Eur. J. Biochem. 207, 97–102 (1992)

Site-directed mutagenesis of the *Streptomyces* R61 DD-peptidase

Catalytic function of the conserved residues around the active site and a comparison with class-A and class-C β-lactamases

Ayaovi M. HADONOU, Jean-Marc WILKIN, Louis VARETTO, Bernard JORIS, Josette LAMOTTE-BRASSEUR, Daniel KLEIN, Colette DUEZ, Jean-Marie GHUYSEN and Jean-Marie FRÈRE

Centre d'Ingénierie des Protéines, Université de Liège, Institut de Chimie, B6, Belgium

(Received December 30, 1991/March 30, 1992) – EJB 91 1749

The importance of various residues in the *Streptomyces* R61 penicillin-sensitive DD-peptidase has been assessed by site-directed mutagenesis. The replacement of the active Ser62 by a Cys residue yielded an inactive protein which was also unable to recognize penicillin. The activity of the Lys65→Arg mutant with the peptide and thiolester substrates was decreased 100–200-fold and the rate of penicillin inactivation was decreased 20000-fold or more. The mutant thus behaved as a poor, but penicillin-resistant, DD-peptidase.

The other studied mutations, the mutations Phe58→Leu, Tyr90→Asn, Thr101→Asn, Phe164→Ala, Asp225→Glu and Asp225→Asn had little influence on the catalytic and penicillin-binding properties. The Asp225 mutants did not exhibit an increased sensitivity to cefotaxime. The Phe164→Ala mutant was significantly more unstable than the wild-type enzyme.

Active-site serine penicillin-sensitive DD-peptidases and β-lactamases share the same kinetic pathway in their interaction with β-lactam compounds (Scheme 1), the major difference being that the acylated enzyme EC* is stable with the former (low k_3) and very rapidly hydrolysed with the latter (high k_3). With slight modifications, the same model can be utilized to account for the interaction between the DD-peptidases and their substrates (Frère and Joris, 1985). At the present time, the *Streptomyces* R61 extracellular DD-peptidase is the only penicillin-sensitive enzyme for which X-ray-diffraction data are available (Kelly et al., 1989). Although these results have not allowed the building of a high-resolution model yet, they revealed clear similarities with the 3-dimensional structures of class A (Kelly et al., 1986; Samraoui et al., 1986) and class C (Oefner et al., 1990) β-lactamases. With the aid of the crystallographic data, Joris et al. (1988) proposed sequence alignments in which identical or similar residues were generally found in corresponding positions near the active-site cavities in the known structures. The role of some of these residues (Arg103, Trp271 and His298) have been examined in previous contributions (Bourguignon-Bellefroid et al., 1992a and b; Hadonou et al., 1992). In the present work, we have modified seven other residues, including the active-site Ser62 itself and the highly conserved Phe58 and Lys65 residues. Table 1 describes the mutations and the oligodeoxynucleotides which were synthesized and also gives the corresponding residues tentatively identified by Joris et al. (1988) in the two most-studied classes of β-lactamases.

Correspondence to J.-M. Frère, Centre d'Ingénierie des Protéines, Université de Liège, Institut de Chimie, B6, B-4000 Sart Tilman, Liège 1, Belgium

Abbreviation. Ac_2-L-Lys-D-Ala-D-Ala, $N^{\alpha}N^{\varepsilon}$-diacetyl-L-lysyl-D-alanyl-D-alanine.

Enzymes. Streptomyces R61 DD-peptidase (EC 3.4.16.4); β-lactamase (EC 3.5.2.6).

MATERIALS AND METHODS

Mutagenesis

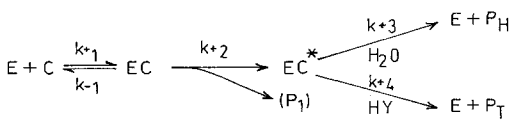
All mutations were performed as described in Hadonou et al. (1992) with the exception of the Ser62→Cys mutant which was obtained using the couple-priming method (Zoller and Smith, 1984). Table 1 lists the oligodeoxynucleotides. All the mutations were in the *Pst*1–*Sph*1 fragment, excepting those of residue Asp225 which were in the *Sph*1–*Eco*RV fragment [see also Fig. 1 in Hadonou et al. (1992)].

