pK_a Calculations for Class A β-Lactamases: Methodological and Mechanistic Implications

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ABSTRACT β-lactamases are responsible for resistance to penicillins and related β-lactam compounds. Despite numerous studies, the identity of the general base involved in the acylation step is still unclear. It has been proposed, on the basis of a previous pK_a calculation and analysis of structural data, that the unprotonated Lys^{73} in the active site could act as the general base. Using a continuum electrostatic model with an improved treatment of the multiple titration site problem, we calculated the pK_a values of all titratable residues in the substrate-free TEM-1 and *Bacillus licheniformis* class A β-lactamases. The pK_a of Lys^{73} in both enzymes was computed to be above 10, in good agreement with recent experimental data on the TEM-1 β-lactamase, but inconsistent with the proposal that Lys^{73} acts as the general base. Even when the closest titratable residue, Glu^{166}, is mutated to a neutral residue, the predicted downward shift of the pK_a of Lys^{73} shows that it is unlikely to act as a proton abstractor in either enzyme. These results support a mechanism in which the proton of the active Ser^{70} is transferred to the carboxylate group of Glu^{166}.

INTRODUCTION

β-Lactamases are widespread in bacteria, where they are responsible for resistance to penicillins, cephalosporins, and related β-lactam compounds, and thus pose a major challenge to antibacterial chemotherapy. These enzymes generally catalyze the hydrolysis of the β-lactam ring amide bond, giving rise to biologically inactive products (Frère, 1995; Waley, 1992). Class A β-lactamases constitute the majority of the penicillin-destroying enzymes and belong to the superfamily of active-site serine penicillin-recognizing enzymes (Joris et al., 1988). Detailed mechanistic understanding of these enzymes can be expected to guide the design of new inhibitors and antibacterial compounds resistant to their action. Several mechanisms have already been proposed for this family of enzymes on the basis of crystallographic, molecular modeling, and mutagenesis studies.

The catalytic pathway of class A β-lactamases involves the acylation of the active serine, Ser^{70} (according to ABL numbering (Ambler et al., 1991), which is used throughout the text), followed by the hydrolysis of the ester bond formed in the first step (Waley, 1992). As in the serine proteases, a general base in the active site is expected to participate in catalysis by accepting the proton of the crucial Ser^{70} residue during the formation of a transient tetrahedral intermediate. Crystal structures indicate that there are two candidate residues that could fulfill this role, Glu^{166} and Lys^{73} (Fig. 1). Glu^{166} is a good candidate but is not within hydrogen bonding distance of the Ser^{70} hydroxyl group. The mechanisms proposed first assumed that the proton of the active-site serine is transferred to the carboxylate group of Glu^{166} 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). An alternative proposal was that the ε-amino group of the Lys^{73} residue could be unprotonated at physiological pH, resulting in an asymmetrical mechanism in which an unprotonated lysine and a glutamate play the role of the general base in the acylation and deacylation steps, respectively (Strynadka et al., 1992). Substrate-induced mechanisms have also been proposed. The first of these requires that Lys^{73} has a low pK_a value in the substrate-free enzyme that is shifted upward upon substrate binding, allowing this residue to abstract the proton from the active serine (Swarén et al., 1995). More recently, a complex mechanism of proton transfer was proposed (Ishiguro and Imajo, 1996) in which, upon binding, the carboxylate group of the substrate would abstract the proton of Ser^{130}, which in turn would be reprotonated by Lys^{73}, leaving the latter residue unprotonated and ready to abstract the proton of the active Ser^{70}.

Two of the proposed mechanisms (Strynadka et al., 1992; Swarén et al., 1995) imply an unusually low pK_a value for the ε-amino group of Lys^{73} in the substrate-free enzyme. Whereas in prior continuum electrostatic calculations the computed pK_a of Lys^{73} was 8.0 in the substrate-free TEM-1 enzyme (Swarén et al., 1995), subsequent NMR titration experiments showed that the pK_a of Lys^{73} is above 10 (Damblon et al., 1996). We here describe continuum dielectric calculations, performed with a different and improved parameterization and treatment of the multiple titration site problem which, in accord with the experimental data, indicate that a downward pK_a shift for Lys^{73} in the TEM-1 enzyme is very unlikely. The same situation holds for another class A enzyme, that from *Bacillus licheniformis*.

