Conserved Water Molecules Stabilize the Ω -Loop in Class A β -Lactamases[†]

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A set of 49 high-resolution (≤ 2.2 Å) structures of the TEM, SHV, and CTX-M class A β -lactamase families was systematically analyzed to investigate the role of conserved water molecules in the stabilization of the Ω -loop. Overall, 13 water molecules were found to be conserved in at least 45 structures, including two water positions which were found to be conserved in all structures. Of the 13 conserved water molecules, 6 are located at the Ω -loop, forming a dense cluster with hydrogen bonds to residues at the Ω -loop as well as to the rest of the protein. This layer of conserved water molecules is packed between the Ω -loop and the rest of the protein and acts as structural glue, which could reduce the flexibility of the Ω -loop. A correlation between conserved water molecules and conserved protein residues could in general not be detected, with the exception of the conserved water molecules at the Ω -loop. Furthermore, the evolutionary relationship between the three families, derived from the number of conserved water molecules, is similar to the relationship derived from phylogenetic analysis.

β -Lactamases (EC 3.5.2.6) hydrolyze the β -lactam ring and therefore are the main cause for resistance against β -lactam antibiotics. A sequence-based classification scheme was initially proposed by Ambler (1) and soon thereafter was extended by others (14, 28). Class A β -lactamases include the plasmid-borne TEM, SHV, and CTX-M β -lactamases, which are found most commonly in gram-negative bacteria. The TEM and SHV β -lactamase families are closely related (with a sequence identity of 67%), while the CTX-M family is more distant (with a sequence identity of 40% to TEM and SHV) (36). The members of the TEM and SHV families differ by only one to seven amino acids, while the members of the CTX-M family are more diverse (80% sequence identity) (13). Because of their importance in antibiotic resistance, the enzymes of class A β -lactamases are well studied and characterized (2, 6, 10, 20, 21, 30), and a vast amount of crystallographic data is available. While the sequence similarity between the protein families is moderate, the structures of all TEM, SHV, and CTX-M β -lactamases show a remarkably high similarity. The enzymes are composed of two domains that are closely packed together: an all- α domain, consisting of eight α -helices, and an α/β domain, consisting of three α -helices and five β -sheets (15). The active site cavity is part of the interface between the two domains and is limited by the Ω -loop. The Ω -loop (residues 161 to 179) is a conserved structural element in all class A β -lactamases and is directly involved in the catalytic reaction of the enzymes because it positions the general base Glu166. Its conformation is anchored by a highly conserved salt bridge formed between Arg164 and Asp179 (19, 37, 38). However, the long Ω -loop has only a few contacts with the

rest of the protein and was therefore speculated to be a flexible element (20). Indeed, a molecular dynamics study (32) attributes some flexibility to a part of the Ω -loop. However, from a recently published nuclear magnetic resonance study of the TEM-1 β -lactamase, it is evident that the protein is one of the most ordered proteins studied by high-resolution nuclear magnetic resonance to date, and there was no indication of increased flexibility of the Ω -loop (34).

One reason for the low flexibility of the Ω -loop in the absence of protein-protein interaction could be stabilization by water molecules. Water is known to have a crucial role in protein structure, flexibility, and activity (9, 12, 22, 31). Water molecules bind via hydrogen bonds to the side chain and backbone atoms of proteins (29), to the polar atoms of substrates and ligands (18), and to other water molecules. This enables water to mediate protein-protein and protein-ligand contacts and to take part in enzyme catalysis. X-ray crystallography has long been used to analyze water at protein surfaces, since crystal structures determined at high resolution provide a detailed picture of protein hydration (25). Comparative studies of crystal structures have shown that there are water binding sites on the surface and in the interior of a protein which are occupied by a water molecule in different crystal structures. Cluster analysis and density-based approaches have been used successfully for the identification and analysis of these conserved waters in multiple crystal structures of a protein or in crystal structures of a protein family (4, 5, 24, 27, 33).

For class A β -lactamases, crystallographic data support the conservation of at least one water molecule in the active site which is known to be essential to the enzymatic reaction (21). But so far, little is known about conserved water molecules (CWMs) outside of the active site. In the present study, we systematically analyzed high-resolution crystal structures of the evolutionarily related TEM, SHV, and CTX-M class A β -lactamase families to determine conserved water molecules and to investigate their role in protein structure and especially the stabilization of the Ω -loop.

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FIG. 1. The number of water molecules (y axis) found to be conserved in a given number of crystal structures (x axis).

