Active-site-directed inactivators of the Zn²⁺-containing D-alanyl-D-alanine-cleaving carboxypeptidase of *Streptomyces albus* G

Paulette CHARLIER,*† Otto DIDEBERG,† Jean-Claude JAMOULLE,‡ Jean-Marie FRÈRE,* Jean-Marie GHUYSEN,* Georges DIVE‡ and Josette LAMOTTE-BRASSEUR†
*Service de Microbiologie, Faculté de Médecine, Université de Liège, Institut de Chimie, B6, B-4000 Sart Tilman, Liège, Belgium, †Service de Cristallographie, Faculté des Sciences, Université de Liège, Institut de Physique, B5, B-4000 Sart Tilman, Liège, Belgium, and ‡Service de Chimie Analytique, Faculté de Médecine, Université de Liège, Institut de Pharmacie, F1, Rue Fusch, 3-5, B-4000 Liège, Belgium

Several types of active-site-directed inactivators (inhibitors) of the Zn²⁺-containing D-alanyl-D-alanine-cleaving carboxypeptidase were tested. (i) Among the heavy-atom-containing compounds examined, $K_2Pt(C_2O_4)_2$ inactivates the enzyme with a second-order rate constant of about $6 \times 10^{-2} M^{-1} . s^{-1}$ and has only one binding site located close to the Zn²⁺ cofactor within the enzyme active site. (ii) Several compounds possessing both a C-terminal carboxylate function and, at the other end of the molecule, a thiol, hydroxamate or carboxylate function were also examined. 3-Mercaptopropionate (racemic) and 3-mercaptoisobutyrate (L-isomer) inhibit the enzyme competitively with a K_i value of $5 \times 10^{-9} – 10 \times 10^{-9} M$. (iii) Classical β-lactam compounds have a very weak inhibitory potency. Depending on the structure of the compounds, enzyme inhibition may be competitive (and binding occurs to the active site) or non-competitive (and binding causes disruption of the protein crystal lattice). (iv) 6-β-Iodopenicillanate inactivates the enzyme in a complex way. At high β-lactam concentrations, the pseudo-first-order rate constant of enzyme inactivation has a limit value of $7 \times 10^{-4} s^{-1}$. 6-β-Iodopenicillanate binds to the active site just in front of the Zn²⁺ cofactor and superimposes histidine-190, suggesting that permanent enzyme inactivation is by reaction with this latter residue.

The transpeptidation and carboxypeptidation reactions involved in the last stages of wall peptidoglycan synthesis in bacteria are catalysed by multiple D-alanyl-D-alanine-cleaving peptidases (in short DD-peptidases) (Ghuysen *et al.*, 1981). The serine DD-carboxypeptidases/transpeptidases operate via acyl-enzyme formation and are susceptible to inactivation by β-lactam antibiotics. One of them has been crystallized and the penicillin-binding site has been located (Kelly *et al.*, 1982). A metallo DD-peptidase is also known that requires a Zn²⁺ cation bound to the active site for catalysis (Dideberg *et al.*, 1980*b*). This enzyme functions solely as a carboxypeptidase and is highly resistant to penicillins and cephalosporins (Ghuysen *et al.*, 1981). Its primary structure (Joris *et al.*, 1983) and crystal structure at 0·25 nm (2.5 Å) resolution (Dideberg *et al.*, 1982) are known. Attempts were made to find effective inhibitors (inactivators) of the Zn²⁺ DD-carboxypeptidase.

The results obtained during this first exploratory phase are described below.

Materials and methods

β-Lactam compounds

Cephalosporin C, cephalothin and cephaloglycine were from Eli Lilly and Co., Indianapolis, IN, U.S.A. The β-iodopenicillanate was a gift from Dr J. T. Henderson and Dr J. E. G. Kemp from Pfizer Central Research, Sandwich, Kent, U.K.; 7-[2-(p-iodophenyl)acetamido]cephalosporanate was that previously used (Dideberg *et al.*, 1982) and was synthesized by Dr. L. Christiaens, Department of Organic Chemistry, University of Liège.

Heavy-metal-containing compounds

$K_3UO_2F_5$ was a gift from Dr. D. Stuart, Oxford, U.K. K_2PtCl_4, $(NH_4)_2IrCl_6$ and $NaAuCl_4$ were

from Aldrich Chemical Co., Milwaukee, WI, U.S.A., $NaUO_2(CH_3CO_2)_3$ was from Merck, Darmstadt, Germany, and $K_2Pt(C_2O_4)_2$ was synthesized as described in Pascal (1958).

Zn^{2+}-directed inactivating reagents

Compounds (2), (3), (4) and (10) of Fig. 4 (see the Results section) were generously given by Dr. M. Ondetti of the Squibb Institute for Medical Research, Princeton, N.J., U.S.A. Compounds (1), (5), (6), (7), (8), (9) and (11) were from Aldrich Europe Division, Janssen Pharmaceutica N.V., B-2340 Beerse, Belgium. Compounds (12), (13) and (14) were synthesized during the present work.
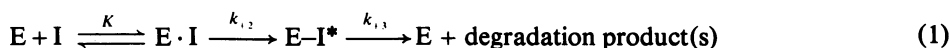
Buffers

Buffer A was 10mM-Tris/HCl, pH 8.3, containing 5mM-$MgCl_2$, and buffer B was 10mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH, pH 8.0, containing 5mM-$MgCl_2$.

Enzyme

The Zn^{2+} DD-carboxypeptidase was purified to protein homogeneity as described in Duez et al. (1978). The enzyme activity was determined by incubating the enzyme with 1.8mM-$N^\alpha N^\epsilon$-diacetyl-L-lysyl-D-alanyl-D-alanine at 37°C in a total volume of 30 μl of either buffer A or buffer B and measuring enzymically the amount of C-terminal D-alanine liberated (Frère et al., 1976; De Coen et al., 1981).