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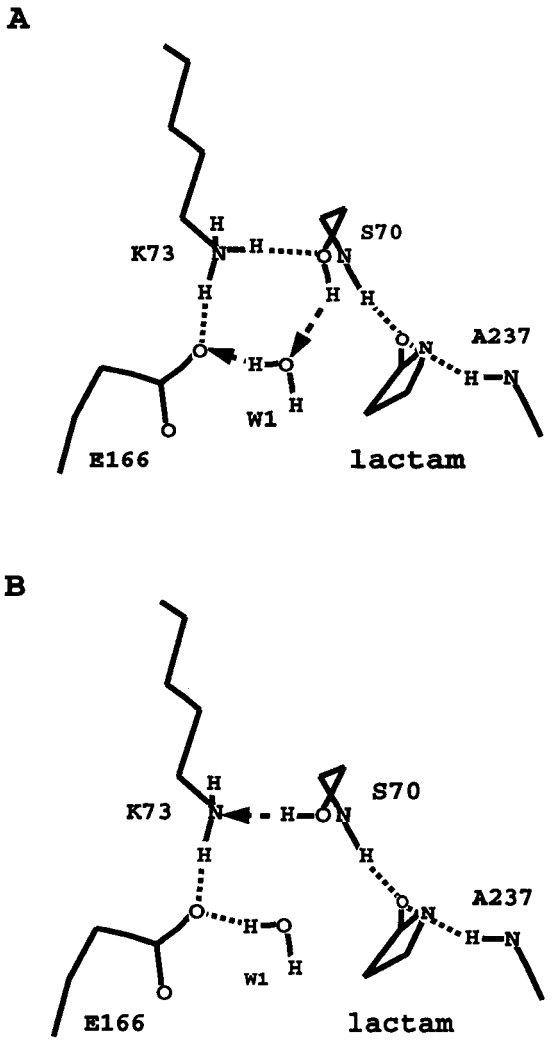

FIGURE 1 Proposed catalytic pathways for the acylation step in class A β-lactamases. The proton of the active serine (Ser^{70}) is transferred, as shown by the arrows, (*A*) to the carboxylate group of Glu^{166} via the W1 water molecule (note that protein flexibility may permit the proton to be transferred directly to Glu^{166} (see text)); (*B*) to the unprotonated Lys^{73} (note that this lysine could be unprotonated in the free enzyme or deprotonation could be induced upon substrate binding (see text)).

Moreover, even in the mutant proteins in which Glu^{166}, which is the closest titratable group to Lys^{73}, is replaced by a neutral residue, our calculations predict that the downward shift of the pK$_a$ of Lys^{73} is not sufficient to generate a neutral ϵ-amino group capable of being an alternative proton abstractor at physiological pH.

MATERIALS AND METHODS

Materials

The crystallographic structures of the *Escherichia coli* TEM-1 β-lactamase (1xpb) (Fonzé et al., 1995), *Bacillus licheniformis* β-lactamase (4blm) (Moews et al., 1990), *B. licheniformis* E166A β-lactamase mutant (1mbl) (Knox et al., 1993), *E. coli* triose phosphate isomerase (1tre) (Noble et al., 1993), *Trypanosoma brucei* triose phosphate isomerase (5tim) (Wierenga et al., 1991), bovine ribonuclease A (3rn3) (Howlin et al., 1989), hen egg white lyzosyme (2lzt) (Ramanadham et al., 1990), bovine pancreatic trypsin inhibitor (4pti) (Marquart et al., 1983), and cytochrome P450cam (2cpp) (Poulos et al., 1987) proteins were used. A model was used for the TEM-1 E166N mutant (Guillaume et al., 1997).

InsightII (Biosym, version 95; Molecular Simulations, San Diego, CA) and Quanta (Release 4.0, 1992, Molecular Simulations) molecular modeling software packages were used for manipulation of protein structures and addition of the polar hydrogen atoms.