Production and purification

Table 2 summarizes the production in the culture supernatants of *Streptomyces lividans* TK24 and the yields of the various purifications. Productions and purifications were achieved as described in Hadonou et al. (1992).

Kinetic analysis

The catalytic properties of the mutants were studied using a tripeptide N^{α},N^{ε}-diacetyl-L-lysyl-D-alanyl-D-alanine (Ac_2-L-



Scheme 1. Kinetic pathway describing the interaction between a DD-peptidase (E) and a substrate or inactivator (C). EC is the Henri-Michaelis complex and EC* the acylenzyme, P_1 is D-Ala with the peptide substrate and thioglycolate with the thiolester. No product is released at this stage with β-lactams. HY is a nucleophilic acceptor substrate, D-Ala in the present work. With β-lactams, two water molecules can be involved in the k_3 step, which results in fragmentation of the antibiotic molecule.

Table 1. Studied mutations, sequences of the oligo-d-nucleotides utilized for obtaining the modified enzymes and corresponding residues in class-A [ABL numbering of Ambler et al. (1991)] and class-C [numbering of Galleni et al. (1988)] β-lactamases. For the first three residues, the equivalence is well established; for the four other ones, it is only tentative.

Mutation	S. R61 DD-peptidase sequence (5′→3′) of the primers	Corresponding residues in β-lactamases	
		class A	class C
Phe58→Leu	GAG CGG TTG* CGC GTC G	Phe66	Phe60
Ser62→Cys	GCG TCG GGC T*GC GTC AC	Ser70	Ser64
Lys65→Arg	C GTC ACC AG*G AGC TTC T	Lys73	Lys67
Tyr90→Asn	TG AAC ACC A*AT CTG CCC	Tyr105	Tyr92
Thr101→Asn	GAC CGG ATC AA*C GTG CGT	Thr108	Thr98
Phe164→Ala	AAC ACG AAC A*A*C GTC GTC GCG	Asn165	Ile155
Asp225→Glu	GCC CTG GTC G*AC TCC ACC CAG	Glu166	Asp217
Asp225→Asn	CC CTG GTC A*AC TCC ACC		

Table 2. Summary of the production and purification of the modified enzymes. The substrate was Ac_2-L-Lys-D-Ala-D-Ala at a concentration of 2 mM; the values are thus different from the V_{max}. The SD on enzyme activity determinations was ±10%. The total amount of enzyme in the supernatant was first estimated on the basis of the intensity of the Coomassie-blue stained enzyme band after SDS-PAGE using a known quantity of WT (wild type) enzyme as standard. The values were in good agreement with those calculated by dividing the total enzymatic activity by the specific activity of the purified protein. Under the same conditions, the specific activity of the wild type enzyme is 11 μmoles/min × mg of enzyme.

Mutant	Volume of culture	Total enzyme in culture	Amount of purified enzyme	Purification yield	Final specific activity
	l	mg		%	μmol D-Ala · min^{-1} · (mg enzyme)$^{-1}$
Phe58→Leu	2.5	200	130	65	0.6
Ser62→Cys	5	200	100	50	n.d.
Lys65→Arg	10	50	2	4	0.08
Tyr90→Asn	2.5	250	120	48	0.75
Thr101→Asn	2.5	390	100	26	1.15
Phe164→Ala	2.5	200	150	75	3.10
Asp225→Glu	2.5	200	150	75	8.7
Asp225→Asn	2.5	500	300	60	18.0

Lys-D-Ala-D-Ala) and a thiolester (C_6H_5-CO-NH-CH_2-CO-S-CH_2-COO^-) substrates. The parameters characteristic of the acylation by β-lactams and of the subsequent deacylation reaction were also determined. The chemicals and techniques have been detailed in Hadonou et al. (1992).

Reaction product(s)

The nature of the products released upon spontaneous degradation of the β-lactam–enzyme adduct was studied in 10 mM sodium phosphate, pH 7.0. The enzymes were inactivated by a stoechiometric amount of [^{14}C]benzylpenicillin (Amersham, UK) and the product was identified by thin-layer chromatography (Frère et al., 1976) after 60 min and 120 min incubation.

