \pm 0.5$ mM and $k_{cat.}/K_m \approx 75 \pm 10\ M^{-1} \cdot s^{-1}$. At variance with that observed with peptide 1, increasing concentrations of Gly-Gly or D-Ala caused a commensurate increase in both $k_{cat.}$ (Figure 1a) and K_m so that $k_{cat.}/K_m$ (Figure 1b) remained constant (75 $M^{-1} \cdot s^{-1}$) and equal to that of the hydrolysis pathway. The amino acceptor concentrations $[A]_{50}$ at which $k_{cat.}$ was half-maximum were 0.98 mM Gly-Gly and 0.91 mM D-Ala. At saturating concentrations of Gly-Gly, $k_{cat.}$ was $0.53 \pm 0.05\ s^{-1}$ and K_m was 7 ± 0.7 mM. At saturating concentrations of D-Ala, $k_{cat.}$ was $0.8 \pm 0.09\ s^{-1}$ and K_m was 11 ± 0.2 mM. With $a = k_{cat.}$ in the absence of amino acceptor (hydrolysis pathway), $b/c = k_{cat.}$ at saturating concentrations of the amino acceptor and $1/c = [A]_{50}$, the curve $k_{cat.}$ versus [A] of Figure 1(a) fitted the empirical equation

$$k_{cat.} = (a + b[A])/(1 + c[A])$$

The significance of this equation is discussed in [9].

As shown by HPLC analysis of the products of the transfer reaction, with A = D-alanine, thiol ester 9 underwent partitioning between hydrolysis (reaction product, H. = benzoyl-D-alanine) and aminolysis (reaction product, T. = benzoyl-D-alanyl-D-alanine, i.e. peptide 8). At a fixed concentration (1 mM) of thiol ester and under conditions in which less than 10% of the thiol ester was consumed, the T/H ratio was directly proportional to the D-alanine concentrations, from 0.1 to 3.5 mM (Figure 2a). At concentrations of D-alanine larger than 3.5 mM, hydrolysis was

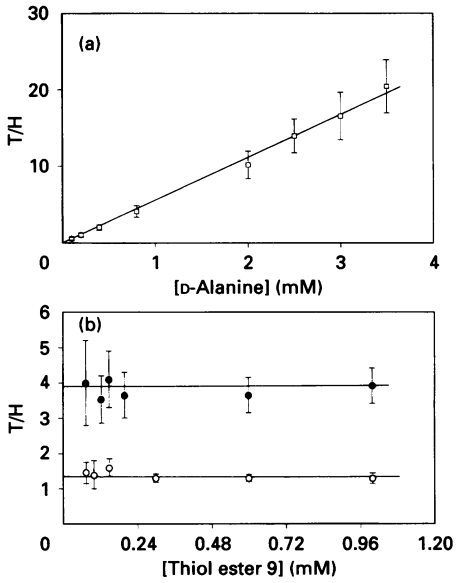
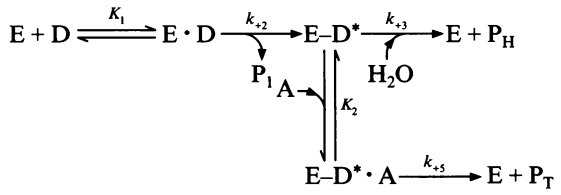


Figure 2 Ratios between the aminolysis product (T) and hydrolysis product (H) produced by the *Streptomyces K15* acyltransferase on the system benzoyl-D-alanyl-thioglycollate/D-alanine

(**a**) Effects of increasing concentrations of D-alanine at a fixed 1 mM carbonyl donor concentration; (**b**) effects of increasing concentrations of the carbonyl donor at fixed concentrations of D-alanine: ○, 0.25 mM; ●, 0.60 mM. Enzyme concentration was 2 μM and incubation was for 45 min at 37 °C. The error bars represent S.D. values (three determinations).



Scheme 3

negligible and the thiol ester was quantitatively converted into benzoyl-D-alanyl-D-alanine. Conversely, at a fixed concentration (0.25 or 0.6 mM) of D-alanine, the T/H ratio was independent of the thiol ester concentration (Figure 2b).

On the basis of these kinetic data, it followed that the amino acceptor did not influence the rate of acylation of the enzyme by the thiol ester. Rather, it acted, at least predominantly, as an alternative nucleophile at the level of the acyl-enzyme E–D*. The simplest scheme that best explains these observations is that of a branched reaction (Scheme 3), in which k_{+5} is larger than k_{+3} but not larger than k_{+2}.

