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pK_a Calculations for class A β-lactamases: Influence of substrate binding

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Abstract

β-Lactamases are responsible for bacterial resistance to β-lactams and are thus of major clinical importance. However, the identity of the general base involved in their mechanism of action is still unclear. Two candidate residues, Glu166 and Lys73, have been proposed to fulfill this role. Previous studies support the proposal that Glu166 acts during the deacylation, but there is no consensus on the possible role of this residue in the acylation step. Recent experimental data and theoretical considerations indicate that Lys73 is protonated in the free β-lactamases, showing that this residue is unlikely to act as a proton abstractor. On the other hand, it has been proposed that the pK_a of Lys73 would be dramatically reduced upon substrate binding and would thus be able to act as a base. To check this hypothesis, we performed continuum electrostatic calculations for five wild-type and three β-lactamase mutants to estimate the pK_a of Lys73 in the presence of substrates, both in the Henri–Michaelis complex and in the tetrahedral intermediate. In all cases, the pK_a of Lys73 was computed to be above 10, showing that it is unlikely to act as a proton abstractor, even when a β-lactam substrate is bound in the enzyme active site. The pK_a of Lys234 is also raised in the tetrahedral intermediate, thus confirming a probable role of this residue in the stabilization of the tetrahedral intermediate. The influence of the β-lactam carboxylate on the pK_a values of the active-site lysines is also discussed.

Keywords: β-lactamases; mechanism; pK_a calculations; Poisson–Boltzmann electrostatics; protein modeling

β-Lactamases catalyze the hydrolysis of β-lactam antibiotics such as penicillins and cephalosporins, resulting in bacterial resistance to these compounds. The elucidation of their mechanism of action is thus of major clinical importance.

Class A β-lactamases are serine enzymes. Their mechanism involves a first recognition step leading to the formation of a Henri–Michaelis complex between the enzyme and the substrate, followed by the attack on the carbonyl carbon of the β-lactam bond by the active-site serine Oγ atom leading to a negatively charged tetrahedral intermediate, before the formation of the acylenzyme (Fig. 1).

Two residues of the active site, Glu166 and Lys73, are potential candidates to act as the general base by accepting the proton of the nucleophilic Ser70.

Gibson et al. (1990) proposed that the active-site residue Glu166 is required for both acylation and deacylation during the hydrolysis of β-lactam antibiotics. Mechanisms first proposed assumed that the proton of the active-site serine is transferred to the carboxylate of Glu166 either via a water molecule (Lamotte-Brasseur et al., 1991) or directly as a result of the flexibility of the protein (Vijayakumar et al., 1995). However, the replacement of Glu166 by an asparagine in the TEM-1 β-lactamase yielded an enzyme forming a stable acyl-enzyme complex with β-lactam antibiotics (Strynadka et al., 1992; Guillaume et al., 1997). Although acylation of the modified proteins by benzylpenicillin remained relatively fast, it was significantly impaired when compared to observations of the wild-type enzyme (Guillaume et al., 1997). The same behavior was observed in the case of the TEM-1 E166Y mutant (Delaire et al., 1991), i.e., the acylation rate is greater than the deacylation rate. But examination of the k_{cat}/K_m values showed slower acylation rates than for the wild-type enzyme. Kinetic studies of the Glu166A mutant of the *Bacillus licheniformis* β-lactamase also showed that accumulation of the acyl-enzyme could result from a more marked decrease of the deacylation compared to the acylation rate (Escobar et al., 1991).

The Glu166 residue has been shown to have a critical role in catalyzing the deacylation step in the class A β-lactamases, but there is no consensus on its role in the acylation step.

