Biochem. J. (1991) 279, 213–221 (Printed in Great Britain)

Mechanism of acyl transfer by the class A serine β-lactamase of *Streptomyces albus* G

Josette LAMOTTE-BRASSEUR, Georges DIVE, Otto DIDEBERG, Paulette CHARLIER, Jean-Marie FRÈRE and Jean-Marie GHUYSEN*

Centre d'Ingénierie des Protéines, Université de Liège, Institut de Chimie, B6, B-4000 Sart Tilman (Liège 1), Belgium

Optimization by energy minimization of stable complexes occurring along the pathway of hydrolysis of benzylpenicillin and cephalosporin C by the *Streptomyces albus* G β-lactamase has highlighted a proton shuttle that may explain the catalytic mechanism of the β-lactamases of class A. Five residues, S70, S130, N132, T235 and A237, are involved in ligand binding. The γ-OH group of T235 and, in the case of benzylpenicillin, the γ-OH group of S130 interact with the carboxylate group, on one side of the ligand molecule. The side-chain NH_2 group of N132 and the carbonyl backbone of A237 interact with the exocyclic CONH amide bond, on the other side of the ligand. The backbone NH groups of S70 and A237 polarize the carbonyl group of the scissile β-lactam amide bond. Four residues, S70, K73, S130 and E166, and two water molecules, W1 and W2, perform hydrolysis of the bound β-lactam compound. E166, via W1, abstracts the proton from the γ-OH group of S70. While losing its proton, the O-γ atom of S70 attacks the carbonyl carbon atom of the β-lactam ring and, concomitantly, the proton is delivered back to the adjacent nitrogen atom via W2, K73 and S130, thus achieving formation of the acyl-enzyme. Subsequently, E166 abstracts a proton from W1. While losing its proton, W1 attacks the carbonyl carbon atom of the S70 ester-linked acyl-enzyme and, concomitantly, re-entry of a water molecule W'1 replacing W1 allows E166 to deliver the proton back to the same carbonyl carbon atom, thus achieving hydrolysis of the β-lactam compound and enzyme recovery. The model well explains the differences found in the $k_{cat.}$ values for hydrolysis of benzylpenicillin and cephalosporin C by the *Streptomyces albus* G β-lactamase. It also explains the effects caused by site-directed mutagenesis of the *Bacillus cereus* β-lactamase I [Gibson, Christensen & Waley (1990) Biochem J. **272**, 613–619].

INTRODUCTION

The serine β-lactamases hydrolyse the endocyclic amide bond of penicillin by transfer of the penicilloyl moiety to the essential serine residue with formation of an ester-linked acyl-enzyme, which is subsequently hydrolysed with release of penicilloate. The reaction is analogous to the catalysed rupture of an acyclic peptide bond by the serine peptidases of the trypsin and subtilisin families. The β-lactamases fall into three classes, A, C and D. Members of a given class have similar primary structures (Joris *et al.*, 1988).

The class A β-lactamases of *Streptomyces albus* G (Dideberg *et al.*, 1987), *Staphylococcus aureus* (Herzberg & Moult, 1987) and *Bacillus licheniformis* (Moews *et al.*, 1990) are of known three-dimensional structure. As a result of the folding of the polypeptide chain, four motifs are brought close to each other

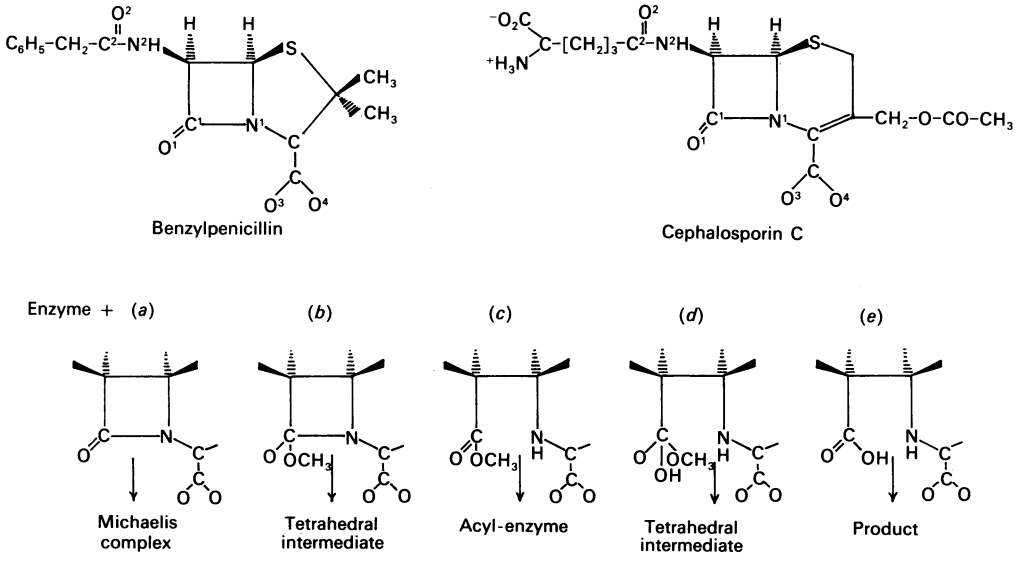

Fig. 1. Benzylpenicillin, cephalosporin C and the corresponding stable entities (*a*)–(*e*) used for geometry optimization and modelling of the *Streptomyces albus* G β-lactamase-catalysed reaction

* To whom correspondence should be addressed.