MATERIALS AND METHODS

Data preparation. Crystal structures of the TEM, SHV, and CTX-M β -lactamase families were retrieved from the Protein Data Bank (PDB) (3). β -Lactamase variants with core-disrupting mutations and β -lactamases bound to inhibitory proteins were excluded. Only structures with an atomic resolution of 2.2 Å or higher were included, as 90% of the available β -lactamase structures have a resolution of 2.2 Å or higher, and it was found that the amount of crystal water depends on the atomic resolution of the crystal structure (7). With this cutoff, only a few structures with a lower resolution are excluded, and not much information is lost, as the excluded structures have fewer water molecules. PDB entries containing more than one β -lactamase were preprocessed to extract the coordinates of single proteins including water molecules within a radius of 6 Å around the protein. This preprocessing was necessary for most of the CTX-M crystal structures. Thus, 25 protein structures of the TEM β -lactamase family (PDB entries 1AXB, 1BT5, 1BTL, 1ERM, 1ERO, 1ERQ, 1ESU, 1FQG, 1JWJ, 1JWP, 1JWV, 1JWZ, 1LHY, 1LI0, 1LI9, 1M40, 1NXY, 1NY0, 1NYM, 1NYY, 1TEM, 1XPB, 1YT4, 1ZG4, 1ZG6), 10 protein structures of the SHV β -lactamase family (PDB entries 1N9B, 1ONG, 1Q2P, 1RCJ, 1SHV, 1TDG, 1TDL, 1VM1, 2A3U, 2A49), and 14 protein structures of the CTX-M β -lactamase family (PDB entries 1YLJ, 1YLP, 1YLT, 1YLW, 1YLY, 1YLZ, 1YMI, 1YMS, 1YMX) were analyzed. A detailed table of all 49 structures with their original references, information on atomic resolution, free R factor, number of water molecules, mutations, and active site occupancy is available in the supplemental material.

Identification of conserved water molecules. All structures including the crystal water molecules were superimposed by multiple-structure fitting of the C_{α} atoms, using the McLachlan algorithm (23) as implemented in the ProFit program (version 2.5.4; A. C. R. Martin [http://www.bioinf.org.uk/software/profit]). The subsets of atoms to be considered in the fitting process were initially determined by a Needleman-Wunsch sequence alignment and then iteratively updated during the fitting process. Subsequently, a complete linkage hierarchical cluster analysis of the water molecules in all 49 superimposed structures was performed using WatCH software (33). The clustering process groups together water molecules from different structures that physically overlap and represent one specific water position. The default cutoff value of 2.4 Å for the maximum interwater distance was used. With this cutoff value, water molecules with a center-to-center distance of 2.4 Å will overlap by 50% if an approximate effective radius of 1.6 Å is assumed. It ensures that clusters do not contain several water molecules from the same crystal structure. The cluster analysis identifies water molecule positions with a conservation rate ranging from 0.02% (found in 1 out of 49 structures) to 100% (found in all structures). Water positions found to be occupied in at least 45 out of 49 analyzed structures (conservation of >90%) were named CWMs. The probability of finding a CWM under the assumption of a random distribution of water molecules (49 X-ray structures with 400 crystal water molecules each) to 1,900 water sites is on the order of 10^{-22} ; thus, the identification of CWMs is highly significant.

Visualization of results and identification of hydrogen bonding partner. Visualization of the results and preparation of the figures were done using PyMOL

(8) and LIGPLOT (39) software. Hydrogen bonding partners of the CWMs were identified for each family by determining protein nitrogen and oxygen atoms as well as other CWMs within a distance of 4.0 Å in all structures of the respective family. To avoid bias due to small differences in the individual structures, only hydrogen bonds that were detected in more than 90% of the structures were further considered. The conservation of hydrogen bonding partners was derived from the multiple sequence alignment obtained by T-COFFEE (26) of all 49 proteins (see the supplemental material). To identify crystal contacts mediated by CWMs, all structures were analyzed for hydrogen bonds between CWMs and atoms of symmetry-related proteins.

RESULTS

Identification of conserved water molecules in class A β -lactamase crystal structures. Forty-nine crystal structures from three class A β -lactamase families with a resolution of at least 2.2 Å were superimposed and analyzed for CWMs: 25 structures from the TEM family, 10 structures from the SHV family, and 14 structures from the CTX-M family. In 32 of the 49 structures, an inhibitor or substrate molecule is bound to the active site. The number of crystal water molecules in the structures of the TEM and SHV families ranges from 63 to 566 and 81 to 414, with an average of 271 and 280, respectively. The structures of the CTX-M family contain more crystal water molecules: 304 to 489, with an average of 408. Subsequent cluster analysis resulted in a total of 1,993 water molecule positions. Only 13 of them were found in at least 45 out of 49 analyzed structures (Fig. 1) and were named CWM1 to CWM13 (Table 1), including two water positions which were found to be conserved in all analyzed structures.