It has also been suggested that the side-chain amine of Lys73 could act as a general base, and that its buried side chain was kept

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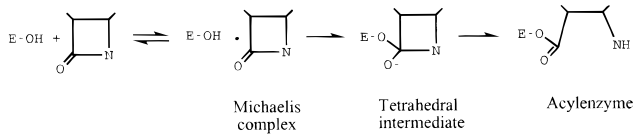

Fig. 1. Schematic representation of part of the reaction pathway between active-site serine β-lactamases and β-lactam antibiotics. E-OH is the enzyme, and the β-lactam substrate is depicted by a β-lactam ring.

in the neutral state by the active-site environment. This requires an unusually low pK_a for Lys73 in the substrate free enzyme (Strynadka et al., 1992). On the other hand, from NMR and chemical modification studies it has been concluded that the pK_a of Lys73 is above 10 (Damblon et al., 1996). Recently, continuum electrostatic calculations, in accord with the experimental data, indicated that a downward pK_a shift for Lys73 in the TEM-1 and *B. licheniformis* enzymes is very unlikely (Raquet et al., 1997b). In the mutant proteins in which Glu166 is replaced by a neutral residue, calculations predicted that the downward shift of the pK_a of Lys73 was not sufficient to generate a neutral ϵ-amino group capable of being a possible proton abstractor at physiological pH.

Finally, a substrate-induced mechanism has been proposed (Swaren et al., 1995; Zawadzke et al., 1996), in which the pK_a of Lys73 would be dramatically reduced upon substrate binding.

To estimate the pK_a of Lys73 in the presence of substrates, we have performed continuum electrostatic calculations for several wild-type and mutant β-lactamases in the absence and presence of different types of β-lactam antibiotics. In all cases, the pK_a of Lys73 was computed to be above 10, which is inconsistent with the proposal of a downward pK_a shift upon substrate binding. The influence of the β-lactam carboxylate on the pK_a values and the possible role of the active-site residues during the acylation process are also discussed.

Results

Ligand-free enzymes

Raquet et al. (1997b) computed the pK_a values for Lys73 and Lys234 in the active site of both TEM-1 and *B. licheniformis* β-lactamases to be in the range 11.1–11.7 (Table 1).

We computed the pK_a values of Lys73 and Lys234 in the *Streptomyces albus* G, *Staphylococcus aureus* PC1 and *Enterobacter cloacae* NMC-A wild-type class A β-lactamases, and again found pK_as of about 11 in all five β-lactamases (data not shown), although they have theoretically different electrostatic environments. Thus, despite being totally buried in the protein (the solvent accessible surface area of these residues is 0, Raquet et al., 1997b), the pK_as of these lysines are conserved at a value resulting in charged lysine side chains at neutral pH. This conserved property is also an indicator of the consistency and robustness of the method used in the calculations.

Henri–Michaelis complexes between TEM-1 and β-lactam compounds

The β-lactam molecules shown in Figure 2 were docked into the TEM-1 active site as described by Lamotte-Brasseur et al. (1991). The β-lactam carbonyl oxygen atom was located in the oxyanion hole formed by the backbone nitrogen atoms of Ser70 and residue 237 (Fig. 3). Hydrogen bonds were formed between the C6(C7) side-chain amide group of the β-lactam and the side-chain nitrogen atom of Asn132 on one hand and the main-chain oxygen atom of residue 237 on the other. In the case of the good substrates considered (benzylpenicillin and cephalothin), which bear a carboxylate, this group was oriented to make a weak hydrogen bond to the ϵ-amino group of Lys234. A cephalosporin lactone and, to a lesser extent, the methyl ester of benzylpenicillin have been shown to bind to β-lactamases despite the fact that they do not possess a formal anionic group on C4 or C3, respectively (Laws & Page,

Table 1. *Calculated pK_as of key residues of the TEM-1 and B. licheniformis β-lactamases in the absence and presence of ligands*[a]

β-Lactam	K73	E166	K234	Carboxyl
—	11.7	4.4	11.1	—
	11.7	*4.4*	*11.3*	
Benzylpenicillin	12.0	4.6	11.5	3.6
	12.1	*4.4*	*11.8*	*3.3*
Cephalothin	12.1	4.6	11.6	3.3
Benzylpenicillin methyl ester	11.7	4.4	11.1	—
Cephalosporin lactone	11.7	4.5	11.1	—
Benzylpenicillin tetrahedral intermediate	12.2	4.9	11.8	3.0
	12.4	*4.8*	*12.0*	*3.2*

[a] The β-lactam substrates as bound in the Henri–Michaelis complex and benzylpenicillin as in the tetrahedral intermediate. The computed pK_a values of the β-lactam carboxylic group are also given. Values for TEM-1 are given in plain text and those *for B. licheniformis* in italics.