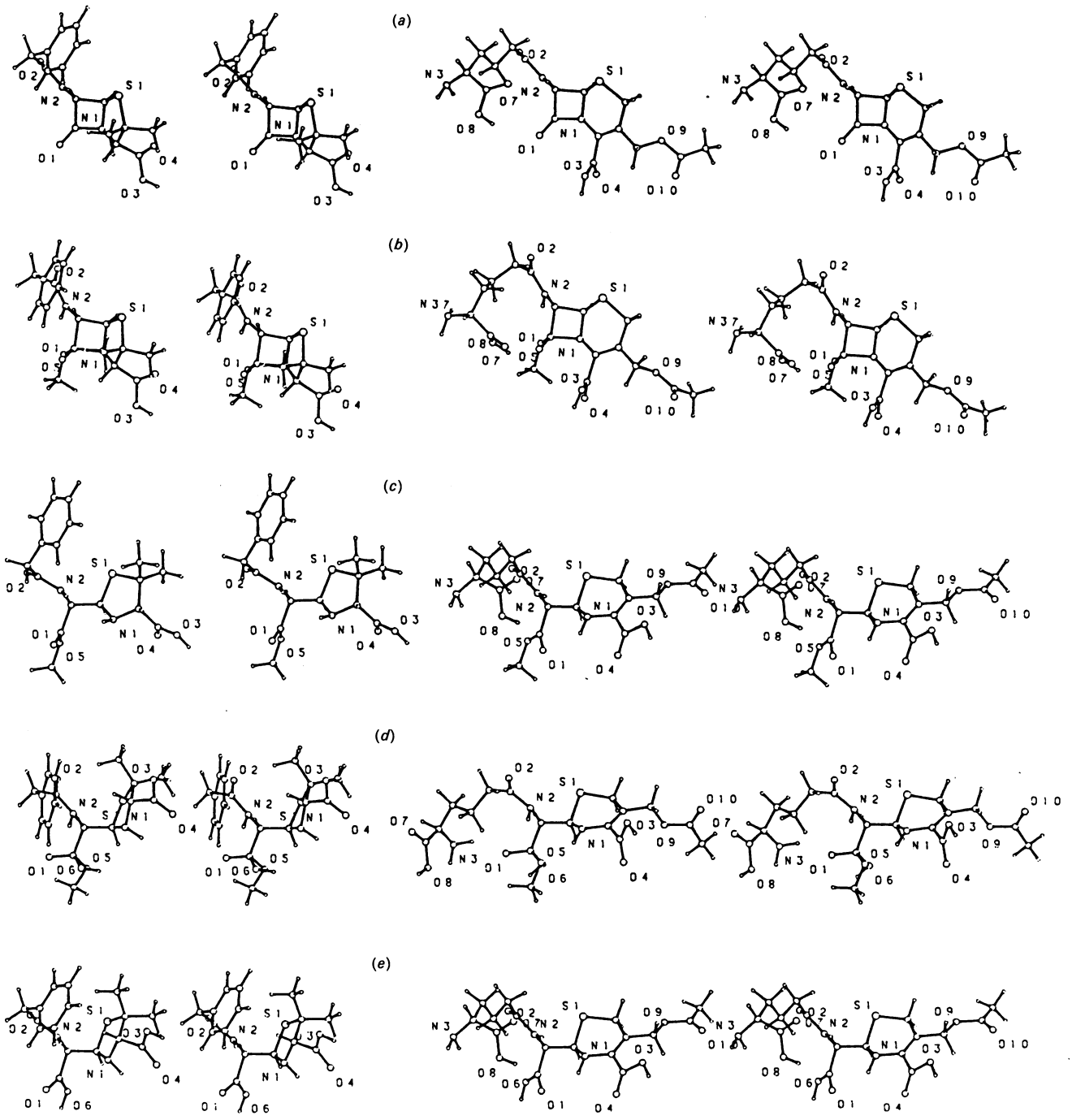

Fig. 2. Optimized structures of the stable entities (*a*)–(*e*) shown in Fig. 1.

and form the active site at the junction between an all-α domain and an α/β domain, the five-stranded β-sheet of which is protected by additional α-helices on both faces. In the standard ABL numbering scheme (Ambler *et al.*, 1991), the S70XXK73 motif, where S70 is the essential serine residue, is at the *N*-terminus of helix α2 of the all-α domain and occupies a central position in the enzyme cavity. The S130DN132 motif connects helices α4 and α5 of the all-α domain and forms one side of the cavity. The E166XELN170 motif is located at the entrance of the cavity. The K234S(or T)GA237 motif is on the innermost β-3 strand of the β-sheet and forms the other side of the cavity.

The mechanism of proton abstraction and donation during hydrolysis of β-lactam compounds by the β-lactamases is still the subject of considerable discussion. In order to shed light on the proton shuttle, discrete steps along the reaction pathway have been examined separately by docking the five stable entities (*a*)–(*e*) shown in Fig. 1 in the active site of the *Streptomyces albus* G β-lactamase and energy-minimizing each of the relevant complexes. Comparison of the optimized complexes in the indicated order (Fig. 1) has highlighted a proton shuttle that may explain the catalytic mechanism of the β-lactamase. The selected β-lactam ligands are benzylpenicillin (K_m = 1 mM; $k_{cat.}$ = 2800 s^{-1}) and cephalosporin C (K_m = 4.5 mM; $k_{cat.}$ = 170 s^{-1}). The numbering (Fig. 1) of atoms O-1, C-1, N-1, O-2, N-2, O-3 and O-4 of the ligands is arbitrary.

MATERIALS AND METHODS

Optimization of the enzyme active site

The three-dimensional structure of the *Streptomyces albus* G β-lactamase has been resolved at 0.17 nm (1.7 Å) by X-ray crystallography (Dideberg *et al.*, 1987, and unpublished work). The enzyme active site was optimized by total energy minimization with the use of the AMBER molecular mechanics V3 framework (Weiner *et al.*, 1984). Standard point charges on the amino acid residues and a distance-dependent dielectric constant $\epsilon = R_{ij}$ were used for the calculation of the Coulombic term. The CH, CH_2 and CH_3 groups were treated as united atoms. Minimization of the X-ray structure was carried out until the root-mean-square gradient was less than 4.2 kJ/nm (0.1 kcal/Å). In this process, each atom was first restrained from its initial position by a weight of 42000 nm²·kJ/mol (100 Å²·kcal/mol) ($E_{rest} = \sum_{atoms} 100\,D^2$ where D = distance between current and initial Cartesian co-ordinates), thus allowing relaxation of the whole structure and elimination of a few contacts that were abnormally short. Convergence was achieved after 54 iterations. Subsequently, a constraint of 4200 nm²·kJ/mol (10 Å²·kcal/mol) was applied to the α-carbon atoms, providing a reasonable representation of separate sections of the protein while still allowing limited motion of the active-site region. Convergence required 9000 iterations. Finally, a Monte Carlo water bath generated 28 water molecules, five of which occurred in the active site. Their atomic positions were refined together with the enzyme and ligand coordinates.