CWMs are found everywhere in the protein structure and can be assigned to two groups (Fig. 2). Group 1 consists of six CWMs interacting with the Ω -loop. Group 2 comprises the remaining seven CWMs, which are located in the interior of the protein and at the surface of the enzyme. None of the CWMs of group 1 is involved in forming crystal contacts between symmetry-related proteins. Even those CWMs of group 2 that are located at the protein surface mediate crystal contacts in only a minor fraction of the analyzed structures.

Conserved water stabilizing the Ω -loop (group 1). Six CWMs interact with the Ω -loop and are located in a narrow,

TABLE 1. Water molecules with more than 90% conservation in all crystal structures^a

CWM	Group	No. (%) of structures in which the CWM is conserved	No. of structures per family in which the CWM is conserved			Hydrogen bonding partner(s) to the CWM		
			TEM (<i>n</i> = 25)	SHV (<i>n</i> = 10)	CTX-M (<i>n</i> = 14)	TEM	SHV	CTX-M
1	2	49 (100.00)	25	10	14	N, O Pro67 N, O Thr265 O Arg244	N, O Pro67 N, O Leu265 O Arg244	N, O Pro67 N, O Phe265
2	1	49 (100.00)	25	10	14	N Met68 N Met69 O Leu169 O Asp179 O Ala172	N Met68 N Met69 O Leu169 O Asp179 O Ala172	OD1, ND2 Asn245 N Met68 N Cys69 O Leu169 O Asp179 O Ala172 N Pro174 OG1 Thr266
3	1	48 (97.96)	24	10	14	OG1 Thr266	NE, NH2 Arg266 OD1, OD2 Asp267 NH1, NH2 Arg164	NH2 Arg164 OG1 Thr171 N Ala172 N Ile173 OD2 Asp176 NH2 Arg164 O Leu169 N, OG1 Thr171 N Ala172 N, O Ile173 N, O Asp176
4	1	48 (97.96)	24	10	14	NH2 Arg164 N Ala172 N Ile173 OD2 Asp176	N Ala172 N Leu173 OD2 Asp176	NH2 Arg164 OG1 Thr171 N Ala172 N Ile173 OD2 Asp176 NH2 Arg164 O Leu169 N, OG1 Thr171 N Ala172 N, O Ile173 N, O Asp176
5	1	48 (97.96)	24	10	14	O Leu169 N Glu171 N Ala172 O Ile173 N, O Asp176	O Leu169 N Glu171 N Ala172 N, O Leu173 O Asp176 NH2 Arg266	O Leu169 N, OG1 Thr171 N Ala172 N, O Ile173 N, O Asp176
6	1	48 (97.96)	24	10	14	O Leu169 N Glu171 N Ala172 O Ile173 N, O Asp176	O Leu169 N Glu171 N Ala172 N, O Leu173 O Asp176 NH2 Arg266	O Leu169 N, OG1 Thr171 N Ala172 N, O Ile173 N, O Asp176
7	2	48 (97.96)	24	10	14	O Lys192 O Leu193 N, O Gly196 N Leu198 N, O Leu199 NE Arg204	O Lys192 O Leu193 N Ser196 N Gln197 N Arg198 N Leu199 OE1, NE2 Gln204	O Gln192 O Leu193 O Gly196 N His197 N Ala198 N, O Leu199
8	2	47 (95.92)	23	10	14	N Leu91	N Leu91 OE1, OE2 Glu92 NH1, NH2 Arg164	O Lys88 N Leu90 N Leu91
9	1	47 (95.92)	24	9	14	O Asp176 O Arg178 O Asp101 ND2 Asn136 O, OG Ser124 N Ala125 OG1 Thr128	O Asp176 O Arg178 O Asp101 ND2 Asn136 O Ala124 N Ala125 OG1 Thr128	O Asp176 O Arg178 O Asp101 ND2 Asn136 N Ala125
10	2	47 (95.92)	25	8	14	N Pro62 N Glu63 N Glu64 N Arg61	N Ala62 N Asp63 N Glu64 N Arg61	N Gly62 N Asp63 N Glu64 N Arg61
11	2	46 (93.88)	22	10	14			
12	2	46 (93.88)	23	9	14			
13	2	46 (93.88)	23	9	14			