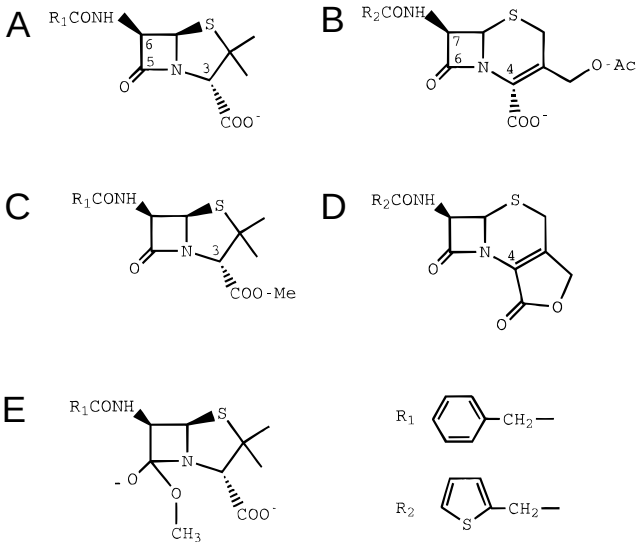

Fig. 2. Structure of the β-lactam molecules considered in the calculations: (**A**) benzylpenicillin, (**B**) cephalothin, (**C**) benzylpenicillin methyl ester, (**D**) deacetylcephalosporin lactone, and (**E**) tetrahedral intermediate with benzylpenicillin. The catalytic serine is represented by a CH_3-O group.

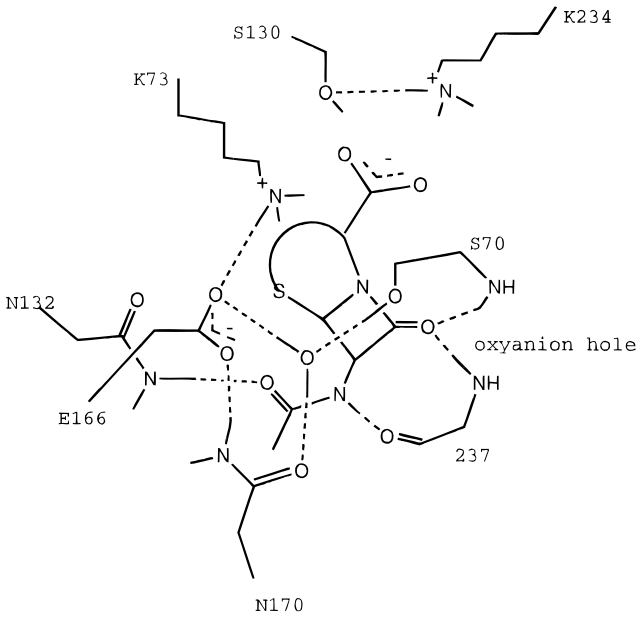

Fig. 3. Schematic diagram of the interactions in the noncovalent Henri–Michaelis complex between a native class A β-lactamase and a β-lactam antibiotic. Dashed lines represent hydrogen bonds.

1989; Varetto et al., 1991). These compounds were also considered here to evaluate the influence of the carboxylate on the pK_as.