Optimization of stable entities along the reaction pathway

The entities (*a*)–(*e*) (Fig. 1) were optimized by the AM1 semi-empirical method (Fig. 2). Each structure was docked into the enzyme active site (79 amino acid residues and three water molecules; see the Results section), and the corresponding complexes were optimized as described for the native enzyme with the use of CNDO charges to compute the Coulombic term. Modelling of the tetrahedral intermediates and acyl-enzyme in which the ligand is covalently linked to the active-site serine residue was done after substituting the O–CH_3 group of entities (*b*), (*c*) and (*d*) by the O–CH_2– side chain of the serine residue. The bond lengths, bond angles and ring dihedral angles of entities (*a*)–(*e*) were constrained to the AM1 values while allowing rotation around the free bonds.

Graphics

The molecular structures were built with the help of a Data General GDC 2400 colour graphic processor using the ULYSSE program, a home-made version of PAKGGRAF (Honig *et al.*, 1973). This program allows the user to modify the relative disposition of parts of the molecular structures, check interatomic distances, adjust torsional angles and produce red/green stereo views of the molecules. The geometric features of the enzyme and the various complexes studied were analysed by using a FRODO version running on a MEGATEK 9300 graphic raster processor (Lamotte-Brasseur *et al.*, 1988) connected to a VAX 11/780.

RESULTS

Active site of the native enzyme

Figs. 3 and 4 give the primary and tertiary structure of the protein respectively. On the basis of the refined crystallographic data, the published amino acid sequence (Dehottay *et al.*, 1987) was corrected by deleting G128 and replacing A140ζL by GM and G265 by A. The numbering shown above the sequence (Fig. 3) refers to the protein precursor. That shown below the sequence refers to the ABL scheme (derived from the alignment of all the class A β-lactamases of known primary structure) (Ambler *et al.*, 1991). The ABL numbering has been used throughout this paper. Figs. 3 and 4 also identify the secondary structures α1–α11 and β1–β5, and the segments S1–S7 that, together, contain the 79

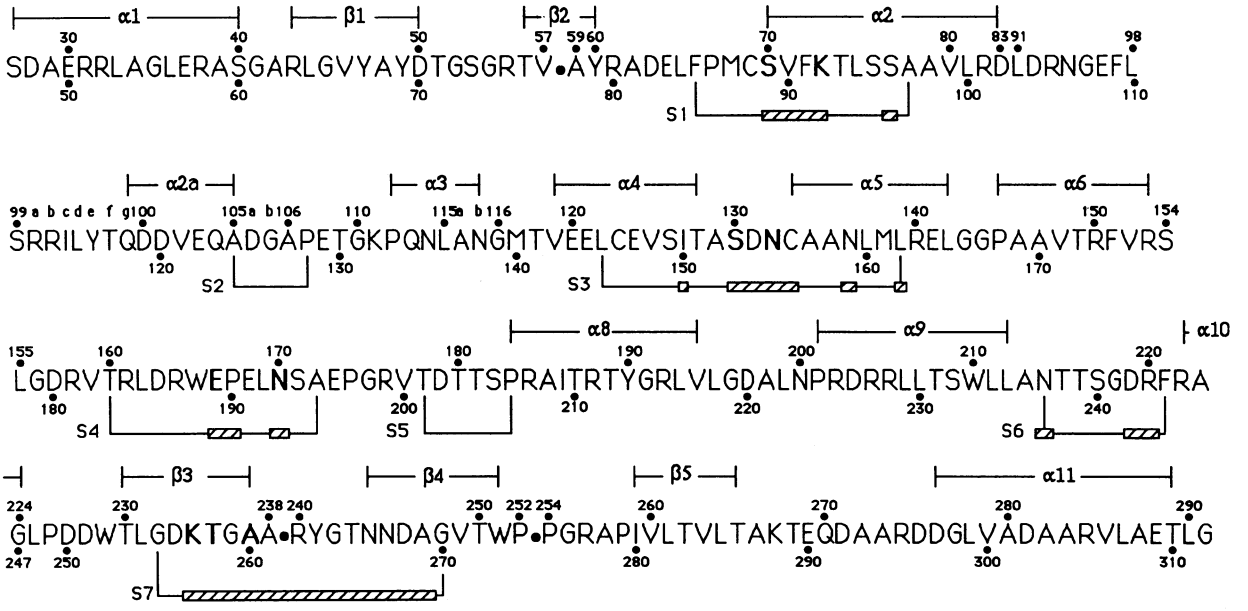

Fig. 3. Primary structure of the *Streptomyces albus* G β-lactamase

The amino acid numbering is that of the ABL scheme or that of the precursor. α1–α11 and β1–β5 indicate regions of α-helices and β-strands respectively. S1–S7 indicate segments containing the 79 amino acid residues that occur with a 1.5 nm (15 Å) radius around the C-α atom of the active-site serine residue S70. The hatched areas in segments S1, S3, S4, S6 and S7 contain the 30 amino acid residues that form the inner shells of the active site (see Fig. 5). The nine amino acid residues shown in **bold** type in the sequence form the innermost boundary of the active site (see Fig. 6*a*).

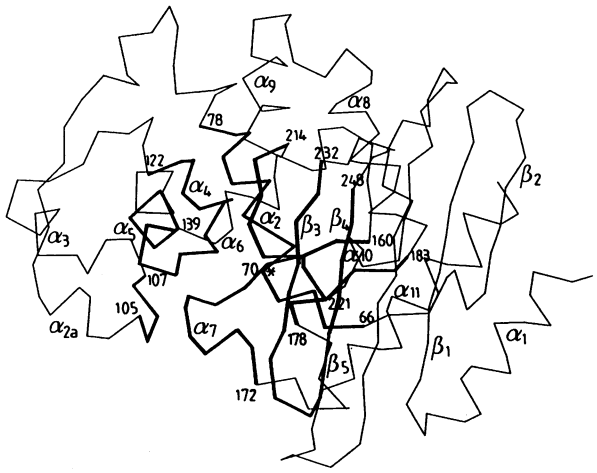

Fig. 4. Folding of the polypeptide chain of the *Streptomyces albus* G β-lactamase

The secondary structures are labelled and the segments S1–S7 (see Fig. 3) are identified (heavy lines).

Table 1. Disposition of the water molecules in the active site of the *Streptomyces albus* G *B*-lactamases

Distances are between the oxygen atom of the water molecule and the oxygen or nitrogen atom of the relevant amino acid residue.