Interaction between the Zn^{2+} DD-carboxypeptidase and β-lactam compounds (Frère et al., 1978)

The β-lactam compounds react with the enzyme according to:

lactam compound by dialysis, A_t is the activity after incubation of the enzyme–β-lactam complex E–I* at 37°C, and A is the activity of an enzyme sample treated as above but without β-lactam compound).

Determination of the apparent rate constants (k_a values) for the formation of complexes E–I and K and k_{+2} values.* The enzyme (3.4 μM) and the β-lactam compound (at concentrations ranging from 1 to 15mM) were incubated together at 37°C in 60 μl of buffer B. Formation of complex E–I* was determined on 1 μl samples removed after increasing times of incubation (and further incubated for 15 min at 37°C with 1.8mM-Ac$_2$-L-Lys-D-Ala-D-Ala). Plots of $\ln(A_t/A_0)$ versus time yielded straight lines (A_t is the residual enzyme activity at time t and A_0 is the initial enzyme activity), permitting calculation of k_a. Plots of k_a versus [I] showed deviation from linearity at high [I] values. Individual values of K and k_{+2} were obtained from the reciprocal plots $1/k_a$ versus $1/$[I].

Effect of the presence of Ac$_2$-L-Lys-D-Ala-D-Ala. The enzyme (1.2 μM), the β-lactam compound (at concentrations ranging from 0.05 to 10mM) and Ac$_2$-L-Lys-D-Ala-D-Ala (at concentrations ranging from 0.275 to 1.1mM) were incubated together for 2 min at 37°C in 100 μl of buffer A, and the amount of released D-Ala was determined. Lineweaver–Burk plots ($1/v$ versus $1/$[S] for various [I] values) allowed determination of the type of enzyme inhibition (competitive or non-competitive).

Interaction between the Zn^{2+} DD-carboxypeptidase and non-β-lactam inhibitors (inactivators)

All the experiments were performed in buffer A.

$$E + I \underset{}{\overset{K}{\rightleftharpoons}} E \cdot I \xrightarrow{k_{+2}} E\text{--}I^* \xrightarrow{k_{+3}} E + \text{degradation product(s)} \qquad (1)$$

where E is enzyme, I is β-lactam compound, E–I* is inactive complex, K is the dissociation constant and k_{+2} and k_{+3} are first-order rate constants. The Zn^{2+} DD-carboxypeptidase shows high intrinsic resistance to all β-lactam compounds tested because of low k_{+2}/K values.

Determination of the k_{+3} values. The enzyme (33 μM) and the β-lactam compound (20mM) were incubated together for 2 h at 37°C in 25 μl of buffer B. The reaction mixture was 100-fold diluted in buffer B, dialysed against the same buffer for 2 h at 4°C and then incubated at 37°C. Samples were removed after increasing times of incubation, and the extent of enzyme recovery (or breakdown of complex E–I*) was determined after an additional incubation of 15 min at 37°C with 1.8mM-Ac$_2$-L-Lys-D-Ala-D-Ala. Plots of $\ln[1-(A_t-A_0)/(A-A_0)]$ versus time gave rise to straight lines (A_0 is the residual activity after removal of the unbound β-

As a first approximation, the inhibiting (inactivating) potency of a compound was expressed by the concentration necessary to decrease the enzyme activity by 50% (ID$_{50}$ value) when enzyme, substrate and inhibitor were incubated together at 37°C. Pseudo-first-order rate constants (k_a) of enzyme inactivation were determined as indicated above. Lineweaver–Burk plots and Dixon plots ($1/v$ versus [I] for various [S] values) were used to determine the type of enzyme inhibition and to measure the K_i values.

Enzyme crystals

Crystallization of the Zn^{2+} DD-carboxypeptidase was carried out as described in Dideberg et al. (1979).

Preparation of enzyme crystal derivatives

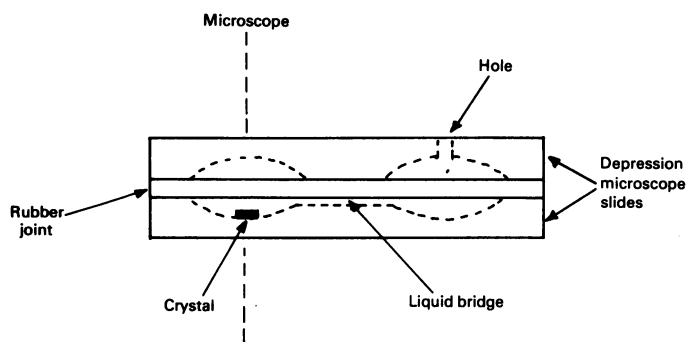A cell consisting of two microscope slides with depressions as shown in Fig. 1, and fixed to each

Fig. 1. *Schematic representation of the cell used for the preparation of complexes of the Zn^{2+} DD-carboxypeptidase crystal*
For further details see the text.
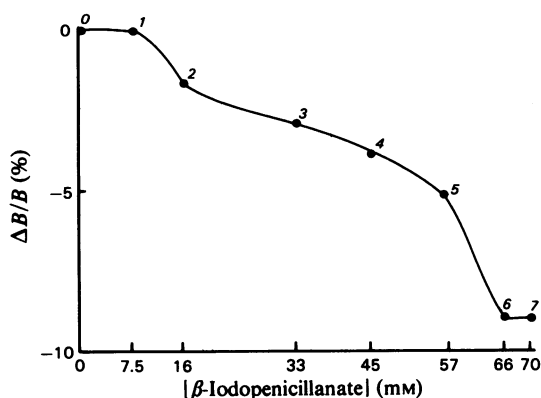

Fig. 2. *Relative variation of the birefringence of a crystal of the Zn^{2+} DD-carboxypeptidase as a function of increasing concentrations of β-iodopenicillanate*