The pK_as were computed for all titratable residues in the Henri–Michaelis complex models of the TEM-1 enzyme by the method described in Raquet et al. (1997b) for the free enzymes. The pK_as of the active-site residues are shown in Table 1. With benzylpenicillin and cephalothin, the pK_a values of the active-site lysines were computed to increase, the shift varying from 0.3–0.5 pK_a units, depending on the β-lactam and on the residue considered. With the carboxylate-free β-lactams bound, the pK_a values were unchanged when compared to those of the same residues in the free enzyme. The presence of the carboxylate thus induced an upward shift of the Lys73 and Lys234 pK_as, which could be correlated with the fact that an anionic group on C3(C4) of the antibiotics is generally thought to be essential for the effective acylation of the β-lactamases. A further upward shift of the pK_as of Glu166 and Lys234 was also calculated for the tetrahedral intermediate as described below.

Benzylpenicillin tetrahedral intermediate

In the Henri–Michaelis complex, the β-lactam carbonyl bond is polarized by the oxyanion hole and the carbonyl carbon C5 is positioned about 3 Å away from the Ser70 OH group, and is ready to undergo nucleophilic attack by the active serine. Upon formation of the Ser70 O_γ-C5 bond, a negatively charged adduct appears in which C5 adopts a tetrahedral geometry.

Recently, the crystal structure of the TEM-1 β-lactamase with a phosphonate transition state analogue bound at the active site was determined (Maveyraud et al., 1998). This work indicated movements of the Lys73 amino, Glu166 carboxyl, and Ser130 hydroxyl groups. The distance between the Lys73 N_ζ and both Ser70 O_γ and Glu166 O_ϵ atoms increased by 0.3 Å. On the other hand, the Ser130 O_γ atom became hydrogen bonded to one oxygen atom of the inhibitor, which was located in the active site at a position equivalent to that of the lactam nitrogen of benzylpenicillin. According to modeling results (Lamotte-Brasseur et al., 1991), the side chains in the active site of the *S. albus* G β-lactamase also reorient upon substrate binding. Similar modifications of the TEM-1 β-lactamase active site appeared after modeling of the tetrahedral intermediate formed with benzylpenicillin (J. Lamotte-Brasseur, unpubl. results). In addition, the hydrogen bond distance between the Lys234 ammonium group and the β-lactam carboxylate was shortened by 0.5 Å.

The calculated pK_as corresponding to this model are shown in Table 1. Lys73 and Lys234 were computed to have slightly raised pK_a values, thus indicating a possible role for Lys234 in the transition-state stabilization. This possibility was already proposed by Brannigan et al. (1991), who showed that a positively charged histidine residue could successfully replace the native Lys234 in the *S. albus* G β-lactamase. The pH dependence of the kinetic parameters demonstrated that the positive charge on residue 234 was more important for the stabilization of the tetrahedral intermediate than for the complementarity between the enzyme and the substrate ground states, which is responsible for the initial binding (Brannigan et al., 1991).

E166X β-lactamases mutants

It is now generally agreed that the Glu166 carboxylate acts as a general base catalyst during the deacylation step where an occluded water molecule is believed to be the nucleophile. This water molecule (W292 in TEM-1) is found centrally in the active sites of all wild-type β-lactamase structures determined so far. Its position is conserved, and it bridges the Glu166 and Asn170 side chains and the hydroxyl group of the essential Ser70. However, the various structural data all indicate that the "catalytic" water molecule is significantly displaced in the mutants: in the absence of the glutamic acid side chain, this molecule is displaced by about 1 Å toward Asn170 and away from Ser70, which both bridge in the native enzymes.

In addition, the Ω-loop (residues 161–179) conformation is different in some mutants. The active site is enlarged by a displacement of this Ω-loop in the TEM-1 E166Y protein (Maveyraud et al., 1996), while, according to Knox et al. (1993), the position of the Ω-loop is not strongly modified in the *B. licheniformis* E166A mutant. The situation is the same in the TEM-1 E166N mutant (Strynadka et al., 1992).

As for the wild-type enzymes, calculations were performed for ligand-free, Henri–Michaelis complexes, tetrahedral intermediates formed with benzylpenicillin for the TEM-1 E166N and E166Y, and the *B. licheniformis* E166A mutant β-lactamases. The results of these calculations are summarized in Table 2.