^a The table lists the overall water molecule conservation; the conservation in the TEM, SHV, and CTX-M families; and the protein hydrogen bonding partners.

tunnel-shaped cavity lined by the Ω -loop (residues 164 to 179) and residues 65 to 69, which form a loop near the catalytic Ser70 and Lys73 residues. The CWMs 2, 3, 4, 5, 6, and 9 form a hydrogen bond network between themselves, the residues of the Ω -loop, and the loop near the catalytic Ser70 (Fig. 3 and 4). The structure of TEM-64 (PDB entry 1JWZ) is the only structure that does not contain any of the conserved Ω -loop water molecules besides CWM2.

CWM2 is one of the two water molecules that are conserved in all structures. It forms four hydrogen bonds to backbone atoms: two with residues of the Ω -loop (Leu169 and Asp179) and two with residues of the protein core (Met68 and Met/

Cys69), as well as an additional hydrogen bond to the adjacent CWM5. CWM5 forms three hydrogen bonds to the backbone atoms of the Ω -loop (Leu169, Glu/Thr171, and Ala172) and two hydrogen bonds to the adjacent CWM4 and CWM9. In the CTX-M family, additional hydrogen bonds are formed to side chain atoms (Arg164 and Thr171). CWM4 forms four hydrogen bonds to residues of the Ω -loop: two to backbone atoms (Ala172 and Ile/Leu173) and two to side chain atoms (Arg164 and Asp176). An additional hydrogen bond is formed to the adjacent CWM9. In the CTX-M family, the side chain of Thr171 is also a hydrogen bond partner. CWM9 forms two hydrogen bonds to backbone atoms of the Ω -loop (Asp176 and

FIG. 2. Water molecules conserved in more than 90% of all analyzed structures, shown together with a representative structure of the TEM β -lactamase family (gray). The Ω -loop is highlighted in blue, the active Ser70 is shown as sticks, and the CWMs are shown as red spheres numbered 1 to 13 according to Table 1.

Arg178) and additional hydrogen bonds to the adjacent CWM4, 5, and 6. In the SHV family, also side chain atoms of Arg164 are hydrogen bond partners. CWM6 forms two hydrogen bonds to residues of the Ω -loop: two to backbone atoms (Ile/Leu173 and Asp176) and, in the SHV family, one additional hydrogen bond to the side chain atoms of Arg266. CWM3 forms hydrogen bonds to the backbone atoms of the Ω -loop (Ala172 in all families and additionally Pro174 in the CTX-M family) and to the side chain atoms of the protein core (Thr266 in the TEM and CTX-M families and Arg266 and Asp267 in the SHV family) and with a water molecule. In the TEM and CTX-M families, this water molecule is located at a position at which, in the SHV family, an arginine side chain

(Fig. 5a) is located. This water molecule is conserved inside TEM and CTX-M to 52% and 100%, respectively. In contrast, in SHV, this water molecule is located at a position where, in TEM and CTX-M, a threonine side chain is located (Fig. 5b) and is conserved to 100% inside this family.

Conserved water molecules not associated with the Ω -loop (group 2). Of the remaining seven CWMs, three are located in the protein interior and are involved in the stabilization of other loop regions. CWM1 is buried in the inside of the protein and is conserved in all structures (Fig. 6a). It forms hydrogen bonds to backbone atoms (Pro67 in all families, Thr/Leu/Phe265 in TEM/SHV/CTX-M, and Arg244 in TEM/SHV) and, in CTX-M, to the side chain atoms of Asn245. It bridges the

FIG. 3. The Ω -loop (residues 164 to 179, blue) and the rest of the protein (residues 65 to 70, gray) form the two outer layers of a sandwich-like structure (highlighted in light green) with a layer of six highly conserved water molecules between them, serving as structural glue. Front (a) and side (b) views are shown. CWMs are numbered according to those listed in Table 1.

FIG. 4. Schematic view of the interactions formed by the CWMs located at the Ω -loop. The CWMs (CWM2-6 and CWM9, cyan) form a hydrogen bond network (green dashed lines) between themselves, the Ω -loop (residues 164 to 179), and the rest of the protein (residues 68, 69, and 266).

loop of residues 65 to 69 with the second β -strand in the α/β domain. CWM10 forms two hydrogen bonds: one to the carbonyl of Asp101 and one to the side chain of Asn136 (Fig. 6b), and it links the long loop of residue 91 to 119 to the helix on which Asn136 is located. Asp101 and Asn136 are completely conserved in the three families. CWM7 is located inside a short loop region between two perpendicular helices (Fig. 6c). This turn is stabilized by hydrogen bonds to the backbone atoms of the loop residues 195 to 201.