Kinetic background and properties of the wild-type enzyme

With β-lactams, deacylation and acylation can be separatedly studied, yielding the values of k_3 and k_2/K', respectively, where $K' = (k_{+2} + k_{-1})/k_{+1}$. With the substrates, the steady-state parameters k_{cat} and K_m were measured. In the absence of acceptor (HY), $k_{cat} = k_2k_3/(k_2 + k_3)$, $K_m = k_3K'/(k_2 + k_3)$, and $k_{cat}/K_m = k_2/K'$.

With the peptide substrate, k_2 is much smaller than k_3 (Varetto et al., 1987a), thus $k_{cat} = k_2$ and $K_m = K'$. Conversely, with the thiolester, k_2 is much larger than k_3, so that $k_{cat} = k_3$ and $K_m = k_3K'/k_2$ (Jamin et al., 1991). The presence of low-acceptor concentrations does not significantly modify the rate of peptide utilization (Frère et al., 1973) but increases the values of both k_{cat} and K_m for the thiolester, the k_{cat}/K_m ratio remaining unchanged. Under these conditions, k_{cat} increases linearly with the acceptor concentration [A] and the relative acceleration is defined as $\Delta k_{cat}/(k_{cat,o} \times \Delta[A])$ where $k_{cat,o}$ is that in the absence of acceptor (Jamin et al., 1991). The methods for obtaining the various constants have been described in detail by Hadonou et al. (1992).

RESULTS AND DISCUSSION

Conformation of the proteins

The fluorescence spectra of all the modified proteins were not significantly different from that of the wild-type enzyme. Interestingly, the maximum of fluorescence emission was at 320 nm in all cases, indicating an unchanged environment for the Trp fluorophores. Thermal denaturation was followed by monitoring the decrease of fluorescence at 320 nm and only the Phe164→Ala mutant exhibited a severe decrease of stability (Table 3).

Kinetic properties

Lys62→Ser

The Ser62→Cys protein was completely inactive with both substrates. It also failed to covalently bind penicillins. At-

Table 3. First-order rate constants (k_d) for the inactivation of the proteins at 60°. The apparent first-order rate constants (k_d) were determined by following the time course of the fluorescence decrease at 320 nm as described before (Hadonou et al., 1992).

Enzyme	$10^3 \times k_d$
	s^{-1}
Wild type	3.3 ± 0.4
Phe58→Leu	3.25 ± 0.3
Ser62→Cys	3.5 ± 0.8
Lys65→Arg	14 ± 2
Tyr90→Asn	2.55 ± 0.2
Thr101→Asn	2.6 ± 0.4
Phe164→Ala	100 ± 7
Asp225→Glu	4.12 ± 0.2
Asp225→Asn	6.7 ± 0.4

Table 4. Kinetic parameters with the peptide substrate.

Enzyme	Parameter		
	k_{cat}	K_m	k_{cat}/K_m
	s^{-1}	mM	$M^{-1} \cdot s^{-1}$
Wild type	55[a]	12[a]	4600
Phe58→Leu	10 ± 0.7	6 ± 0.2	1700
Lys65→Arg	> 2[b]	>10[b]	20 ± 3
Tyr90→Asn	15 ± 2	8 ± 0.5	1900
Thr101→Asn	34 ± 2	13 ± 1	2600
Phe164→Ala	15 ± 0.5	14 ± 1	1100
Asp225→Glu	14 ± 1	7 ± 0.5	2000
Asp225→Asn	26 ± 2	2.5 ± 0.2	10000

[a] From Frère et al. (1976).
[b] The initial rate of peptide utilization remained strictly proportional to the substrate concentration up to 10 mM.

Table 5. Kinetic parameters for the hydrolysis of the thiolester substrate.

Enzyme	k_{cat}	K_m	k_{cat}/K_m
	s^{-1}	μM	$M^{-1} \cdot s^{-1}$
Wild type	6[a]	50[a]	120000
Phe58→Leu	3.19 ± 0.07	75 ± 3	42500
Lys65→Arg	>5[b]	>7000[b]	600 ± 50
Tyr90→Asn	4.06 ± 0.3	53 ± 4	77000
Thr101→Asn	6.8 ± 0.3	47 ± 5	145000
Phe164→Ala	2.14 ± 0.02	60 ± 2	36000
Asp225→Glu	1.85 ± 0.08	42 ± 2	44000
Asp225→Asn	2.6 ± 0.1	42 ± 2	62500

[a] From Adam et al. (1990).
[b] At 7 mM, the disappearance of the substrate remained strictly first order.