Compared to the pK_as calculated for the wild-type enzyme residues, three points should be noted: (1) As observed by Raquet et al. (1997b), Lys73 and Lys234 retain pK_as of about 11 in the TEM-1 E166N and *B. licheniformis* E166A ligand-free mutants. These calculations show that the pK_as of Lys73 and Lys234 are in the same range in the E166Y mutant, despite the fact that the substrate binding site is enlarged in this enzyme. (2) The pK_a of Tyr166, a titratable residue, was also calculated and found to be normal both in the free enzyme and in the complexes. (3) Substrate binding to the three mutants also induced a slight upward shift of the pK_as of the two active-site lysines.

Table 2. *pK$_a$ values computed for Lys73, residue 166 and Lys234 in TEM-1 and B. licheniformis E166X mutant enzymes, free or with benzylpenicillin as bound in the Henri–Michaelis complex or tetrahedral intermediate*

β-Lactamase		K73	X166	K234
TEM-1 E166N	Ligand	11.2	—	10.9
TEM-1 E166Y		11.4	9.2	11.0
B. licheniformis E166A	Free	11.3	—	10.9
TEM-1 E166N	Michaelis	11.5	—	11.5
TEM-1 E166Y		11.6	9.3	11.7
B. licheniformis E166A	Complex	11.5	—	11.4
TEM-1 E166N	Tetrahedral	11.8	—	11.8
TEM-1 E166Y		12.1	9.7	12.0
B. licheniformis E166A	Intermediate	11.9	—	11.8

Discussion

The pK_a of Lys73 has previously been computed to be above 10 in the free TEM-1 and *B. licheniformis* class A β-lactamases (Raquet et al., 1997b), in good agreement with the NMR results obtained with the TEM-1 β-lactamase, but in contrast to the proposal that Lys73 acts as the general base involved in the acylation step. Even in the mutant proteins where Glu166 is replaced by a neutral residue, the downward shift of the Lys73 pK_a was calculated to be insufficient to make this residue uncharged at pH 7.

Using the same continuum electrostatic model, additional calculations were carried out to check the influence of substrates. Upon binding of penicillin or cephalosporin (both as the Henri–Michaelis complex or the tetrahedral intermediate), the pK_a of the Lys73 is computed to increase. This residue is thus protonated at neutral pH, even when a β-lactam substrate is bound in the enzyme active site. This shows that Lys73 is unlikely to act as a proton abstractor.

The pK_a of Lys234 is also raised in the tetrahedral intermediate, thus confirming a possible role for this residue in the stabilization of the tetrahedral intermediate.

At neutral pH, the Glu166 and Tyr166 residues are computed to have normal protonation states even in the tetrahedral intermediate models. This result does not support a mechanism in which residue 166 would act directly as the general base during the acylation step.

The conserved water molecule is another key element of the active site. It could act as the central relay during the proton transfer from the active serine to the β-lactam ring nitrogen (Lamotte-Brasseur et al., 1991). The importance of this water molecule is highlighted by the behavior of penicillins and cephalosporins containing a methoxy group at C6 (penicillins) or C7 (cephalosporins), which acylate the active serine very slowly or not at all, while similar molecules lacking the methoxy group do so with high efficiency. Both types of β-lactams can be easily fitted into the enzyme active site but the methoxy group of the former displaces the water molecule, interrupting the proton transfer process (Matagne et al., 1993).

Some of the pK_a calculations were repeated with this crystallographically observed water molecule explicitly added as a titratable site (data not shown). These calculations indicated that on formation of the tetrahedral intermediate, the acidic pK_a of the water molecule shifted upward more than that of Glu166 (1.5–3 U vs. 0.5 U). This result can be explained by the fact that the water is located closer to the negatively charged tetrahedral intermediate than the Glu166 carboxylic group. This hypothesis would permit the proposal of a common mechanism for class A and class C β-lactamases. Indeed a conserved water molecule is also found close to the nucleophilic serine in the class C enzymes (Lobkovsky et al., 1993). It is located on the β face of the antibiotic, whereas for the class A enzymes, the water molecule is on the α face (Massova & Mobashery, 1998). Glu166 in class A β-lactamases or the conserved Tyr150 (Matagne et al., 1998) in the class C enzymes would then be important for activating the water by constraining it to the most efficient position to facilitate the proton transfer.