Water molecule	Atom of amino acid residue	Distance (nm)	
		Optimized model	Crystal structure
W1	E166Oε1	0.274	0.266
	N170Oδ1	0.280	0.279
	S70Oγ	0.274	0.262
W2	S130CO	0.288	0.282
	N132Oδ1	0.274	0.352
	S130Oγ	0.277	0.542
W3	I127CO	0.280	0.305
	N214Oδ1	0.273	0.277
	K234Nζ	0.299	0.276

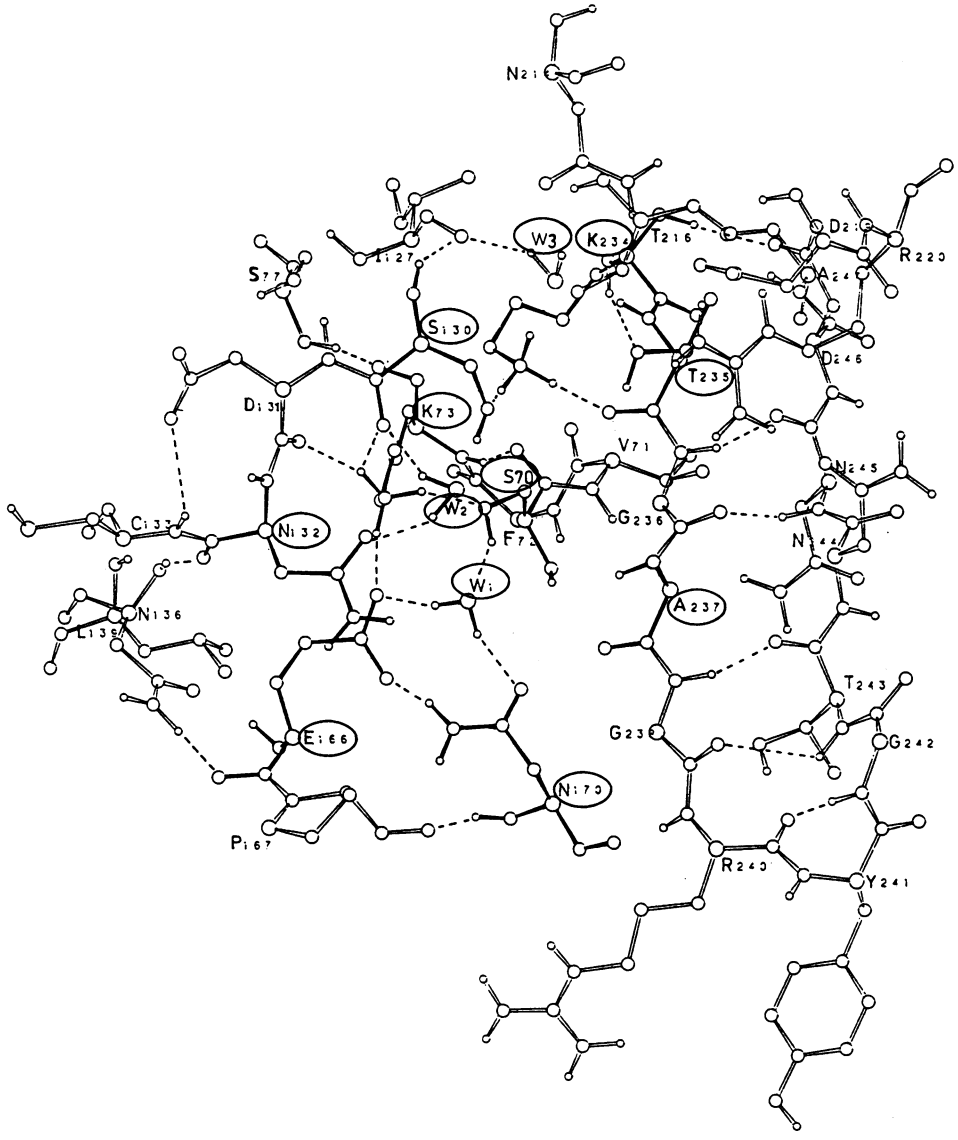

Fig. 5. Spatial disposition of the 30 amino acid residues that form the inner shells of the active site of the *Streptomyces albus* G β-lactamase

The nine amino acid residues that form the innermost boundary of the active site are encircled (Fig. 6*a*).

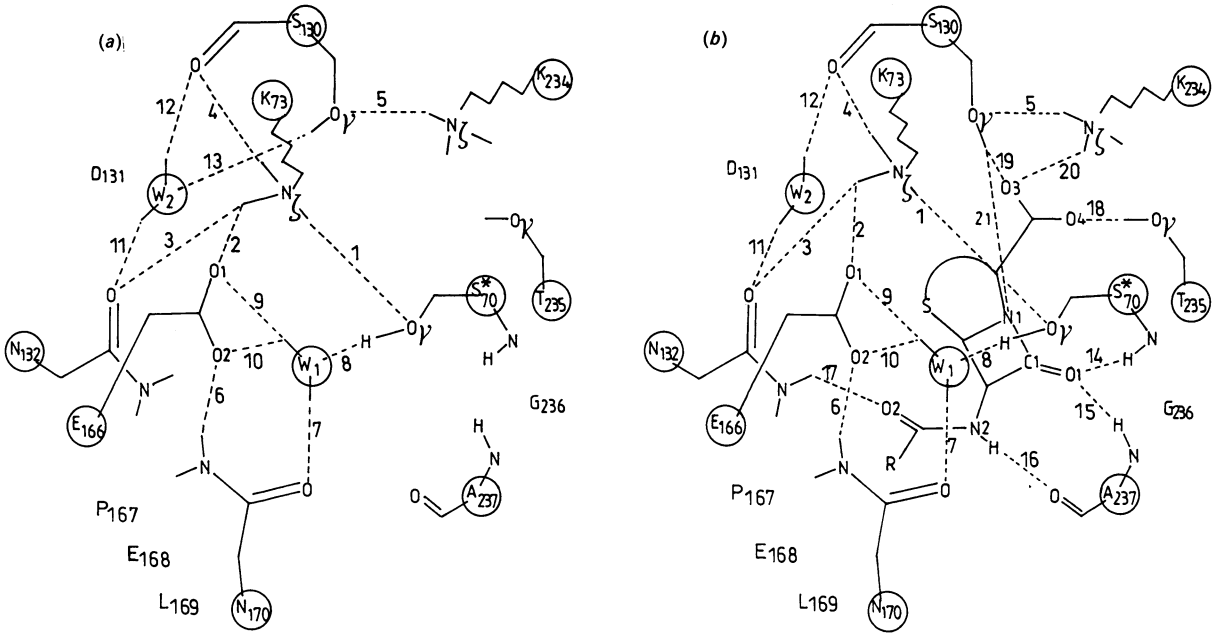

Fig. 6. Schematic diagram of the hydrogen-bonding network within the active site of the *Streptomyces albus* G β-lactamase

(*a*) The native cavity. (*b*) The Michaelis complex formed with benzylpenicillin and cephalosporin C. The bond lengths and angles are given in Table 2.

amino acid residues that occur within a 1.5 nm (15 Å) radius around S70Cα and define the active site.