Four CWMs are located at the surface of the enzyme. CWM8 forms one hydrogen bond to the backbone nitrogen of Leu91 in all families. Leu91 is the only conserved amino acid in the loop region between residues 87 and 99. The structures of the CTX-M family have more hydrogen bonding partners, as

their loop conformation is distinct from the structures of the TEM and SHV families. In eight structures of the SHV family, CWM8 is involved in a crystal contact to Arg222 of a symmetry-related protein. CWM11 is situated at the bottom of a small dent at the protein surface, which is formed by the residues 124, 125, and 128. Hydrogen bonds are formed primarily to backbone atoms but also to side chain atoms of these residues. An additional hydrogen bond is formed to a water molecule which is conserved in 44 of 49 structures and also occupies the dent. In two TEM structures, CWM11 forms a crystal contact to Glu104. CWM12 is located at the N-terminal helix and forms hydrogen bonds to backbone atoms of residues 62 to 64. CWM12 forms no crystal contacts. CWM13 is located 4.8 Å away from CWM12 and forms one hydrogen bond to the back-

FIG. 5. CWM3 (red sphere) forms hydrogen bonds with the carbonyl atom of Ala172 on the Ω -loop, with the side chain atom of the protein core (Thr266 in TEM and CTX-M, and Arg266 in SHV), and with a water molecule (blue sphere). (a) In TEM and CTX-M, this water molecule is located at a position at which, in SHV, an arginine side chain is located. (b) In contrast, in SHV, this water molecule is located at a position at which, in TEM and CTX-M, a threonine side chain is located.

bone atom of Arg61. Crystal contacts are formed to His96 and Tyr97 in 21 structures of the TEM family.

DISCUSSION

Identification of conserved waters. Conserved waters were identified in a set of 49 high-resolution crystal structures of three class A β -lactamase families. Despite being diverse in sequence, all class A β -lactamases have a similar structure which allowed complete linkage cluster analysis to be applied after all structures were superpositioned into a common reference frame. Compared to the method of visual inspection, computational methods have the advantage of speed and objectivity when conserved waters in the crystal structures of related proteins are studied (5). A total of 13 water molecules were conserved in more than 90% of the structures, including the two water molecules present in all structures. About the same number of conserved waters was found in previous studies of different proteins (5, 24, 27, 33), despite the fact that those studies differ in the method applied, the number of structures, and their resolution. In general, the number of water molecules in crystallographic models increases with resolution (7), while crystallization conditions have only a moderate effect (22). For a reliable analysis of conserved waters, it is therefore desirable to investigate a large number of highly resolved structures derived from proteins crystallized under different conditions. It has been shown that artifacts due to crystal contacts can be reduced by comparing structures from different space groups (5, 16). The 49 β -lactamase structures investigated here have been crystallized in two different space groups ($P2_12_12_1$ and $P2_1$). Thus, the influence of crystal contacts on conservation can be excluded for most CWMs, with the exception of CWM8, CWM11, and CWM13 at the enzyme surface, which form crystal contacts in some structures.

Stability of the Ω -loop. Besides the four CWMs that are located at the protein surface, all other CWMs are associated with loops. Nearly half of all CWMs (6 out of 13) are present in the catalytically relevant Ω -loop. From the presence of this large number of highly CWMs, we conclude that it is this

conserved water cluster that stabilizes the Ω -loop and links it to the protein. The occurrence of CWMs especially in loop regions is in accordance with the results of previous studies of other proteins that have shown the involvement of conserved waters in positioning loops to β -strands (17), the stabilization of hairpin structures (17), and the stabilization of a twisted β -turns (27). This is further supported by a statistical analysis of high-resolution protein structures which concluded that well-resolved internal water molecules preferentially reside near residues that are not part of an α -helix or a β -strand and help to satisfy the intramolecular hydrogen bond needs of backbone atoms (29). Indeed, in our study, the large majority of all hydrogen bonds formed by CWMs at the Ω -loop involve backbone atoms. The Ω -loop and the rest of the protein form the two outer layers of a sandwich-like structure, with a CWM layer between them serving as a structural glue which could reduce flexibility of the Ω -loop. A stabilization of protein interfaces by water-mediated hydrogen bonds has been reported for many protein-protein complexes (see reference 24 and references therein). Acting as the structural glue by hydrating the main chain atoms of turns, loops, and coils and, thus, stabilizing loops seems to be the key structural role of CWMs in β -lactamases.