$\Delta B/B$ is the relative variation of birefringence. Diffusion of β-iodopenicillanate was carried out within a total period of time of 24 h. β-Iodopenicillanate was added stepwise after 30 min (*1*), 90 min (*2*), 180 min (*3*), 270 min (*4*), 18 h (*5*), 20 h (*6*) and 24 h (*7*). For further details see the text.

other by a rubber joint, was used. Before the cell was mounted, an enzyme crystal (of thickness 0.05 mm) and a 30 μl sample of mother liquor were deposited in the left-hand well, and another 30 μl sample of mother liquor was deposited in the right-hand well. The two droplets were connected by a thin liquid bridge by means of a fine needle. After the cell had been sealed, a solution of the selected compound (heavy atom or organic inhibitor) was injected in the right-hand well and the compound concentration was increased stepwise, the system being allowed to equilibrate between each step. Formation of the enzyme derivative was monitored by measuring the changes in birefringence of the crystal with time, with a Berek compensator. Fig. 2 shows the results obtained with β-iodo-

penicillanate. All the above operations were performed at 20°C.

X-ray-diffraction studies

The experiments were performed with a Hilger–Watts diffractometer fitted with an He-filled tunnel and with the use of Ni-filtered Cu K_α radiation (40 kV, 26 mA). Absorption corrections were deduced from ϕ scans (North *et al.*, 1981), and the decreased intensity due to radiation damage was corrected by a time function. The maximum decay correction was 20%. The positions of the heavy atoms were determined from F_{HLE} Patterson functions, and parameters were refined by a least-squares procedure. For the complexes formed with $Pt(C_2O_4)^{2-}$, $UO_2F_5{}^{3-}$ and $AuCl_4{}^-$, the process was followed by three cycles of phase refinement at 0.25 nm resolution.

The crystal data for the native enzyme and for the enzyme–β-iodopenicillanate complex are given in Table 1. The complete 0.28 nm data set (*hkl* and *hk̄l*) was measured on one crystal. The structure factor amplitudes of the enzyme–β-lactam complex were scaled to those of the native enzyme. The $R_{dif.}$ factor was 0.11. A difference electron-density map was computed from the coefficients $m||F_c| - |F_p||$ and the multiple isomorphous-replacement phases (Dideberg *et al.*, 1982). Reflexions of resolution lower than 1 nm were omitted from these summations.

Results

Inactivation of the Zn^{2+} DD-carboxypeptidase by heavy-metal-containing compounds

Pseudo-first-order kinetics of enzyme inactivation were observed with $(NH_4)_2IrCl_6$, $NaAuCl_4$, $K_2Pt(C_2O_4)_2$ and K_2PtCl_4. The corresponding second-order rate constants were 2.4×10^{-2}, 5.7×10^{-2}, 6.2×10^{-2} and $18.6 \times 10^{-2} \text{M}^{-1} \cdot \text{s}^{-1}$ respectively. With the two uranyl compounds

Table 1. *Crystal data for the native Zn^{2+} DD-carboxypeptidase and the complex formed with β-iodopenicillanate*

$$R_{sym} = \sum_{h}\sum_{i}|I_{hi} - \bar{I}_{h}|/\sum_{h}\bar{I}_{h}$$

where I_{hi} is the intensity of an *hkl* reflexion and $\bar{I}_{h}$ is the mean intensity.

$$R_{dif.} = \sum||F_{c}| - |F_{p}||/\sum|F_{p}|$$

where $|F_{c}|$ is the structure-factor amplitude of the β-iodopenicillanate–Zn^{2+} DD-carboxypeptidase complex and $|F_{p}|$ that of the native protein.

	a (nm)	b (nm)	c (nm)	β (°)	R_{sym} (%)
Native	5.11	4.97	3.87	100.60	3.2
Complex	5.119	4.964	3.866	100.56	4.6

Table 2. *Position of heavy atoms in the Zn^{2+} DD-carboxypeptidase crystal structure*

Heavy-atom compound	Site no. (as shown in Fig. 3)	Relative occupancy	Position
$K_2Pt(C_2O_4)_2$	X	1.02	**Active site, close to Zn^{2+} and His-190**
K_2PtCl_4	P3	0.85	Between Cys-140 of one enzyme molecule and Asp-39 of a second enzyme molecule
	P2	0.50	Close to Met-153
	X	0.27	**Active site, close to Zn^{2+} and His-190**
	P4	0.26	Between the side chains of Met-124 and Glu-165
$NaAuCl_4$	X	1.34	**Active site, close to Zn^{2+} and His-190**
	A2	1.26	Close to His-177
$K_3UO_2F_5$	U2	0.72	Close to Asp-62
	X	0.47	**Active site, close to His-152 and Asn-141**

[20mM-$NaUO_2(CH_3CO_2)_3$ and 30mM-$K_3UO_2F_5$], rapid but partial enzyme inactivation (35% and 25% respectively) occurred during the first 5–10min of incubation. During subsequent incubation, enzyme inactivation by $NaUO_2(CH_3CO_2)_3$ continued to proceed, but at a very low rate ($0.93 \times 10^{-2} M^{-1} \cdot s^{-1}$). $K_3UO_2F_5$ had no further detectable effect, at least within 60min of treatment.

The positions of the aforementioned heavy atoms [except $(NH_4)_2IrCl_6$ and $NaUO_2Ac_3$] in the Zn^{2+} DD-carboxypeptidase and the relative occupancies of the binding sites were determined (Table 2 and Fig. 3). Compound $K_2Pt(C_2O_4)_2$ gave rise to one single binding site, which was located within the enzyme cavity. The other heavy-atom-containing compounds gave rise to multiple (two to four) binding sites, but in each case only one of these was also located within the enzyme active site.

Compound K_2PtCl_4 was the most potent inactivator (as expressed by the second-order rate constant of enzyme inactivation), although binding to the active site was rather weak (as expressed by the relative occupancy value). However, K_2PtCl_4 was the only heavy-atom-containing compound that occupied four distinct binding sites, and the enzyme–K_2PtCl_4 derivative was the only one that showed lack of isomorphism.