The optimized active site differs only slightly from that derived from the X-ray data [mean distance between pair of C-α atoms: 0.057 nm (0.57 Å)]. Moreover, three water molecules occupy positions that are almost identical with those found in the crystal structure (Table 1). Figs. 5 and 6 show the hydrogen-bonding network at two levels of completeness of the active site. Fig. 5 contains the 30 innermost amino acid residues (identified in Fig. 3 by the hatched area of segments S1, S3, S4, S6 and S7) and the water molecules W1, W2 and W3. Fig. 6(*a*) represents the nine amino acid residues, i.e. S70 and K73, S130 and N132, E166 and N170, K234, T235 and A237 (see the Introduction), that, together with W1 and W2, form the immediate boundary of the cavity.

The main feature of the molecular structure is the presence of three hydrogen-bonding sub-networks in which the side-chain amino groups of K73, W1 and W2 occupy central positions (Fig. 6*a* and Table 2). In the NζK73 sub-network the side-chain amino group of K73 is in hydrogen-bonding interactions with the O-γ atom of S70 (bond 1), the oxygen atom O-1 of the carboxylate group of E166 (bond 2), the carbonyl side chain of N132 (bond 3) and the carbonyl backbone of S130 (bond 4). In the W1 sub-network the water molecule interacts with the carbonyl side chain of N170 (bond 7), the γ-OH group of S70 (bond 8), the oxygen atom O-1 of the carboxylate group of E166 (bond 9) and, much more loosely, the oxygen atom O-2 of the same E166 (bond 10). In the W2 sub-network the water molecule interacts with the side-chain carbonyl group of N132 (bond 11) and both the backbone carbonyl group (bond 12) and the γ-OH group (bond 13) of S130.

The reaction profile proposed below derives from the sequential changes that occur in the optimized complexes obtained by docking entities (*a*)–(*e*) in the enzyme active site (see the Materials and methods section). For the optimization of the non-covalent complexes formed by docking entities (*a*) and *e*) in the enzyme active site, the *xyz* guess geometry was defined by a relative enzyme–ligand disposition such that the carbonyl carbon atom C-1 of the ligand lies in the vicinity of the O-γ atom of S70, and the carboxylate group of the ligand is oriented towards the bottom of the cavity, i.e. towards K234. The reaction profile is illustrated in Fig. 7. The lengths and angle values of the bonds involved in the interaction are given in Table 2.

Michaelis complex

Binding of the β-lactam ligand to the active site hardly modifies the W1 sub-network. Though slightly displaced, W1 remains the connecting bridge between the γ-OH group of S70 and, preferentially, the O-ε1 atom of E166 (bonds 8, 9 and 10). Bonds 11 and 12 of the W2 sub-network are also unmodified but bond 13 disappears. W2 no longer interacts with the γ-OH group of S130, which is now oriented towards the nitrogen atom N-1 of the β-lactam ring (bond 21) (Figs. 6*b* and 7*a*).

The main feature of the enzyme–ligand interactions is an anti-parallel hydrogen-bonding between the innermost β-3 strand of the β-sheet and the functional groups of the ligand-binding entity (as defined previously; Lamotte-Brasseur *et al.*, 1991). The carbonyl oxygen atom O-1 of the β-lactam ring is in hydrogen-bonding interactions with the backbone NH groups of S70 (bond 14) and A237 (bond 15), which create the required oxyanion hole. The N-2–H group and O-2 atom of the exocyclic amide bond of the ligand interact with the A237 backbone carbonyl group (bond 16) and the N132 side-chain amino group (bond 17) respectively. The oxygen atoms O-3 and O-4 of the carboxylate group of benzylpenicillin interact with the γ-OH groups of both T235 (bond 18) and S130 (bond 19). With cephalosporin C, bond 18 only occurs, but additional interactions are provided by the CH_2–O–$COCH_3$ group borne by the dihydrothiazine ring, which extends towards T216, D219 and R220, and by the acyl side chain borne by the β-lactam ring, which extends towards R240 of strand β-3 and T243 of strand β-4. These additional interactions are expressed by an increased value of the van der Waals component of the total energy of interaction when compared

Table 2. H···X distances and YH···X bond angles, where X and Y are the heteroatoms involved in hydrogen-bonding, in the native active site of the *Streptomyces albus* G β-lactamase and in the complexes occurring along the reaction pathway

Abbreviations: T.I., tetrahedral intermediate; Pen, benzylpenicillin; Ceph, cephalosporin C.