One of the Ω -loop-stabilizing waters (CWM4) is hydrogen bonded to the N_ϵ of the Arg164 side chain and the carboxyl oxygen of Asp176. We therefore speculate that this interaction can have an additional stabilizing effect on the Ω -loop in the TEM, SHV, and CTX-M wild-type β -lactamases, as it links together the two opposed residues. It has been shown that upon disruption of the Arg164-Asp179 salt bridge via substitutions by uncharged amino acids, the structure of the Ω -loop breaks down and more bulky substrates can bind to the active site (37), which leads to the extended-spectrum β -lactamases activity of these mutants. Indeed, a conformational change of the Ω -loop in the region between residues 167 and 175 is seen in the crystal structure of the variant TEM-64, where the salt bridge is disrupted by an Arg164Ser substitution. In this structure, a part of the Ω -loop moves up to 4.5 Å, and thus, the

FIG. 6. Loop-stabilizing CWMs. (a) CWM1 (red sphere) is buried in the inside of the protein and forms hydrogen bonds to the main chain atoms of residues 67 and 265. The Ω -loop is shown in blue. (b) CWM10 (red sphere) forms hydrogen bonds to the carbonyl atom of Asp101 and to the side chain nitrogen atom of Asn136. Both residues are 100% conserved throughout the three families. (c) CWM7 (red sphere) is situated in a small loop comprising residues 195 to 201. The loop region forms a turn of more than 90 degrees between two helices.

active site is enlarged (40). Interestingly, the crystal structure of TEM-64 is the only structure in our analysis that contains none of the water molecule CWM3, 4, 5, and 6. We attribute this to the additional change in backbone conformation of residues 171 to 175 which displaces the four CWMs.

Conserved waters and conserved amino acids. Previously, it was concluded that residues forming a conserved water binding site are generally conserved (5, 17). In class A β -lactamases, the situation is more ambiguous. Most hydrogen bonds of the CWMs are formed to main chain atoms; thus, side chains could be variable as long as substitutions do not disturb the local backbone geometry. Nevertheless, there seems to be no correlation between CWMs and residue conservation. Nearly the same number of main chain interactions to residues conserved in all families and to residues conserved only inside each family were found. Often, the same CWMs can interact with conserved as well as with nonconserved residues. However, the situation at the Ω -loop is different. Most of the residues that interact with the CWMs are conserved throughout all families, with the exception of residues 69, 171, and 173. A high conservation at the amino-acid-sequence level is compulsory in protein-water interaction when side chain atoms are involved. CWM4 at the Ω -loop and CWM10 are hydrogen bonded to the side chains of Asp176 and Asn136, respectively, which are conserved in all of the structures analyzed. Nevertheless, we also observed the substitution of a residue which is involved in side chain interactions with a CWM. The Ω -loop-associated CWM3 is the center of a hydrogen bond network to residues 172 and 266 and a water molecule. While the orientation of residue 266 depends on the type of side chain, the structure of this hydrogen bond network is conserved: upon replacing Thr266 with arginine, the side chain and the water molecule change places, while the positions of CWM3 and of residue 172 are maintained. The role of conserved waters in maintaining hydrogen bond networks has been demonstrated previously for protein-protein interfaces and protein-ligand and protein-cofactor binding (4, 35, 41). The hydrogen bond network of CWM3 is an example of an intraprotein network which is stable upon side chain substitution between protein families.

Evolutionary aspects of conserved waters. It has been proposed to treat the conservation of protein-bound water analogously to the conservation of amino acid position in a multiple sequence alignment (5). Likewise, by analogy to sequence motifs, which often denote structurally or functionally important residues and are derived from multiple sequence alignments, motifs of CWMs could be defined from comparative crystal structure analysis. In the case of the class A β -lactamases, the cluster of Ω -loop-associated CWMs would justify for such a CWM motif, as it has the function of stabilizing the Ω -loop, and it is found in all of the analyzed structures of the class A β -lactamase families, except for the structure with the changed Ω -loop conformation.