Attempts to construct 'bi-product' analogue inactivators

A general application of the concept of 'bi-product' analogues (Byers & Wolfenden, 1972) makes use of functional groups able to interact regio- and stereo-specifically with the different enzyme binding areas and to anchor the molecule firmly within the enzyme active site. This approach led to the synthesis of specific inhibitors of the Zn^{2+}-containing carboxypeptidases A and B, thermolysin and angiotensin-converting enzyme (Cushman et al., 1977).

The Zn^{2+} DD-carboxypeptidase active site probably possesses at least three subsites (Fig. 4). Subsites S_2, S_1 and, with a less strict requirement, subsite S_1' must be suitably complemented by the substrate side chains, and Arg-136 is probably involved in charge-pairing. Several bifunctional compounds were tested (Fig. 4). Most of the active compounds possessed a C-terminal carboxylate function capable of binding to the cationic centre Arg-136, and either a thiol, hydroxamate or carboxylate function capable of binding to the Zn^{2+} cofactor. Except in compound (10), the back-
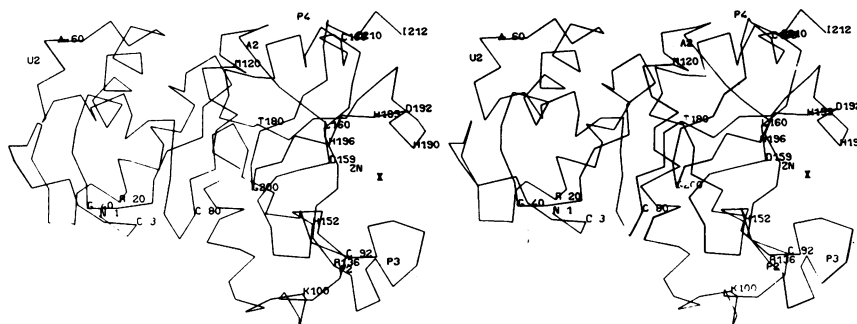
Fig. 3. *Stereoscopic view of the three-dimensional structure of the Zn^{2+} DD-carboxypeptidase*
The α-carbon positions were derived from a Kendrew model. For the labelling of the heavy-atom-binding sites, see Table 2. The amino acid residues were designated by the one-letter code (IUPAC–IUB Commission on Biochemical Nomenclature, 1969). Numbering of the amino acid residues is that derived from X-ray data (Dideberg *et al.*, 1982). At present, there are still minor discrepancies between this sequence and that derived from chemical and enzymic degradation (Joris *et al.*, 1983). Strong experimental evidence suggests (i) that His-152, His-193 and His-196 serve as Zn^{2+} ligands, (ii) that charge-pairing may occur between the C-terminal carboxylate function of the bound substrate and Arg-136, and (iii) that His-190 may play the role of a proton donor in catalysis. The model was drawn with the use of the Packgraph programme (Katz & Levinthal, 1972).

bones of the compounds were too short to permit interaction with subsites S_1 and S_2. Unless otherwise specified, racemic compounds were used. From this preliminary exploration, the following are apparent.

1. Inactivating potency requires a C-terminal anionic group [compare compounds (13) and (14)] and greatly depends on the nature of the function that is directed towards the Zn^{2+} cofactor. The comparable pairs compounds (1) and (12) and compounds (2) and (11) showed that replacing a thiol by a hydroxamate or a carboxylate at this position caused a 1000-fold and 100000-fold decreased affinity respectively.

2. As shown by Lineweaver–Burk plots of $1/v$ versus $1/[S]$ for various concentrations of I (where v is the initial velocity of substrate consumption, S is the substrate Ac₂-L-Lys-D-Ala-D-Ala and I is the inactivator), compounds (1)–(5) were potent competitive inhibitors. Contrary to the expectations, comparable effectiveness was achieved whether the lateral chain assumed to complement subsite S_1' was a proton, a methyl group in the L configuration or a methyl group in the D configuration, and whether one single or two carbon atoms were disposed between the terminal thiol and carboxylate functions. Moreover, the most potent inactivators were 3-mercaptopropionate ($K_i = 5 \times 10^{-9}$M) and the L-isomer of 3-mercaptoisobutyrate ($K_i = 10 \times 10^{-9}$M), whereas the D-isomer of 3-mercaptoisobutyrate was somewhat but distinctly less potent ($K_i = 60 \times 10^{-9}$M). Such small truncated molecules did not offer the possibility of complementing subsites S_1 and S_2, and, as the above observations strongly suggested, subsite S_1' alone

seemed not to play any significant role in the binding process. Considering the flexibility of these molecules, enzyme inactivation may be caused by bidentate interaction with formation of a penta- or hexa-co-ordinate Zn^{2+} ion (Fig. 5).

3. As models showed, folding the above molecules to form a penta- or hexa-co-ordinate Zn^{2+} ion oriented the lateral chain towards His-190, which may play the role of proton donor in catalysis. A methyl group, especially in an L configuration, had no prominent steric effect, but more bulky substituents on the α-carbon atom [compounds (6)–(10)] were much less favourable, thus considerably decreasing the binding efficacy.