		Bond length (nm) and bond angle (°)										
			Michaelis complex		Enzyme acylation				Enzyme deacylation			
					T.I.		Acyl-enzyme		T.I.		Product	
	Bond	Native enzyme	Pen	Ceph	Pen	Ceph	Pen	Ceph	Pen	Ceph	Pen	Ceph
1	K73Nζ H→Oγ S70	0.1732 148.5	0.1686 169.1	0.1696 166.4	0.2309 112.1	0.203 149.5	0.2011 155.6	0.2149 143.7	0.1998 137.1	0.2005 142.7	0.1676 155	0.1686 161.4
2	K73Nζ H→Oε1 E166	0.1668 145.4	0.1698 139.4	0.1689 141.1	0.2589 117.5	0.2459 112.9	0.1697 140.2	0.1707 138.7	0.2415 129.6	0.2556 115.1	0.1711 137.9	0.1685 142.5
3	K73Nζ H→Oδ N132	0.219 114.1	0.2031 122.2	0.2055 119	0.1752 138.6	0.1715 157	0.2055 115.8	0.2013 121.9	0.1716 144.7	0.1688 161.5	0.1955 121.1	0.2052 116.8
4	K73Nζ H→OCS130	0.2082 121.1	0.1695 166	0.1713 156.8	0.1726 143.4	0.2496 131.2	0.1729 147.3	0.1713 163.4	0.1742 139.1	0.1732 145.5	0.1718 151.5	0.1698 168.5
5	K234Nζ H→Oγ S130	0.1719 147.2	0.1685 174.6	0.1674 166.2	0.1769 173.6	0.1717 168.8	0.1708 154.5	0.1698 165.2	0.1706 157.2	0.1709 166.8	0.17 164	0.1695 162.4
6	N170Nζ H→Oε2 E166	0.1661 161.3	0.1669 171.4	0.1672 169	0.1815 153.8	0.1795 157.8	0.1662 168.3	0.1689 170.4	0.1757 153.6	0.2006 147.5	0.167 173.3	0.1682 173.5
7	W1H2→Oδ N170	0.1895 156.2	0.1794 165.9	0.1785 168.5	0.176 175.6	0.1805 156.7	0.181 165.1	0.1782 171	0.181 152.5	0.1946 140	0.1814 163.7	0.1799 162.4
8	S70Oγ H→W1	0.1863 148.5	0.207 133.4	0.2061 135							0.3418 159.8	0.2118 129.7
8*	W1H1→Oγ S70				0.1856 155.8	0.1897 141			0.3118 174.2	0.5295 64.8		
9	W1H1→Oε1 E166	0.1792 164	0.1703 160.7	0.1692 162.6			0.1731 164.5	0.2217 127.8			0.2912 134.3	0.2227 131.2
9*	E166Oε1 H→W1				0.2104 140.1	0.2117 134.1			0.2791 105.6			
10	W1H1→Oε2 E166	0.2526 136.3	0.2194 132.2	0.2276 132.1			0.2128 126.9	0.1706 165.2			0.1657 165.6	0.1713 161.6
10*	E166Oε2 H→W1									0.2469 129.7		
11	W2H2→Oδ N132	0.1929 144.5	0.1836 154.9	0.1831 156.1	0.1803 163.6	0.1809 159.3	0.1894 146.3	0.2373 110.3	0.2373 115	0.2663 105	0.1826 155.1	0.245 107
12	W2H1→OCS130	0.1947 142.1	0.1816 158	0.1819 158	0.2565 108.5	0.1883 151.3	0.1807 161.3	0.1811 167.9	0.1802 167.5	0.1796 173	0.1807 157.6	0.2814 165.8
13	S130Oγ H→W2	0.1936 164.8	0.4222 104.1	0.3889 132.5	0.4559 85.2	0.4485 91	0.4918 76.1	0.4786 85.9	0.4978 76	0.48 86.9	0.3959 95.3	0.4794 88.4
14	S70NH→O1		0.186 160.8	0.1932 163.8	0.186 154.2	0.1858 150.2	0.1804 157.8	0.1791 151.3	0.1798 154	0.2025 121.9	0.1874 172.7	0.201 164.6
15	A237NH→O1		0.1887 143.8	0.1924 142.9	0.1786 150.8	0.1803 149.1	0.1835 152.3	0.1896 149.5	0.1796 151.2	0.1823 146.4	0.183 150.4	0.1879 144
16	N2H→OCA237		0.1961 133.4	0.1918 168.7	0.1922 157.9	0.1947 161.2	0.1876 159.7	0.1873 167.5	0.1899 156.8	0.1901 170.5	0.1873 148.3	0.1894 161
17	N132Nδ H→O2		0.1843 158.9	0.1822 169.6	0.1805 158.2	0.1818 172.1	0.1813 158.6	0.1811 171.1	0.1813 158	0.1808 172	0.1852 156	0.186 168.2
18	T235Oγ H→O4		0.191 160.2	0.184 157.4	0.1804 167.2	0.1821 159.2	0.194 172.4	0.2883 84.9	0.1803 163.2	0.2887 83.4	0.1783 166.4	0.2953 83.6
19	S130Oγ H→O3		0.1963 131.3	0.2769 81.3	0.1833 140.8	0.189 135.6	0.184 156.7	0.1855 145.7	0.1823 151.6	0.189 146.1	0.1855 145.4	0.1839 150.1
20	K234Nζ H→O3		0.3032 112.6	0.2623 116	0.2577 105	0.2631 106.8	0.3273 98.9	0.3122 106.5	0.3083 99.1	0.3169 108.5	0.3058 105.3	0.3268 109.9
21	S130Oγ H→N1		0.311 168.5	0.336 131.8	0.249 141.8	0.278 131.7	0.243 125.9	0.341 156.3	0.24 129.5	0.358 160.3	0.297 145.2	0.376 159.1

with that of benzylpenicillin (Table 3). Note that the carboxylate head of both benzylpenicillin and cephalosporin C is oriented towards but only weakly hydrogen-bonded to the side-chain amino group of K234 (bond 20).

Enzyme acylation

In the Michaelis complex (Figs. 6*b* and 7*a*), the β-lactam carbonyl C-1=O-1 is polarized by the oxyanion hole (bonds 14 and 15); the β-lactam carbonyl carbon atom C-1 is 0.28 nm (2.8 Å) from the O-γ atom of S70, the angle C-β–O-γ (of S70)···C-1 (of the β-lactam ring) is 107° with cephalosporin C and 109° with benzylpenicillin, and the γ-OH group of S70 is hydrogen-bonded to W1, which is itself hydrogen-bonded to the O-ε1 atom of E166. Hence the conditions are settled for proton abstraction of the γ-OH group of S70 by the O-ε1 atom of E166 via W1, attack of carbon atom C-1 by the oxygen atom while it