Table 6. Transpeptidation properties of the wild-type and modified proteins; relative acceleration in the presence of D-Ala. The donor and the acceptor substrates were the thiolester and D-Ala, respectively. The acceptor concentrations ranged over 0–5 mM and the initial donor concentrations were 2–3-times K_m. The k_{cat} and K_m values were determined by analysing complete time courses. The SD values are less than 10%. n.d., not determined. For the Lys65→Arg mutant, the K_m value was too large to allow a significant analysis.

Enzyme	$\Delta k_{cat}/(k_{cat} \cdot \Delta[A])$
	mM^{-1}
Wild type	0.78
Phe58→Leu	0.62
Lys65→Arg	n.d.
Tyr90→Asn	n.d.
Thr101→Asn	0.83
Phe164→Ala	0.65
Asp225→Glu	1.12
Asp225→Asn	1.38

tempts to demonstrate a non-covalent, reversible binding of those antibiotics remained unsuccessful for technical reasons. This result is in contrast with those obtained with class-A β-lactamases, where the replacement of the active Ser residue by a Cys yielded enzymes exhibiting a significant residual activity, sometimes with a modified specificity profile (Sigal et al., 1984; Dalbadie-McFarland et al., 1982; Knap and Pratt, 1989) and with a class-C β-lactamase, where the same modification yielded an enzyme whose k_{cat} values were much more significantly modified for cephalosporins than for penicillins (D. Monnaie, A. Dubus, C. Jacobs and J. M. Frère, unpublished results).

Lys65→Arg

Of all the other mutants (Tables 4, 5 and 6), only the Lys65→Arg protein exhibited a markedly altered activity; the k_{cat}/K_m values were decreased about 200-fold. With the thiolester, this was clearly due to a dramatically increased K_m value, results which might be explained by either a lower k_2 or a higher K' since $K_m = k_3K'/k_2$. A 200-fold decrease of k_2 would yield a k_{cat} value lower than 5 s^{-1} (M. Jamin and J. M. Frère, unpublished results) in disagreement with the measured k_{cat} which is significantly larger than 5 s^{-1}. Similarly, with the peptide substrate for which $k_{cat} = k_2$, the decrease of the k_{cat}/K_m value cannot be completely attributed to k_2, which decreases at most by a factor 20 (Table 4), if it does decrease at all. Thus, although a decrease of k_2 might be involved, it is safe to assume that K' has been significantly increased for both substrates. This increase might be sufficient to completely explain the mutant's strongly decreased k_{cat}/K_m, assuming that k_2 remains unchanged. Alternatively, a contribution of both a decreased k_2 and an increased K' cannot be rejected. The mutation probably disrupts the architecture of the active site, resulting in an impaired recognition of the substrates. An even larger decrease was observed for the second-order rate constant for acylation by benzylpenicillin (Table 7). Replacement of the corresponding Lys73 by Ala in the class-A β-lactamase of *Bacillus licheniformis* (Imanaka et al., 1989) yielded a protein unable to bind and hydrolyse the substrate, while the Lys73→Arg mutation in the closely related enzyme of *Bacillus cereus* (Gibson et al., 1990) did not completely abolish its activity. Since the Ser*-Xaa-Xaa-Lys sequence is at the N-terminus of the α2 helix in the β-lactamases of classes A and C (Herzberg, 1991; Knox and Moews, 1991; Oefner et al., 1990) and in the *Streptomyces* R61 DD-peptidase (Kelly et al., 1989), these results underline the requirement for a postively charged residue one helix turn after the active Ser*, but a Lys is always 200- (substrates)–20000- (benzylpenicillin)-fold more efficient than an Arg. In the case of the *B. cereus* Lys73→Arg mutant, the drastically reduced k_{cat}/K_m ($= k_2/K'$) values could be attributed to the sole decrease of k_2, in contrast with our observations. Conversely, the same mu-

Table 7. Interactions with benzylpenicillin.