4. Alternative mechanisms for the loss of enzyme activity on treatment with 3-mercaptopropionate were envisaged. One of them might have been by opening of the disulphide bridges [which occur in three places in the protein (Dideberg *et al.*, 1982; Joris *et al.*, 1983)]. This possibility was eliminated by the following experiment. The enzyme (1.1 μM) and the inhibitor (100 μM) were incubated together for 5 min at 37°C in buffer A, which treatment caused complete enzyme inhibition. The reaction mixture was then supplemented with 1 mM-dithiobis-2-nitrobenzoate, which caused enzyme recovery with a first-order rate constant of about 6.4×10^{-4} s⁻¹ (90% recovery was observed after 60 min). Another alternative was that loss of enzyme activity might have been by removal of the Zn^{2+} cofactor from the active site by the inhibitor. In turn, this possibility was eliminated by the following experiment. The enzyme (5 nM) and the inhibitor (100 nM) were incubated together at 37°C in buffer A until 80% of

Enzyme cavity

	S$_2$	S$_1$	Zn	S$_1'$	Arg-136

CH$_3$
CO
NH
[CH$_2$]$_4$ O   CH$_3$ O   CH$_3$ O$^-$
CH$_3$—CO—NH—CH—C—NH—CH—C—NH—CH—C=O
                L   D   D

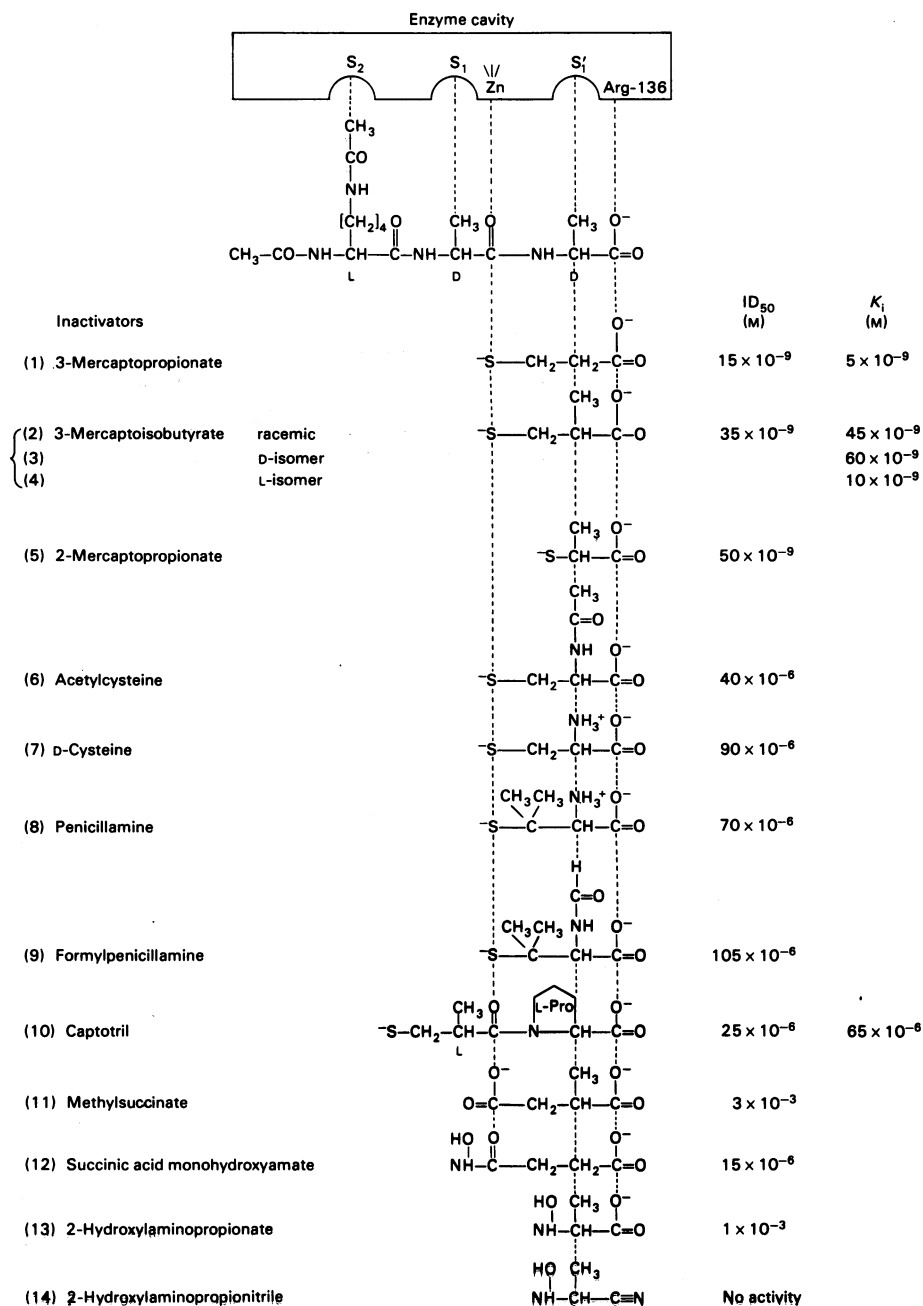| Inactivators			ID$_{50}$ (M)	K_i (M)
(1) 3-Mercaptopropionate		$^-$S—CH$_2$—CH$_2$—C=O	15×10^{-9}	5×10^{-9}
(2) 3-Mercaptoisobutyrate	racemic	$^-$S—CH$_2$—CH—C—O (CH$_3$, O$^-$)	35×10^{-9}	45×10^{-9}
(3)	D-isomer			60×10^{-9}
(4)	L-isomer			10×10^{-9}
(5) 2-Mercaptopropionate		$^-$S—CH—C=O (CH$_3$, O$^-$)	50×10^{-9}	
(6) Acetylcysteine		$^-$S—CH$_2$—CH—C=O	40×10^{-6}	
(7) D-Cysteine		$^-$S—CH$_2$—CH—C=O (NH$_3^+$, O$^-$)	90×10^{-6}	
(8) Penicillamine		$^-$S—C—CH—C=O (CH$_3$ CH$_3$, NH$_3^+$, O$^-$)	70×10^{-6}	
(9) Formylpenicillamine		$^-$S—C—CH—C=O (CH$_3$ CH$_3$, NH, O$^-$)	105×10^{-6}	
(10) Captotril		$^-$S—CH$_2$—CH—C—N—CH—C=O (L-Pro)	25×10^{-6}	65×10^{-6}
(11) Methylsuccinate		O=C—CH$_2$—CH—C=O (O$^-$, CH$_3$, O$^-$)	3×10^{-3}	
(12) Succinic acid monohydroxyamate		NH—C—CH$_2$—CH$_2$—C=O (HO, O)	15×10^{-6}	
(13) 2-Hydroxylaminopropionate		NH—CH—C=O (HO, CH$_3$, O$^-$)	1×10^{-3}	
(14) 2-Hydroxylaminopropionitrile		NH—CH—C≡N (HO, CH$_3$)	No activity	