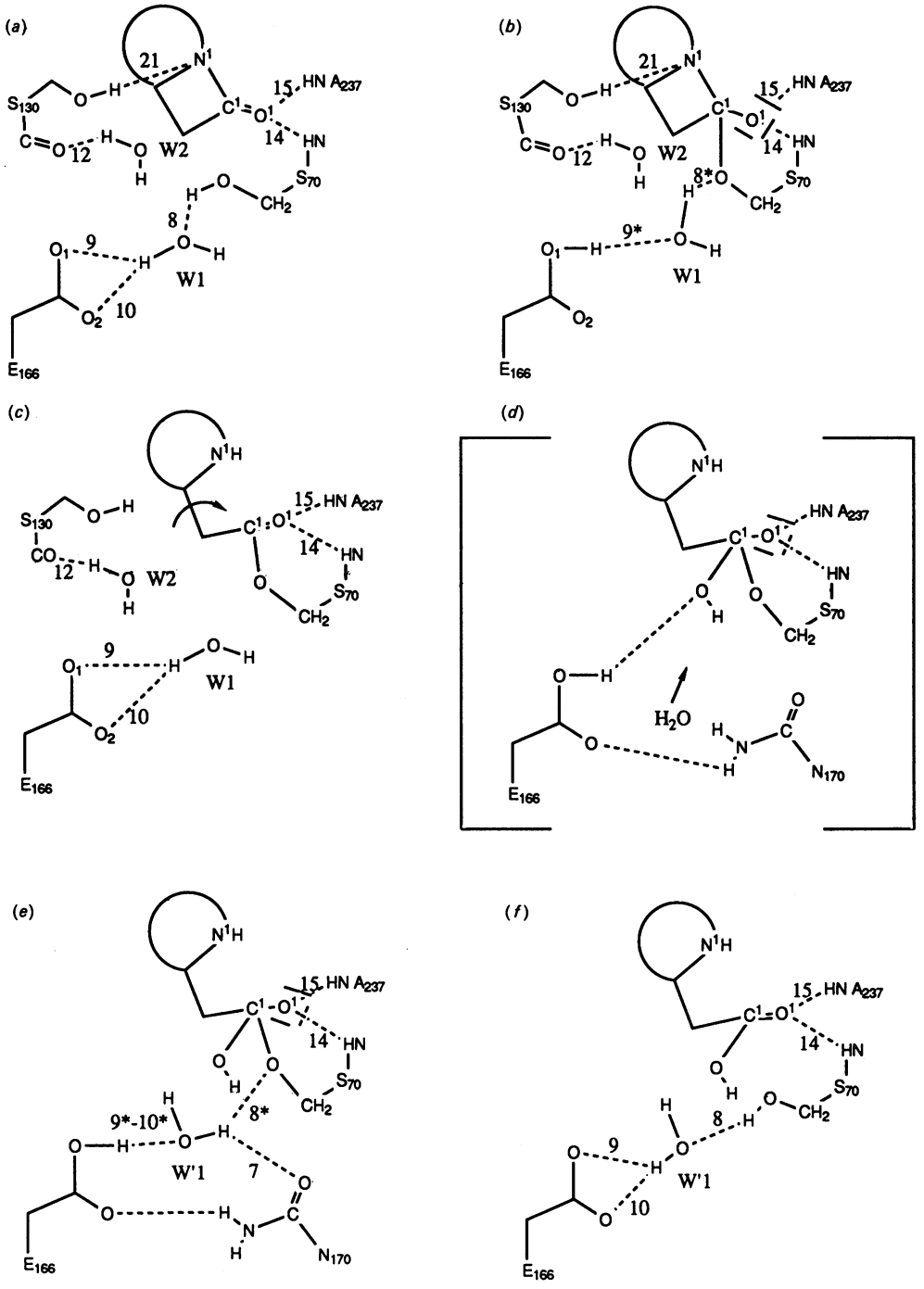

Fig. 7. Schematic representation of the proton shuttle during hydrolysis of benzylpenicillin or cephalosporin C by the *Streptomyces albus* G β-lactamase

(*a*) Michaelis complex; (*b*) tetrahedral intermediate; (*c*) acyl-enzyme; (*d*) and (*e*) tetrahedral intermediate; (*f*) product.

is losing its proton, and formation of a negatively charged adduct in which the C-1 atom adopts a tetrahedral geometry (Fig. 7*b*).

Generation of the tetrahedral adduct has the following consequences. (1) As a result of the transfer of the proton from the γ-OH group of S70 to the nearest oxygen atom O-ε1 of E166, the W1 sub-network undergoes the following rearrangements: bond 8 is converted into bond 8*, bond 9 is converted into bond 9* and bond 10 disappears. (2) The NζK73 and W2 sub-networks also undergo re-arrangements. Bonds 1, 2 and 12 (benzylpenicillin) and 2 and 4 (cephalosporin C) become loose and distorted whereas bond 3 is improved. (3) Strong hydrogen-bonding occurs between the γ-OH group of S130 and the O-3 atom of the carboxylate group of cephalosporin C (i.e. bond 19, which was not present in the Michaelis complex, is formed).

Collapse of the tetrahedral adduct (Fig. 7*b*) is made by back-delivery of the abstracted proton (now located on the O-ε1 atom of E166) on the β-lactam nitrogen atom N-1, giving rise to the acyl-enzyme (Fig. 7*c*) with restoration of a non-tetrahedral carbonyl carbon atom C-1. Given that the γ-OH group of S130, which has been displaced from W2 during binding of the ligand to the enzyme, is oriented towards N-1 (bond 21), back-delivery of the proton probably occurs through the W2 and NζK73 sub-

Table 3. Decomposition of the energy of interaction during formation of the Michaelis complex formed between the *Streptomyces albus* G β-lactamase and benzylpenicillin and cephalosporin C

Energy component	Energy of interaction (kJ/mol)	
	Penicillin	Cephalosporin C
van der Waals	−109	−151
Electrostatic + hydrogen bond	−42	−46
Total	−151	−197

networks, with the γ-OH group of S130 serving as final carrier. On the basis of the angle values of bond 21, the process should occur more easily with benzylpenicillin (168.5°) than with cephalosporin C (131.8°).

Collapse of the tetrahedral adduct has the following consequences. (1) The sub-network W1 undergoes re-arrangements such that bond 8* disappears, bond 9* is reconverted into bond 9 and bond 10 is restored. Depending on the β-lactam ligand, W1 is differently disposed. In the penicilloyl-enzyme, bond 9 (W1H1→Oε1E166) is shorter and better oriented than bond 10 (W1H1→Oε2E166). In the cephalosporoyl-enzyme bond 10 is shorter and better oriented than bond 9. These differences have important consequences on the ensuing steps of the reaction pathway. (2) Sub-networks NζK73 and W2 re-adopt a conformation comparable with that found in the Michaelis complex. (3) The carboxylate group of the penicilloyl moiety remains hydrogen-bonded to the γ-OH groups of T235 (bond 18) and S130 (bond 19) as it was in the Michaelis complex and the tetrahedral adduct. In contrast, the carboxylate group of the cephalosporoyl moiety interacts with the γ-OH group of S130 (bond 19, which was present in the tetrahedral adduct but not in the Michaelis complex) but no longer interacts with the γ-OH group of T235 (bond 18, which was present in both the Michaelis complex and the tetrahedral adduct). (4) The constraint imposed on the carboxylate group of the penicilloyl (bonds 18–19) and cephalosporyl (bond 19) moieties considerably limits the rotation that the thiazolidine or dihydrothiazine ring undergoes as a consequence of the rupture of the β-lactam amide bond: 30° versus 60° in the isolated entities (*c*).