However, the pK_a calculation methodology used has not yet been calibrated for calculating the pK_a values of individual water molecules and this hypothesis must be considered with caution. The upward shift is not sufficient for the water molecule to become the base, but many important factors for the pK_a of this water molecule may be missing in the model. A more detailed model might show that the water molecule could be the transient intermediate proton sink, rather than Glu166. A combined QM/MM potential is probably the most suitable method to incorporate the solvent quantum effects into simulations of enzymatic systems and investigate the different roles of Lys73, Glu166, and the water molecule. Application of this method to the system described above would be of particular interest.

In conclusion, the results of the pK_a calculations presented here show that Lys73 is unlikely to act as a proton abstractor, even when a β-lactam is bound. The results also confirm a probable role of Lys234 in the stabilization of the penicillin tetrahedral intermediate. However, the results did not permit clarification of the nature of the proton abstractor during the acylation step, if a classical general base catalysis is involved in this process.

Materials and methods

Materials

Crystallographic structures from the Brookhaven Protein Data Bank were used for the TEM-1 (1XPB) (Fonzé et al., 1995), *B. licheniformis* (4BLM) (Moews et al., 1990), and *S. aureus* PC1 (3BLM) (Herzberg, 1991) wild-type β-lactamases and for the *B. licheniformis* E166A (1MBL) (Knox et al., 1993) mutant protein. Refined coordinates of the *S. albus* G (Dideberg et al., 1987) and TEM-1 E166Y (Maveyraud et al., 1996) β-lactamases were provided by Dr. O. Dideberg and L. Maveyraud, respectively.

Modeled coordinates were used for the *E. cloacae* NMC-A carbapenemase (Raquet et al., 1997a) and TEM-1 E166N mutant (Guillaume et al., 1997).

The protein structures were manipulated with Insight II (Biosym, version 97, Molecular Simulations, Inc., San Diego, California). Polar hydrogen atoms were added using Whatif (Hooft et al., 1996). The positions of the water molecules and hydrogen atoms were optimized with the AMBER united atom force field (Pearlman et al., 1995).

The University of Houston Brownian Dynamics (UHBD) program, version 6.1 (Madura et al., 1995), was used for electrostatic calculations together with supplemental utilities for pK_a calculations (Antosiewicz et al., 1996) and modifications for parametrization (Demchuk & Wade, 1996) and solution of the multiple titration site problem (Raquet et al., 1997b).

Modeling of β-lactam structures

The geometries of the antibiotic structures were optimized by the AM1 semi-empirical method, as described by Lamotte-Brasseur et al. (1991). CNDO partial atomic charges on the ligands were generally used. To check the influence of the values assigned to the partial atomic charges, AM1 and MNDO charges were also considered for the TEM-1–benzylpenicillin complexes. This led to similar results (data not shown).

Modeling of protein structures

Initial assignments of the protonation states of titratable residues were made as follows: the protonation state of histidine residues was chosen by geometrical analysis of potential hydrogen bonds. Other titratable sites were assigned their usual protonation state at pH 7.0. N- and C-termini were assumed to be protonated and deprotonated, respectively. Polar hydrogen atoms were then added to the crystal structures using the Whatif (Hooft et al., 1996) software package. Initial positions of the "catalytic" water molecules were those observed in the crystal or model structures. The positions of the water molecules and hydrogen atoms were optimized with the AMBER united atom force field (Pearlman et al., 1995) first by steepest descent energy minimization of atoms with forces above 500 kcal mol^{-1} $Å^{-1}$ and then by conjugate gradient energy minimization until the RMS gradient was less than 0.1 kcal mol^{-1} $Å^{-1}$.