As a consequence of the analogy between conserved waters and conserved residues, phylogenetic analysis based on conserved waters can be performed. The three class A β -lactamase families analyzed in this work have similar structures but only moderate sequence similarities. Phylogenetic analysis has shown that the CTX-M family diverged earlier in evolution than the SHV and TEM families (11). A similar evolutionary relationship is observable when looking at the number of water

molecules that are more conserved between the TEM and SHV families than those of the CTX-M family. The structures of the TEM and SHV families have in common five conserved waters which are not present in the structures of the CTX-M family. Between the CTX-M and the TEM families, two water molecules are highly conserved which are not found in the SHV family, and between the SHV and CTX-M families, only one water molecule is conserved. The more distant relationship of CTX-M is further supported by analyzing the differences between the families. Water molecules that are conserved by more than 90% in a specific family but not found in the other families indicate how far the families have diverged from each other. The TEM, SHV, and CTX-M families have 5, 7, and 45 uniquely conserved water molecules, respectively. These molecules are located mostly at the surface of the protein, and their occurrence in only one family reflects changes of the local environment in that particular region of the protein structure. We hope that future comparative studies of other protein families will provide further insight into the evolutionary aspects of water conservation.

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REFERENCES

- Ambler, R. P. 1980. The structure of β -lactamases. *Philos. Trans. R. Soc. Lond. B* **289**:321–331.
- Babic, M., A. M. Hujer, and R. A. Bonomo. 2006. What's new in antibiotic resistance? Focus on beta-lactamases. *Drug Resist. Updat.* **9**:142–156.
- Berman, H. M., J. Westbrook, Z. Feng, G. Gilliland, T. N. Bhat, H. Weissig, I. N. Shindyalov, and P. E. Bourne. 2000. The Protein Data Bank. *Nucleic Acids Res.* **28**:235–242.
- Bottoms, C. A., P. E. Smith, and J. J. Tanner. 2002. A structurally conserved water molecule in Rossmann dinucleotide-binding domains. *Protein Sci.* **11**:2125–2137.
- Bottoms, C. A., T. A. White, and J. J. Tanner. 2006. Exploring structurally conserved solvent sites in protein families. *Proteins* **64**:404–421.
- Bradford, P. A. 2001. Extended-spectrum β -lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin. Microbiol. Rev.* **14**:933–951.
- Carugo, O., and D. Bordo. 1999. How many water molecules can be detected by protein crystallography? *Acta Crystallogr. Sect. D* **55**:479–483.
- DeLano, W. L. 2002. The PyMOL molecular graphics system. DeLano Scientific, San Carlos, CA.
- Despa, F. 2005. Biological water: its vital role in macromolecular structure and function. *Ann. N. Y. Acad. Sci.* **1066**:1–11.
- Fisher, J. F., S. O. Meroueh, and S. Mobashery. 2005. Bacterial resistance to β -lactam antibiotics: compelling opportunism, compelling opportunity. *Chem. Rev.* **105**:395–424.
- Hall, B. G., and M. Barlow. 2004. Evolution of the serine β -lactamases: past, present and future. *Drug Resist. Updat.* **7**:111–123.
- Helms, V. 2007. Protein dynamics tightly connected to the dynamics of surrounding and internal water molecules. *Chemphyschem* **8**:23–33.
- Jacoby, G. A. 2006. β -Lactamase nomenclature. *Antimicrob. Agents Chemother.* **50**:1123–1129.
- Jaurin, B., and T. Grundstrom. 1981. *ampC* cephalosporinase of *Escherichia coli* K-12 has a different evolutionary origin from that of β -lactamases of the penicillinase type. *Proc. Natl. Acad. Sci. USA* **78**:4897–4901.
- Jelsch, C., F. Lenfant, J. M. Masson, and J. P. Samama. 1992. β -Lactamase TEM1 of *E. coli*. Crystal structure determination at 2.5 Å resolution. *FEBS Lett.* **299**:135–142.
- Loris, R., U. Langhorst, S. De Vos, K. Decanniere, J. Bouckaert, D. Maes, T. R. Transue, and J. Steyaert. 1999. Conserved water molecules in a large family of microbial ribonucleases. *Proteins* **36**:117–134.