The NACCESS program (S. Hubbard) was used for solvent-accessible surface area calculations, with the standard Lee and Richards parameters (Lee and Richards, 1971).

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), which we modified for solution of the multiple titration site problem.

Modeling of protein structures

To assign proton coordinates, initial assignments of the protonation states of titratable residues were made as follows. The protonation state of histidine residues was chosen by geometric analysis of potential hydrogen bonds. Other titratable sites were assigned their usual protonation state at pH 7.0. N- and C-terminii were assumed to be ionized. Polar hydrogen atoms were then added to the crystal structures by using the Quanta software package (Molecular Simulations). The positions of the hydrogen atoms were optimized with the CHARMm force field (Brooks et al., 1982), first by simplex energy minimization of atoms with forces above 500 kcal · mol^{-1} Å^{-1} and then by conjugate gradient energy minimization, until the r.m.s. gradient was less than 0.1 kcal · mol^{-1} Å^{-1}. The positions of the hydrogen atoms were optimized in the presence of some crystallographic water molecules (those that are hydrogen-bonded to the protein) and in the presence of a crystallographically assigned sulfate ion located within the active site. The water molecules and the sulfate ion were then omitted in further calculations.

Electrostatics calculations

Partial atomic charges and atomic radii were assigned from the OPLS (Jorgensen and Tirado-Rives, 1988) and CHARMm19 (Brooks et al., 1982) parameter sets. The representation of the 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 proton positions 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 3–78.5 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 and 1.4 Å for calculations with the CHARMm 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 conjugate gradient method (Davis and McCammon, 1989). The electrostatic potentials were calculated by means of focusing grids on each of the titratable residues. Focusing was done in four steps, and four cubic grids with spacings of 2.5, 1.2, 0.75, and 0.25 Å and 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 is 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 and McCammon, 1991).

pK_a calculations

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 in solution were 4.0 for Asp, 12.0 for Arg, 3.8 for C-terminal carboxylate, 4.4 for Glu, 6.3 for His, 10.4 for Lys, 7.5 for the N-terminal amine, and 9.6 for Tyr (Antosiewicz et al., 1996). Ionization of these titratable groups was modeled by the addition of a unit point charge of appropriate sign placed at the position of the C^{γ} atom in Asp, the C^{ζ} atom in Arg, the C atom in the C-terminal carboxylate group, the C^{δ} atom in Glu, the $N^{\epsilon 2}$ atom in neutral $N^{\delta 1}$-protonated His (attributed to all His residues of β-lactamases from a careful analysis of their potential hydrogen-bonds) or the $N^{\delta 1}$ atom in neutral $N^{\epsilon 2}$-protonated His, the N^{ζ} atom in Lys, the N atom in the N-terminal amine, and the O^{η} atom in Tyr.

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 1.4 Å was used in calculations.

RESULTS AND DISCUSSION

Sensitivity of computed pK_as to methodology and parameters

Solution of the multiple titration site problem

The folding of a polypeptide chain into a functional protein results in multiple interactions between ionizable groups, which often leads to different pK_a values for the same residue in the folded and unfolded conformations of the protein. The pK_a perturbations are primarily the result of the electrostatic properties of the protein and its solvent environment. Current methods of predicting the total charge on a protein and the pK_as of its residues range in complexity from the simplest "null model" in which all residues are assumed to adopt their standard pK_as in solution, to full atomic representation molecular dynamics methods (see references in Demchuk and Wade, 1996). Although ideally a detailed dynamic model would be used, this is too computationally demanding for frequent use. The Poisson-Boltzmann continuum dielectric model (Bashford and Karplus, 1990) provides a description of intermediate detail that captures the electrostatic features important for pK_a determination and makes it possible to calculate pK_as with sufficient computational efficiency that it can be applied to many titratable sites. Despite considerable effort, most methods have failed to beat the null model in reproducing pK_a values for sets of titratable residues (Antosiewicz et al., 1994, 1996). Recently, however, improvements have been made to the continuum dielectric approach, resulting in models that give better predictions than the null model (Antosiewicz et al., 1994, 1996; Demchuk and Wade, 1996). The methodology is based on numerical solution of the Poisson-Boltzmann equation and relies for its success on the use of a rather high protein dielectric constant with values generally in excess of 10. This methodology was used here to calculate the pK_as of titratable residues in two different class A β-lactamases and their mutants.