Scheme 3 is reminiscent of that proposed for the transfer reactions catalysed by the *Streptomyces* R61 DD-peptidase/PBP on the system benzoyl-glycyl-thioglycollate/D-alanine except that, in this latter case, a second molecule of thiol ester would bind to the ternary complex E–D*·A to form a quaternary complex E–D*·A·D also productive in hydrolysis [15]. Note that the affinity of the *Streptomyces* K15 enzyme for the amino acceptor Gly-Gly ($[A]_{50}$ = 0.92 mM) is at least 20 times greater than that of the *Streptomyces* R61 enzyme for the same acceptor ($[A]_{50}$ = 22.5 mM). In agreement with this result, the *Streptomyces* R61 enzyme behaves mainly as a carboxypeptidase.

In contrast with that described for peptides 1–4, carbonyl-donor activity of the benzoyl thiol esters 9–12 required a D-alanine residue at position 2 and a thioglycollate at position 3 or a glycine residue at position 2 and a D-thiolactate at position 3. Activity was much decreased when positions 2 and 3 were occupied by a glycine and a thioglycollate respectively (thiol ester 11) or when positions 2 and 3 were occupied by a D-alanine and a D-thiolactate respectively (thiol ester 12). Substrate requirements at positions 2 and 3, however, varied depending on the nature of the residue at position 1. The result of the replacement of the benzoyl moiety at position 1 by a benzyl moiety (thiol esters 13 and 14) or by an Ac_2-L-Lys moiety (thiol esters 15 and 16) was that high carbonyl-donor activity required a D-alanine at position 2 and a D-thiolactate at position 3. Of the carbonyl donors 1–16 of Table 1, thiol ester 14, benzyl-D-alanyl-D-thiolactate, had the highest activity, acetylating the enzyme with a second-order rate constant of about 450 $M^{-1} \cdot s^{-1}$.

Transfer reaction on ester carbonyl donor 17

D-lactate, the leaving group of enzyme acylation by the ester, had no acceptor activity with the acyl (Ac_2-L-Lys-D-alanyl)-enzyme. In water, hydrolysis proceeded with a $k_{cat.}$ of 0.13 ± 0.013 s^{-1}, a K_m of 0.7 ± 0.16 mM, and a $k_{cat.}/K_m$ of about 186 $M^{-1} \cdot s^{-1}$, a value comparable with that obtained for the corresponding thiol ester 16. Increasing concentrations of Gly-Gly did not modify the value of $k_{cat.}/K_m$ suggesting that Scheme 3 applied. At saturating concentrations of Gly-Gly, as shown by HPLC analysis of the reaction products, the ester was quantitatively converted into the peptide Ac_2-L-Lys-D-Ala-Gly-Gly.

DISCUSSION

The *Streptomyces* K15 acyltransferase catalyses transfer of the electrophilic group of amide, thiol ester and ester carbonyl donors to exogenous acceptors via formation of a serine ester-linked acyl-enzyme. To achieve enzyme acylation, the donor molecule must bind to the enzyme in a position that allows the proton of the γOH of the active-site serine (S35) to be abstracted, the activated OγS35 to attack the carbonyl carbon of the scissile (CON, COS or COO) bond and the abstracted proton to be back-donated to the adjacent N, S or O atom. The distance between the carbonyl carbon that undergoes the nucleophilic attack and the neighbouring atom to which the proton is back-donated is about 0.14 nm for a peptide or an ester bond but about 0.185 nm for a thiol ester bond, and the rotation barrier is about 12.5 kJ/mol for a thiol ester or an ester bond but about 63 kJ/mol for a peptide bond. Understandably, positioning of the scissile bond with respect to the enzyme active centre allowing for optimal functioning of the enzyme charge relay system, is likely to vary depending on the type of carbonyl donors used. In consequence, it may require substituents of various structures at positions immediately upstream (R^1 and R^2) and downstream (R^3) of the substrate electrophilic centre.

The data presented above show clearly that the specificity profile of the *Streptomyces* K15 enzyme for carbonyl donors and the mode of binding of the amino acceptor along the reaction pathway vary depending on the nature of the scissile bond of the carbonyl donor. These observations well illustrate the concept of how the charge-relay system of an enzyme is created by the interacting partners. Theoretical studies carried out on chymotrypsin (which is mechanistically analogous to the *Streptomyces* K15 enzyme) also support this concept [16]. Optimization at the AM1 quantum level of a 244-atom model of the Henri–Michaelis

complex *N*-acetyl-*N*-tryptophanamide·chymotrypsin reveals that optimal positioning of the carbonyl donor within the enzyme cavity is the result of the combined effects of the enzyme active-site environment, the deformation undergone by the bound molecule, the relaxation undergone by the polypeptide backbone and the freedom of a water molecule.

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