Enzyme deacylation

In the acyl-enzyme (Fig. 7*c*), the oxygen atom of W1 is 0.298 nm (2.98 Å) from the carbon atom C-1 of the ligand and W1 is interacting strongly with the O-ε1 atom of E166 in the case of benzylpenicillin (bond 9) or the O-ε2 atom of E166 in the case of cephalosporin C (bond 10). Hence the conditions are settled for proton abstraction from W1 by the O-ε1 atom or the O-ε2 atom of E166, attack of the C-1 atom by W1 while it is losing one proton and formation of the tetrahedral adduct shown in Fig. 7(*d*). According to this mechanism, E166 fulfils two distinct functions. The first, required for the formation of the tetrahedral adduct leading to protein acylation, is to abstract, via W1, a proton from the γ-OH group of S70, activating a nucleophile for attack on the C-1 atom. The second, required for the formation of the tetrahedral adduct leading to protein deacylation, is to abstract a proton from W1, thus activating a nucleophile for attack of the same carbon atom C-1 now being linked to the O-γ atom of S70.

Release of the reaction product is made by back-delivery of the proton from E166 to the O-γ atom of S70, causing rupture of the O-γ (S70)–C-1 ester bond. The process requires re-entry of a water molecule W'1, replacing W1, which has been consumed in the process. The optimized structure of the tetrahedral adduct after re-entry of W'1 is shown in Fig. 7(*e*). Depending on which oxygen atom, O-ε1 or O-ε2, of E166 bears the proton (bond 9* or 10*), W'1 is much better oriented towards the O-γ atom of S70 (bond 8*) in the case of benzylpenicillin [0.312 nm (3.12 Å); 174.2°] than in the case of cephalosporin C [0.53 nm (5.3 Å); 65°], suggesting that hydrolysis of the cephalosporoyl-enzyme proceeds with more difficulty than that of the penicilloyl-enzyme.

DISCUSSION

In its resting state, the active site of the *Streptomyces albus* G β-lactamase is a hydrogen-bonding network in which nine amino acid residues, S70, K73, S130, N132, E166, N170, K234, T235 and A237, and two water molecules, W1 and W2, form interconnected sub-networks.

Binding of benzylpenicillin or cephalosporin C to the active site gives rise to an integrated functional complex. The γ-OH group of T235 interacts with the carboxylate group, on one side of the ligand molecule. The side-chain NH_2 group of N132 and the carbonyl backbone of A237 interact with the exocyclic CONH amide bond, on the other side of the ligand. The backbone NH groups of A237 and S70 interact with the scissile amide bond, at a central position in the ligand. The relative spatial disposition adopted by the two interacting partners results in the polarization of the carbonyl group of the scissile β-lactam amide bond (by A237 and S70) and in the correct positioning of the carbonyl carbon atom with respect to the O-γ atom of S70; it maintains, via W1, a close connection between the γ-OH group of S70 and the O-ε1 atom of E166; and it causes, through a displacement of W2, the re-orientation of the γ-OH group of S130 towards the nitrogen atom of the β-lactam ring.

Fine tuning of the two-stroke (acylation/deacylation) catalytic machinery strongly depends on the structure of the β-lactam compound and the design of the enzyme active site. As a consequence of the less-favourable disposition adopted by cephalosporin C in the *Streptomyces albus* G β-lactamase active site when compared with that of benzylpenicillin, back-delivery of the proton in both acylation and deacylation steps should occur with less facility. Experimental data support the prediction: the *Streptomyces albus* G β-lactamase performs 2800 rotations/s when acting on benzylpenicillin versus 170 rotations/s when acting on cephalosporin C (Matagne *et al.*, 1990).

Site-directed mutagenesis of the *Bacillus cereus* β-lactamase I, a homologue of the *Streptomyces albus* G β-lactamase, has led to the observations that the rate constants for acylation and deacylation for hydrolysis of benzylpenicillin by the mutant E166D is decreased about 2000-fold and that the rate constant for acylation by the K73R mutant is decreased about 100-fold (Gibson *et al.*, 1990). Hydrolysis of benzylpenicillin by the *Streptomyces albus* G β-lactamase in which E166 has been replaced by D and K73 by R respectively has been studied by molecular modelling. Suffice it to say here that in the E166D mutant W1 no longer connects the OH group of S70 to the dicarboxylic acid, thus severely hampering proton abstraction, and that in the K73R mutant W1 is oriented towards the O-ε2 atom instead of the O-ε1 atom of E166, thus probably decreasing the efficacy of proton abstraction.

Changes in the enzyme active site do not always have deleterious effects but may profoundly modify its specificity profile. The *Streptomyces* N132S β-lactamase mutant has a rather specific decreased hydrolytic activity towards the cephalosporins (Jacob *et al.*, 1990*a*,*b*). Emergence among important bacterial pathogens of 'new' TEM β-lactamases of class A exhibiting high hydrolysing capacity towards third-generation cephalosporins is achieved by alteration of a limited number of amino acid residues

(Sougakoff *et al.*, 1989; Collatz *et al.*, 1990). Key mutations often affect the K234T(or S)GA-containing β-3 strand (Ghuysen, (1991). According to the proposed model, N132 and the β-3 strand mainly serve to anchor the ligand in the enzyme cavity.

The β-lactamases of class C have no dicarboxylic acid with a spatial disposition that would be compatible with a role in the activation of the essential serine residue. It has been proposed that the tyrosine residue of the YAN motif could play a role in a general acid/base catalysis (Oefner *et al.*, 1990). This tyrosine residue in the class C β-lactamases is almost superimposable on S130 of the SDN motif in the class A β-lactamases.

This work was supported in part by the Fonds de la Recherche Scientifique Médicale (Contract n° 3.4537.88), an Action Concertée with the Belgian Government (Convention 86/91-90), the Fonds de Recherche de la Faculté de Médecine ULg and a Convention Tripartite between the Région Wallonne, SmithKline Beecham, U.K., and the University of Liège. G. D. is Chercheur Qualifié of the Fonds National de la Recherche Scientifique, Brussels.

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Received 4 March 1991; accepted 19 April 1991