Fig. 4. *Positioning of the tripeptide Ac$_2$-L-Lys-D-Ala-D-Ala in the active site of the Zn^{2+} DD-carboxypeptidase and inhibiting potency of bifunctional compounds*

ID$_{50}$ is defined in the Materials and methods section. With compounds (1)–(5) the enzyme (5 nM) and the inhibitor (10–200 nM) were incubated for 60 min. With compounds (6)–(10) and (12) the enzyme (0.8 μM) and the inhibitor (33 μM–1 mM) were incubated for 5 min. With compounds (11), (13) and (14) the enzyme (0.8 μM) and the inhibitor (1–10 mM) were incubated for 5 min. All the experiments were performed at 37°C and in buffer A. For further details see the text.
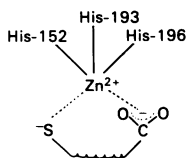
His-193

His-152   His-196

Zn^{2+}

—S   O   C   O

Fig. 5. *Possible bidentate interaction between the Zn^{2+} DD-carboxypeptidase and a bifunctional (thiol/carboxylate) inhibitor*

the initial activity had disappeared (i.e. after 5 min). The reaction mixture was then supplemented with 120 nM-$ZnSO_4$, i.e. at a concentration much higher than that of 3-mercaptopropionate, which treatment failed to regenerate an active carboxypeptidase.

Inactivation by bicyclic ring-fused azetidinone structures

(a) *Classical penams and 3-cephems.* On the basis of reaction (1) (see the Materials and methods section), the lower the K value and the higher the k_{+2} and k_{+3} values, the more potent a β-lactam as an enzyme inactivator. Although, once formed, complexes E–I* are very stable (low k_{+3} values), the 6-β-substituted penicillins were found to be extremely poor inactivators of the Zn^{2+} DD-carboxypeptidase because of very high K values and very low k_{+2} values (150 mM and $8 \times 10^{-4} s^{-1}$ respectively for phenoxymethylpenicillin, which is the most potent penam inactivator so far tested) (Frère *et al.*, 1978). The 7-β-substituted cephalosporins were somewhat better inactivators, mainly because of more favourable K values (1–10 mM).

Lineweaver–Burk plots showed that 7-β-substituted Δ^3-cephems fall into two groups. Cephalothin ($K = 9.5$ mM; $k_{+2} = 5 \times 10^{-4} s^{-1}$; $k_{+3} = 5 \times 10^{-6} s^{-1}$; in buffer B) and cephalosporin C ($K = 2$ mM; $k_{+2} = 1 \times 10^{-4} s^{-1}$; $k_{+3} = 2 \times 10^{-6} s^{-1}$; in buffer B) acted non-competitively. In parallel to this, cephalothin (10 mM) and cephalosporin C (1.66 mM) destroyed the crystal lattice, thus preventing any study from being carried out by X-ray diffraction. In contrast, cephaloglycine ($K = 4.5$ mM; $k_{+2} = 3 \times 10^{-4} s^{-1}$; $k_{+3} = 9 \times 10^{-6} s^{-1}$; in buffer B) and 7-[2-(p-iodophenyl)acetamido]cephalosporanate ($K = 3.5$ mM; $k_{+2} = 6 \times 10^{-4} s^{-1}$; $k_{+3} = 1 + 10^{-6} s^{-1}$; in buffer A) acted competitively. The enzyme complex formed with this latter compound showed satisfactory isomorphism and indicated that the β-lactam was bound to the active site (Dideberg *et al.*, 1980a, 1982).

(b) *β-Iodopenicillanate.* 6-β-Halogenopenams are β-lactamase inactivators, and, in this respect, 6-β-iodopenicillanate has been much studied (Frère *et al.*, 1982, and other references included in that paper). Kinetically, the interaction between

the Zn^{2+} DD-carboxypeptidase and 6-β-iodopenicillanate was found to resemble that observed with the β-lactamases, in that complete and permanent inactivation was observed only at high ($\geqslant 12500$) inactivator/enzyme ratio. When this ratio was decreased, enzyme inactivation was incomplete, and, after subsequent prolonged incubation, partial and spontaneous recovery of enzyme activity was observed. Although more work remains to be done in order to elucidate the underlying mechanism, these observations suggested a branched pathway including (i) normal turnover through an intermediate E–I*, (ii) re-arrangement of this intermediate into a transitorily inactive complex E–I**, and (iii) re-arrangement of the same intermediate into a permanently inactive complex E–I*** (see Fig. 6 legend). The first phases of enzyme inactivation by increasing concentrations of β-iodopenicillanate proceeded according to pseudo-first-order kinetics. From the k_a values thus obtained, a Hanes plot, [I]/k_a versus [I], gave rise to a straight line (Fig. 6) intersecting the ordinate axis at $K_i/k_{a (limit)}$ and the abscissa axis at $-K_i$. From the slope ($1/k_{a (limit)}$), a $k_{a (limit)}$ value of $7 \times 10^{-4} s^{-1}$ was computed. The K_i value was 0.66 mM.