Modeling of complexes

Each β-lactam structure was oriented in the enzyme active site exploiting the requirements of electrostatic, hydrophobic, and shape complementarity between enzyme and substrate, as first suggested by Herzberg and Moult (1987). This mode of binding is shown in Figure 3 and described in Results. Modeling of the benzylpenicillin tetrahedral intermediates in which the ligand is covalently linked to the active-site serine residue was done after substituting the O-CH3 group of the optimized structure (see Fig. 2E) by the O-CH2 side chain of the serine residue. The geometry of the corresponding Henri–Michaelis and tetrahedral intermediate complexes was optimized as described for the native enzyme, constraining bond lengths, angles, and ring dihedral angles to their values from the AM1 calculations.

Electrostatics calculations

Partial atomic charges on the protein and all atomic radii were assigned from the OPLS parameter sets (Jorgensen & Tirado-Rives, 1988). The representation of neutral forms of residues that are normally charged at neutral pH was modified as proposed by Demchuk and Wade (1996), so that the neutral forms of these residues could be represented with the same number of protons as their charged forms. This removed the need to model protons in energetically unfavorable positions, as would be necessary if the standard representation of the neutral forms of these residues were used. Atomic radii were scaled by 1.122 to correspond to the radii at the minimum in the Lennard–Jones potential, and hydrogen atom radii were set to zero. Dielectric constant values assigned were 78.5 for the solvent, and 15 or 78.5 for the protein. The dielectric boundary was positioned at the molecular surface derived by computing the solvent accessible surface with a probe of radius 1.0 Å for calculations with the OPLS parameters. The ionic strength of the solvent was assumed to be 150 mM and to follow a Boltzmann distribution at 298 K. Molecules were surrounded by a 2 Å thick ion exclusion layer.

Electrostatic energies were calculated by numerically solving the finite-difference linearized Poisson–Boltzmann equation, using an incomplete Cholesky preconditioned conjugated gradient method (Davis & McCammon, 1989). The electrostatic potentials were calculated by means of focusing grids on each of the titratable residues. Focusing was done in four steps. Four cubic grids with spacings of 2.5, 1.2, 0.75, and 0.25 Å and with dimensions of 60^3, 25^3, 20^3, and 20^3, respectively, were generally used.

The potential at the boundary of the outer grid was assigned, assuming each protein atom to be a Debye–Hückel sphere. Both the partial atomic charges and the dielectric boundary were discretized onto the finite-difference grids, and the dielectric constant was smoothed at the grid points adjacent to the boundary (Davis & McCammon, 1991).

pK_a Calculations

The pK_as of all the ionizable groups of each protein were estimated from solution of the multiple titration site problem at a range of pHs by assuming that the pK_a of an ionizable group at a chosen pH is given by

$$pK_a = pH - (\ln([I]/[HI]))/2.303 \quad (1)$$

where HI represents the protonated form and I the unprotonated form of the ionizable group (Raquet et al., 1997b).

For each titratable site, a model compound was defined, consisting of the whole amino acid residue in its conformation in the protein. Reference pK_a values for the model compounds and calculation protocol were those indicated in Raquet et al. (1997b). Reference pK_a value for the β-lactam carboxylate was 3.5 (Laws & Page, 1989).

The appropriate dielectric constant for the protein to obtain the best estimates of pK_as was then chosen using a criterion based on desolvation energy (Demchuk & Wade, 1996). This indicated that the most accurate pK_a values should be obtained using a protein dielectric equal to that of the solvent (78.5). When a protein dielectric constant of 15 was used, the calculated pK_as of Lys73 and Lys234 increased and decreased by about 0.7 U, respectively, but the values were in all cases above 10. The results of the calculations led to the same conclusions, i.e., that the lysines are in a protonated state.

Solvent accessibility calculations

The solvent accessible surface was determined from the protein three-dimensional structure by the algorithm of Lee and Richards (1971). A spherical probe radius of 1.0 Å was used in the calculations.

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