Enzyme	k_2/K'	$10^4 \times k_3$
	$M^{-1} \cdot s^{-1}$	s^{-1}
Wild type	13000	1.4
Phe58→Leu	14000 ± 800	1.78 ± 0.1
Lys65→Arg	0.6 ± 0.04	1.27 ± 0.1
Tyr90→Asn	14000 ± 1000	1.65 ± 0.05
Thr101→Asn	15000 ± 1000	1.73 ± 0.08
Phe164→Ala	2400 ± 100	0.6 ± 0.02
Asp225→Glu	24500 ± 2000	2.1 ± 0.3
Asp225→Asn	20000 ± 2000	1.8 ± 0.2

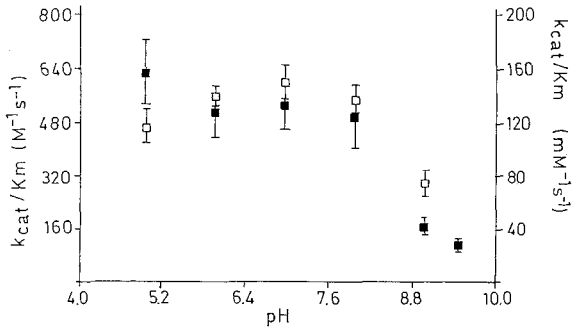


Fig. 1. pH-Dependence of the k_{cat}/K_m values of the Lys65→Arg (□) and wild-type (■) proteins with the thiolester substrate. For the former, the values were obtained from initial rates measured for at least two different substrate concentrations at [S] ≪ K_m and for the latter, from individual values of k_{cat} and K_m. The low activity of the mutant protein precluded measurements at higher pH values because the spontaneous hydrolysis of the substrate became proportionally too large. With the wild-type protein, this could be circumvented by using more concentrated enzyme.

tation in a class-C β-lactamase yielded a 20-fold increase in K_m and only a threefold decrease in k_{cat}, a situation closer to that described here (Tsukamoto et al., 1990a). However, the results obtained with the class-C enzyme should be considered with caution; indeed, the wild-type protein exhibited a very low activity when compared to other enzymes of the same family (0.1–2%) and the specific activities mentioned by the authors in the text did not correspond to the k_{cat} values given in the tables.

The rate of acylation by the peptide and the β-lactams has been shown to decrease upon deprotonation of a group of pK 9–9.5 (Varetto et al., 1987a and b). To investigate the possible role of the Lys65 residue in that decrease, we studied the pH dependence of the k_{cat}/K_m. The results (Fig. 1) show similar activity decreases at pH 9. Thus, the deprotonation of Lys65 does not appear to be responsible for the enzyme loss of activity above pH 9.0.

The most remarkable observation was that the mutant protein became nearly insensitive to inactivation by benzylpenicillin. It also completely failed to recognize carbenicillin (no detectable inactivation after a 30-min incubation with 6 mM antibiotic), although the wild-type enzyme was rapidly inactivated by the same compound ($k_2/K' = 850\ M^{-1}\ s^{-1}$; Table 8). The decrease of the k_2/K' values for penicillin was thus much more drastic than that for the peptide and ester substrates (more than 100-fold). The mutant enzyme thus essentially behaved as a low activity, but penicillin-resistant DD-peptidase.

Asp225

The Asp225→Glu and Asp225→Asn mutations did not yield any major modification in the enzyme activity or the interaction with various penicillins (Tables 4–8). That the Asn225 side chain was not deamidated after the synthesis of the enzyme was demonstrated by the fact that the isoelectric pH of the modified protein was significantly higher than that of the wild type (data not shown). It thus appears unlikely that the Asp225 residue might participate in the catalysis as a general base, as does the corresponding Glu166 residue in class-A β-lactamases (Gibson et al., 1990). A similar conclusion was reached for residue Asp217 of a class-C enzyme by Tsukamoto et al. (1990b). However, these authors observed an increase in the catalytic activity of the mutant versus various oxyiminocephalosporins (cefotaxime and ceftazidime). Our results did not indicate a similar specificity modification, and the identity of the general base which might activate the active-site Ser residue of the DD-peptidase remains mysterious. Finally, and with both modified proteins, the degradation product of benzylpenicillin was phenylacetylglycine and not penicilloic acid, a situation similar to that observed with the wild-type enzyme (Frère et al., 1976).