An enzyme crystal was treated with β-iodopenicillanate as described in the Materials and methods section. The [I]/[E] ratio was estimated to be about 8300, and extensive inactivation occurred. (Indeed, when the measurements described below were completed, the treated crystal was dissolved, and DD-carboxypeptidase assays carried out on a sample of this solution showed that 85% of the enzyme has been permanently inactivated.) The enzyme derivative was perfectly isomorphous, and the difference Fourier synthesis at 0.28 nm resolution gave one main peak that was much higher than any other features of the map. The shape and the height of the peak did not correspond to an iodine atom with a full occupancy, strongly suggesting that iodine had been eliminated. The site of interaction clearly extended from His-190 just in front of the Zn^{2+} cofactor (Fig. 7) to an open cavity formed by the two segments His-190–Gly-189–Pro-188 and Asn-141–Ser-Asn-Val-144–Gly-Gly-Ala-147. These observations strongly suggest that, in the process of permanent inactivation of the Zn^{2+} DD-carboxypeptidase by 6-β-iodopenicillanate, the catalytically active His-190 was alkylated with loss of I^-. Such a mechanism would drastically differ from the inactivation of the serine β-lactamases, whose serine residue is irreversibly acylated by β-bromo-(iodo)penicillanate (Cohen & Pratt, 1980). A minor peak was also found in the difference map close to Arg-136. The shape and the size of the density suggested that this peak was attributable to an ion.
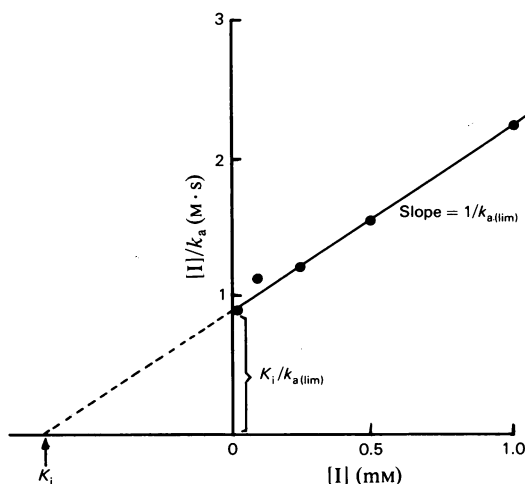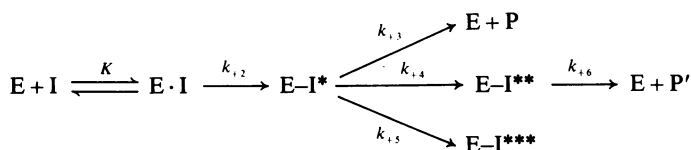
Fig. 6. *Hanes plot for the interaction between the Zn^{2+} DD-carboxypeptidase and β-iodopenicillanate* (I)
k_a (s^{-1}) is the pseudo-first-order rate constant of enzyme inactivation. Assuming that the interaction obeys the scheme:

$$E + I \xrightleftharpoons{K} E \cdot I \xrightarrow{k_{+2}} E-I^* \begin{array}{c} \xrightarrow{k_{+3}} E + P \\ \xrightarrow{k_{+4}} E-I^{**} \xrightarrow{k_{+6}} E + P' \\ \xrightarrow{k_{+5}} E-I^{***} \end{array}$$

where P and P' = degradation products, then:

$$K_i = \frac{k_{+3}K}{k_{+2} + k_{+3}} \quad \text{and} \quad \frac{1}{k_{a\,(\text{lim})}} = \frac{k_{+2} + k_{+3}}{k_{+2}(k_{+4} + k_{+5})}$$

Discussion

Non-β-lactam bifunctional reagents may be potent competitive inhibitors of the Zn^{2+} DD-carboxypeptidase. Enzyme inactivation by these agents (see Fig. 4) cannot be attributed to cleavage of the disulphide bridges and subsequent disorganization of the protein three-dimensional structure, nor to the detachment of the Zn^{2+} ion from the active site. Most probably it is due to the immobilization of the Zn^{2+} cation by co-ordination, thus preventing the cofactor from performing its catalytic function. The bifunctional compounds so far examined complement only part of the binding sites of the enzyme active site. Specificity and perhaps increasing inactivating potency requires more elaborate structures that not only would firmly anchor the inhibitor molecules in the active site but would be apt to interact specifically with the S_1 and S_2 subsite targets.

Classical β-lactams are extremely poor inactivators (as well as substrates) of the Zn^{2+} DD-carboxypeptidase. Some of them (cephaloglycine and 7-[2-(p-iodophenyl)acetamido]cephalosporanate) are weak competitive inhibitors. X-ray-diffraction studies have provided direct evidence that this latter compound binds to the enzyme active site. Other β-lactams (cephalosporin C and cephalothin) are weak non-competitive inhibitors. In parallel to this, these compounds destroy the protein crystal lattice and profoundly alter the scattering behaviour of the protein by inducing large conformational changes and/or aggregation (Labischinski *et al.*, 1984). 6-β-Iodopenicillanate is an unusual penam in that it has a very short side chain. Its interaction with the Zn^{2+} D-carboxypeptidase is complex, but at high β-lactam concentrations it inactivates the enzyme with a pseudo-first-order rate constant of $7 \times 10^{-4} s^{-1}$ (limit value). X-ray analysis shows that this β-lactam compound is located in the enzyme active site just in front of Zn^{2+} and superimposes His-190 (which is thought to act as proton donor during catalysis of a bound peptide substrate), suggesting that His-190 acts as a nucleophile by its lone electron pair of the nitrogen atom and undergoes alkylation by 6-β-iodopenicillanate with loss of iodine.