- Loris, R., P. P. G. Stas, and L. Wyns. 1994. Conserved waters in legume lectin crystal structures. The importance of bound water for the sequence-structure relationship within the legume lectin family. *J. Biol. Chem.* **269**:26722–26733.
- Lu, Y., R. Wang, C. Y. Yang, and S. Wang. 2007. Analysis of ligand-bound water molecules in high-resolution crystal structures of protein-ligand complexes. *J. Chem. Inf. Model.* **47**:668–675.
- Majiduddin, F. K., and T. Palzkill. 2003. An analysis of why highly similar enzymes evolve differently. *Genetics* **163**:457–466.
- Massova, I., and S. Mobashery. 1998. Kinship and diversification of bacterial penicillin-binding proteins and β -lactamases. *Antimicrob. Agents Chemother.* **42**:1–17.
- Matagne, A., J. Lamotte-Brasseur, and J. M. Frere. 1998. Catalytic properties of class A β -lactamases: efficiency and diversity. *Biochem. J.* **330**:581–598.
- Mattos, C. 2002. Protein-water interactions in a dynamic world. *Trends Biochem. Sci.* **27**:203–208.
- McLachlan, A. D. 1982. Rapid comparison of protein structures. *Acta Crystallogr. Sect. A* **38**:871–873.
- Mustata, G., and J. M. Briggs. 2004. Cluster analysis of water molecules in alanine racemase and their putative structural role. *Protein Eng. Des. Sel.* **17**:223–234.
- Nakasako, M. 2004. Water-protein interactions from high-resolution protein crystallography. *Philos. Trans. R. Soc. Lond. B* **359**:1191–1204.
- Notredame, C., D. G. Higgins, and J. Heringa. 2000. T-Coffee: a novel method for fast and accurate multiple sequence alignment. *J. Mol. Biol.* **302**:205–217.
- Ogata, K., and S. J. Wodak. 2002. Conserved water molecules in MHC class-I molecules and their putative structural and functional roles. *Protein Eng. Des. Sel.* **15**:697–705.
- Ouellette, M., L. Bissonnette, and P. H. Roy. 1987. Precise insertion of antibiotic resistance determinants into Tn21-like transposons: nucleotide sequence of the OXA-1 β -lactamase gene. *Proc. Natl. Acad. Sci. USA* **84**:7378–7382.
- Park, S., and J. G. Saven. 2005. Statistical and molecular dynamics studies of buried waters in globular proteins. *Proteins* **60**:450–463.
- Poole, K. 2004. Resistance to β -lactam antibiotics. *Cell. Mol. Life Sci.* **61**:2200–2223.
- Prabhu, N., and K. Sharp. 2006. Protein-solvent interactions. *Chem. Rev.* **106**:1616–1623.
- Roccatano, D., G. Sbardella, M. Aschi, G. Amicosante, C. Bossa, A. Di Nola, and F. Mazza. 2005. Dynamical aspects of TEM-1 β -lactamase probed by molecular dynamics. *J. Comput. Aided Mol. Des.* **19**:329–340.
- Sanschagrin, P. C., and L. A. Kuhn. 1998. Cluster analysis of consensus water sites in thrombin and trypsin shows conservation between serine proteases and contributions to ligand specificity. *Protein Sci.* **7**:2054–2064.
- Savard, P. Y., and S. M. Gagne. 2006. Backbone dynamics of TEM-1 determined by NMR: evidence for a highly ordered protein. *Biochemistry* **45**:11414–11424.
- Sharrow, S. D., K. A. Edmonds, M. A. Goodman, M. V. Novotny, and M. J. Stone. 2005. Thermodynamic consequences of disrupting a water-mediated hydrogen bond network in a protein:pheromone complex. *Protein Sci.* **14**:249–256.
- Tzouvelekis, L. S., E. Tzelepi, P. T. Tassios, and N. J. Legakis. 2000. CTX-M-type β -lactamases: an emerging group of extended-spectrum enzymes. *Int. J. Antimicrob. Agents* **14**:137–142.
- Vakulenko, S. B., P. Taibi-Tronche, M. Toth, I. Massova, S. A. Lerner, and S. Mobashery. 1999. Effects on substrate profile by mutational substitutions at positions 164 and 179 of the class A TEM_{PU}C19 β -lactamase from *Escherichia coli*. *J. Biol. Chem.* **274**:23052–23060.
- Vakulenko, S. B., M. Toth, P. Taibi, S. Mobashery, and S. A. Lerner. 1995. Effects of Asp-179 mutations in TEM_{PU}C19 β -lactamase on susceptibility to β -lactams. *Antimicrob. Agents Chemother.* **39**:1878–1880.
- Wallace, A. C., R. A. Laskowski, and J. M. Thornton. 1995. LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions. *Protein Eng.* **8**:127–134.
- Wang, X., G. Minasov, and B. K. Shoichet. 2002. Evolution of an antibiotic resistance enzyme constrained by stability and activity trade-offs. *J. Mol. Biol.* **320**:85–95.
- Xu, D., C. J. Tsai, and R. Nussinov. 1997. Hydrogen bonds and salt bridges across protein-protein interfaces. *Protein Eng. Des. Sel.* **10**:999–1012.