Phe164

Early crystallographic results (Kelly et al., 1989) suggested that residue Phe164 might impair the diffusion of water molecules to the ester bond formed between the enzyme active Ser and the antibiotic. Although the Ala side chain is much smaller, the activity and kinetic properties of the Phe164→Ala mutant were only slightly modified. In particular, there was no significant increase of the k_3 value for penicillin or the thiolester substrate. Those results indicated that the bulky Phe side-chain was probably not responsible for protecting the ester bond in the acylenzyme from hydrolysis. However, the increased instability of the modified protein suggested a rather important structural role for that residue.

Other mutants

The properties of the three other mutants were not significantly different from those of the wild-type enzyme. The substitutions performed in the present work might have been too conservative for obtaining proteins with significantly altered behaviours. In the known class A β-lactamase structures (Moews et al., 1990; Knox and Moews, 1991; Herzberg, 1991), the corresponding residues appear to be more important for the general folding of the protein than for catalysis. In particular, it seems that residue Phe58 might be replaced by another large hydrophobic side chain without major damages for the architecture of the enzyme.

CONCLUSIONS

Of the mutations analysed in the present study, only Ser62→Cys and the Lys65→Arg produced strongly impaired enzymes. Strikingly, and in contrast with observations made with β-lactamases of classes A and C, the 'cysteine DD-peptidase' was completely inactive, suggesting more drastic requirements for the orientation of the principal catalytic group of the latter enzyme. Conversely, when the highly conserved Lys65 residue of the DD-peptidase was replaced by Arg, similar decreases of the k_{cat}/K_m values were observed for the DD-peptidase (this study) and the β-lactamases of both classes

Table 8. Interaction with other β-lactams. n.d., not determined.

Substrate	Enzyme form							
	Wild type		Asp225→Glu		Asp225→Asn		Phe164→Ala	
	k_2/K'	k_3	k_2/K'	k_3	k_2/K'	k_3	k_2/K'	k_3
	$M^{-1}\,s^{-1}$	s^{-1}	$M^{-1}\,s^{-1}$	s^{-1}	$M^{-1}\,s^{-1}$	s^{-1}	$M^{-1}\,s^{-1}$	s^{-1}
Ampicillin	110[a]	1.4×10^{-4}[a]	160± 10	$2.7\pm0.2\times10^{-4}$	140± 10	$2.3\pm0.3\times10^{-4}$	30± 3	$0.5\pm0.03\times10^{-4}$
Carbenicillin	830[a]	1.4×10^{-4}[a]	1100±100	$1.9\pm0.4\times10^{-4}$	600± 50	$1.6\pm0.1\times10^{-4}$	125±10	n.d.
Cephalosporin C	1500[a]	1×10^{-6}[a]	2000±100	$4\pm0.1\times10^{-6}$	1400±100	$5.1\pm0.1\times10^{-6}$	900±20	$3.2\pm0.6\times10^{-6}$
Cephalothin	840±130	n.d.	800±100	n.d.	800±100	n.d.	450±30	n.d.
Cefuroxime	2400±150	n.d.	2500±200	n.d.	2600±300	n.d.	1200±50	n.d.
Cefotaxime	110± 10	$<4\times10^{-6}$	160± 5	$4\pm0.1\times10^{-6}$	140± 2	$3.6\pm0.2\times10^{-6}$	n.d.	n.d.
Ceftazidime	500± 60	$1.5\pm0.3\times10^{-5}$	500± 30	$6.5\pm3\times10^{-6}$	500± 40	$7\pm2\times10^{-6}$	n.d.	n.d.

[a] From Frère et al. (1976).

(Gibson et al., 1990; Tsukamoto et al., 1990a). These similar variations might, however, not be due to a modification of the same kinetic constant(s) in all cases.

Residue Asp225 does not appear to be the general base responsible for the activation of Ser62. Such an activating residue thus remains to be identified.

Finally, the bulky, hydrophobic side chain of Phe164 is not responsible for the very low rate of hydrolysis of the acylenzyme, but it contributes to the stability of the protein.