The above studies strongly suggest that both the high intrinsic resistance of the Zn^{2+} DD-carboxypeptidase to β-lactam compounds and the extremely low substrate activity of these β-lactam compounds towards the Zn^{2+} DD-carboxypeptidase are
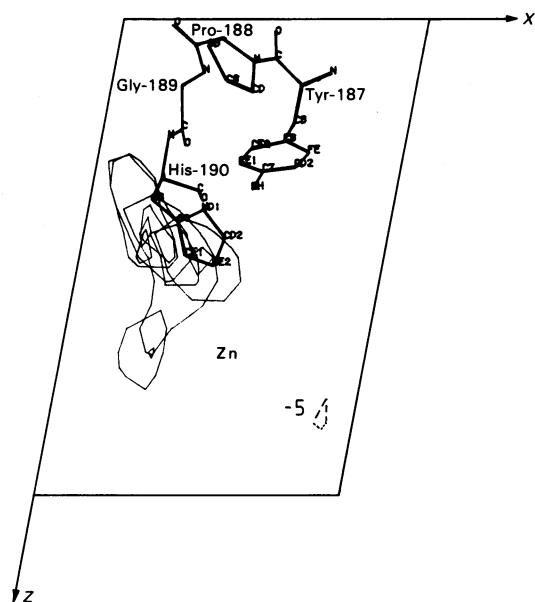
Fig. 7. *Difference electron density between the native Zn^{2+} DD-carboxypeptidase and its complex formed with β-iodopenicillanate, in the vicinity of the Zn^{2+}-cofactor site* The contour levels, shown on an arbitrary scale with maximal value of 10, are −5, 5 and 7. The contour of negative value (−5) is shown as a broken line. The unit cell was divided into 48 × 48 × 48 points. The Figure is a composite of sections of $Y = 19$ to 24, and the area shown extends from $X = 38$ to 48 and from $Z = 14$ to 34. The sequences Tyr-187–Pro-188–Gly-189–His-190 (as positioned in the Kendrew model) are shown as **bold** lines.

caused, most probably, by a particular geometry of the enzyme active site. Production of enzyme–ligand associations of high productiveness occurs only with carbonyl donor peptides (thus, for example, the standard tripeptide substrate Ac₂-L-Lys-D-Ala-D-Ala is hydrolysed with a turnover number of $2.5\,s^{-1}$ at 37°C). In contrast, the β-lactam compounds are little predisposed to align correctly with regard to the active functional groups. In fact, models show that with these compounds it is difficult to align simultaneously both the β-lactam amide bond with the Zn^{2+} ion and the C-terminal carboxylate with Arg-136 because of collisions with other side chains of the enzyme active site (P. Charlier & O. Dideberg, unpublished work).

One way to resolve the crystal structure of a protein by X-ray-diffraction methods rests upon the preparation of heavy-atom complexes with this protein. In this procedure, attachment of the heavy atom to the protein should not perturb the atomic arrangement of the crystal and should involve as few amino acid side chains as possible (Blundell &

Johnson, 1976). Among the heavy-atom-containing compounds tested, $K_2Pt(C_2O_4)_2$ is remarkable in that it has only one binding site, located within the enzyme cavity, close to the Zn^{2+} cofactor and the putative proton donor His-190. Inactivation proceeds with a second-order rate constant of about $6 \times 10^{-2}\,M^{-1}\cdot s^{-1}$.

References

Blundell, T. L. & Johnson, L. N. (1976) *Protein Crystallography*, pp. 183–239, Academic Press, London

Byers, L. D. & Wolfenden, R. (1972) *J. Biol. Chem.* **247**, 606–608

Cohen, S. A. & Pratt, R. F. (1980) *Biochemistry* **19**, 3996–4003

Cushman, D. W., Cheung, H. S., Sado, E. F. & Ondetti, M. A. (1977) *Biochemistry* **16**, 5484–5491

De Coen, J. L., Lamotte-Brasseur, J., Ghuysen, J. M., Frère, J. M. & Perkins, H. R. (1981) *Eur. J. Biochem.* **121**, 221–232

Dideberg, O., Frère, J. M. & Ghuysen, J. M. (1979) *J. Mol. Biol.* **129**, 677–679

Dideberg, O., Charlier, P., Dupont, L., Vermeire, M., Frère, J. M. & Ghuysen, J. M. (1980a) *FEBS Lett.* **117**, 212–214

Dideberg, O., Joris, B., Frère, J. M., Ghuysen, J. M., Weber, G., Robaye, R., Delbrouck, J. M. & Roelandts, I. (1980b) *FEBS Lett.* **117**, 215–218

Dideberg, O., Charlier, P., Dive, G., Joris, B., Frère, J. M. & Ghuysen, J. M. (1982) *Nature (London)* **299**, 469–470

Duez, C., Frère, J. M., Geurts, F., Ghuysen, J. M., Dierickx, L. & Delcambe, L. (1978) *Biochem. J.* **175**, 793–800

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

Frère, J. M., Geurts, F. & Ghuysen, J. M. (1978) *Biochem. J.* **175**, 801–805

Frère, J. M., Dormans, Ch., Duyckaerts, C. & De Graeve, J. (1982) *Biochem. J.* **207**, 437–444

Ghuysen, J. M., Frère, J. M., Leyh-Bouille, M., Dideberg, O., Lamotte-Brasseur, J., Perkins, H. R. & De Coen, J. L. (1981) in *Topics in Molecular Pharmacology* (Burgen, A. S. V. & Roberts, G. C. K., eds.), pp. 63–97, Elsevier/North-Holland Biomedical Press, Amsterdam

IUPAC–IUB Commission on Biochemical Nomenclature (1969) *Biochem. J.* **113**, 1–4

Joris, B., Van Beeumen, J., Casagrande, F., Gerday, Ch., Frère, J. M. & Ghuysen, J. M. (1983) *Eur. J. Biochem.* **130**, 53–69

Katz, L. & Levinthal, C. (1972) *Annu. Rev. Biophys. Bioeng.* **1**, 465–504

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

Labischinski, H., Giesbrecht, P., Fischer, E., Barnikel, G., Bradaczek, H., Frère, J. M., Houssier, Cl., Charlier, P., Dideberg, O. & Ghuysen, J. M. (1984) *Eur. J. Biochem.* **138**, 83–87

North, A. C. T., Philips, D. C. & Matthews, F. S. (1981) *Acta Crystallogr. Sect. A* **24**, 351–359

Pascal, P. (1958) *Nouveau Traité de Chimie Minérale*, vol. 19, pp. 774–775, Masson, Paris