A. M. H. is a fellow of the *Agence Générale pour la Coopération au Développement* (AGCD, Brussels), J. M. W. is a fellow of the *Institut pour l'Encouragement à la Recherche Scientifique dans l'Industrie et l'Agriculture* (IRSIA, Brussels) and B. J. is *Chercheur qualifié* of the *Fonds National de la Recherche Scientifique* (FNRS, Brussels). This work was supported in part by *Actions concertées* with the Belgian Government (conventions 86/91-90 and 89/94-130), the *Fonds de la Recherche Scientifique Médicale* (contract no. 3.4537.88), and a *Convention tripartite* between the *Région wallonne*, SmithKline Beecham, UK, and the University of Liège. The authors are indebted to the *Fonds National de la Recherche Scientifique* for a grant providing the stopped-flow apparatus [*Fonds de la Recherche Fondamentale et Collective* (FRFC, Brussels); contract no. 2.4503.90]. This text presents research results of the Belgian programme on Interuniversity Poles of Attraction initiated by the Belgian State, Prime Minister's Office, Science Policy Programming (PAI no. 19). The scientific responsability is assumed by its authors.

REFERENCES

Adam, M., Damblon, C., Plaitin, B., Christiaens, L. & Frère, J. M. (1990) Chromogenic depsipeptide substrates for β-lactamases and penicillin-sensitive DD-peptidases, *Biochem. J. 270*, 525–529.

Ambler, R. P., Coulson, A. F. W., Ghuysen, J. M., Frère, J. M., Joris, B., Forsman, M., Levesque, R. C., Tiraby, G. & Waley, S. G. (1991) A standard numbering scheme for class A β-lactamases, *Biochem. J. 276*, 269–272.

Bourguignon-Bellefroid, C., Wilkin, J. M., Joris, B., Aplin, R. T., Houssier, C., Prendergast, F. G., Van Beeumen, J. & Frère, J. M. (1992a) Importance of the two tryptophane residues in the *Streptomyces* R61 DD-peptidase. Engineering a PBP with a significant β-lactamase activity, *Biochem. J. 282*, 361–367.

Bourguignon-Bellefroid, C., Joris, B., Van Beeumen, J., Ghuysen, J. M. & Frère, J. M. (1992b) Point mutations of two arginine residues in the *Streptomyces* R61 DD-peptidase, *Biochem. J. 283*, 123–128.

Dalbadie-McFarland, G., Cohen, L. W., Riggs, A. D., Morin, C., Itakura, K. & Richards, J. H. (1982) Oligonucleotide-directed mutagenesis as a general and powerful method for studies of protein function, *Proc. Natl Acad. Sci. USA 79*, 6409–6413.

Frère, J. M. & Joris, B. (1985) Penicillin-sensitive enzymes in peptidoglycan biosynthesis, *CRC Crit. Rev. Microbiol. 11*, 299–396.

Frère, J. M., Ghuysen, J. M., Perkins, H. R. & Nieto, M. (1973) Kinetics of concomitant transfer and hydrolysis reactions catalysed by the exocellular DD-carboxypeptidase-transpeptidase of *Streptomyces* R61, *Biochem. J. 135*, 483–492.

Frère, J. M., Leyh-Bouille, M., Ghuysen, J. M., Nieto, M. & Perkins, H. R. (1976) Exocellular DD-carboxypeptidases-transpeptidases from *Streptomyces*, *Methods Enzymol. 45*, 610–636.

Galleni, M., Lindberg, F., Normark, S., Cole, S., Honoré, N., Joris, B. & Frère, J. M. (1988) Sequence and comparative analysis of three *Enterobacter cloacae* ampC β-lactamase genes and their products, *Biochem. J. 250*, 753–760.

Gibson, R. M., Christensen, H. & Waley, S. G. (1990) Site-directed mutagenesis of β-lactamase I. Single and double mutants of Glu-166 and Lys-73, *Biochem. J. 272*, 613–619.

Hadonou, A. M., Jamin, M., Adam, M., Joris, B., Dusart, J., Ghuysen, J. M. & Frère, J. M. (1992) Importance of the His-298 residue in the catalytic mechanism of the *Streptomyces* R61 extracellular DD-peptidase, *Biochem. J. 282*, 495–500.

Herzberg, O. (1991) Refined crystal structure of the β-lactamase from *Staphylococcus aureus* PC1 at 2 Å resolution, *J. Mol. Biol. 217*, 701–719.

Imanaka, D., Kuroda, A. & Wang, H. (1989) Protein engineering of penicillinase as affinity ligands for bioprocessing, *J. Ferment. Bioeng. 67*, 315–320.

Jamin, M., Adam, M., Damblon, C., Christiaens, L. & Frère, J. M. (1991) Accumulation of acylenzyme in DD-peptidase catalysed reactions with analogues of peptide substrates, *Biochem. J. 280*, 499–506.

Joris, B., Ghuysen, J. M., Dive, G., Renard, A., Dideberg, O., Charlier, P., Frère, J. M., Kelly, J. A., Boyington, J. C., Moews, P. C. & Knox, J. R. (1988) The active-site-serine penicillin-recognizing enzymes as members of the *Streptomyces* R61 DD-peptidase family, *Biochem. J. 250*, 313–324.

Kelly, J. A., Dideberg, O., Charlier, P., Wéry, J., Libert, M., Moews, P. C., Knox, J. R., Duez, C., Fraipont, C., Joris, B., Dusart, J., Frère, J. M. & Ghuysen, J. M. (1986) On the origin of bacterial resistance to penicillin: comparison of a β-lactamase and a penicillin target, *Science 231*, 1429–1431.

Kelly, J. A., Knox, J. R., Zhao, H., Frère, J. M. & Ghuysen, J. M. (1989) Crystallographic mapping of β-lactams bound to a D-alanyl-D-alanine peptidase target enzyme, *J. Mol. Biol. 209*, 281–295.

Knap, A. K. & Pratt, R. F. (1989) Chemical modification of the RTEM-1 thiol β-lactamase by thiol-selective reagents. Evidence for activation of the primary nucleophile of the β-lactamase active site by adjacent functional groups, *Proteins 6*, 316–323.

Knox, J. R. & Moews, P. C. (1991) β-Lactamase of *Bacillus licheniformis* 749/C. Refinement at 2 Å resolution and analysis of hydration, *J. Mol. Biol. 220*, 435–455.

Moews, P. C., Knox, J. R., Dideberg, O., Charlier, P. & Frère, J. M. (1990) β-Lactamase of *Bacillus licheniformis* 749/C at 2 Å resolution, *Proteins 7*, 156–171.

Oefner, C., Darcy, A., Daly, J. J., Gubernator, K., Charnas, R. L., Heinze, I., Hubschwerlen, C. & Winkler, F. K. (1990) Refined crystal structure of the β-lactamase from *Citrobacter freundii* indicates a mechanism for β-lactam hydrolysis, *Nature 343*, 284–288.

Samraoui, B., Sutton, B., Todd, R., Artimyuk, P., Waley, S. G. & Phillips, D. (1986) Tertiary structural similarity between a class A β-lactamase and a penicillin-sensitive D-alanyl carboxypeptidase-transpeptidase, *Nature 320*, 378–380.

Sigal, I. S., Harwood, B. G. & Arentzen, R. (1982) Thiol β-lactamase: replacement of the active-site serine of RTEM β-lactamase by a cysteine residue, *Proc. Natl Acad. Sci. USA 79*, 7157–7160.

Sigal, I. S., De Grado, W. F., Thomas, B. J. & Petteway, S. R. (1984) Purification and properties of thiol β-lactamase, *J. Biol. Chem. 259*, 5327–5332.

Tsukamoto, K., Tachibana, K., Yamazaki, N., Ishii, Y., Ujiie, K., Nishida, N. & Sawai, T. (1990a) Role of lysine 67 in the active site of class C β-lactamase from *Citrobacter freundii* GN346, *Eur. J. Biochem. 188*, 15–22.

Tsukamoto, K., Kikura, R., Ohno, R. & Sawai, T. (1990b) Substitution of aspartic acid-217 of *Citrobacter freundii* cephalosporinase and properties of the mutant enzymes, *FEBS Lett. 264*, 211–214.

Varetto, L., Frère, J. M., Nguyen-Distèche, M., Ghuysen, J. M. & Houssier, C. (1987a) The pH-dependence of the active-site serine DD-peptidase of *Streptomyces* R61, *Eur. J. Biochem. 162*, 525–531.

Varetto, L., Frère, J. M. & Ghuysen, J. M. (1987b) The importance of the negative charge of β-lactam compounds for the inactivation of the active-site serine DD-peptidase of *Streptomyces* R61, *FEBS Lett. 225*, 218–222.

Zoller, M. J. & Smith, M. (1984) Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single stranded DNA template, *DNA 3*, 479-488.