In the continuum electrostatics approach (for more details, see Demchuk and Wade, 1996; Bashford and Karplus, 1990), free energies for ionization of a titratable site are computed twice: once when the site is considered as part of a model compound in solution, and a second time when the site is in the protein environment. This provides the pK_a shift relative to an experimentally determined reference pK_a for the model compound in solution. The pK_a shift can be considered as the sum of terms due to the different electrostatic interactions of the titratable group with its environment. One of these terms is the electrostatic free energy between the charge at the titratable site and charges at the other titratable sites. The evaluation of this term requires solution of the multiple titration site problem, so called because of the coupling between titratable sites, i.e., the charge occupancy of one titratable site at a given pH will depend on that of the others around it. For a protein with N titratable sites, there are 2^N possible ionization states whose contribution to pK_as should be evaluated. For all but very small proteins, a systematic search through all of these states is not feasible, and a more computationally efficient search procedure must be used. Several methods have been developed to solve the multiple titration site problem. These include the Monte Carlo search (Antosiewicz and Porscke, 1989; Beroza et al., 1991; Yang et al., 1993) and cluster (Gilson, 1993) methods, both of which we have used. The Monte Carlo method relies on suitable move definitions and acceptance parameters to achieve efficient and sufficiently thorough searching. In the cluster method, titratable sites are divided into clusters such that, at a given pH, there are only weak interactions between sites in different clusters that can titrate at this pH. Then a mean field treatment is used for intercluster interactions, and a systematic search is performed within clusters. This method is considerably faster than the Monte Carlo method.

The pK_a of an ionizable group is equal to the pH at which it is 50% ionized. Thus pK_as are usually determined by computing titration curves and identifying the pHs at which titratable residues are 50% ionized. This approach requires calculation of the average protonation of all titratable sites over a wide pH range at frequent pH intervals (e.g., approximately every 0.3 pH units; Beroza et al., 1991; Gilson, 1993). It is analogous to experimental procedures for determining pK_as via titration. Whereas this approach is generally computationally tractable with the cluster method (Gilson, 1993), it is not always feasible with the Monte Carlo method. The pK_as of all ionizable groups in a protein can, however, be estimated from solution of the multiple titration site problem at a single pH. This is done 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. This is how pK_as are often determined from Monte Carlo search procedures (Antosiewicz and Porscke, 1989; Madura et al., 1995). However, the states of residues that titrate far from the pH of the calculations may be poorly sampled, and therefore the computed pK_as of these residues may have large errors or may be undetermined (if only one of the protonation states is sampled).

Good agreement between the results of the cluster calculations at multiple pHs and Monte Carlo calculations at a single pH has been observed for small proteins (typically with $N < 30$) (Antosiewicz et al., 1994). However, for larger proteins, including β-lactamases, we observed a systematic upward shift in pK_a values for the basic residues and a downward shift for the acidic residues when using the cluster method and deriving pK_as from the titration curve. There was a clear correlation between the difference of the mean pK_a value of a residue type from its model value and the number of ionizable sites in the protein (Fig. 2). This artifact results from the titration procedure, which forces the modeled system to pH values that are physically unrealistic. At these pH values, the protein adopts highly improbable titration states in which it cannot preserve its native structure.

Fig. 2 *A* shows the mean pK_a values of lysine and arginine residues computed for six different proteins, with the normal cluster method and with a modified cluster method (see below). Because for the proteins shown in Fig. 2 *A*, a larger number of acidic residues (resulting in a larger maximum negative charge) correlated with a more negative net charge, we also computed the mean pK_a values of two homologous triose phosphate isomerase dimers (together with their monomers) that exhibit opposite net charge. Fig. 2 *B* shows that even in the case of a protein with a large positive net charge, the mean pK_a values are shifted upward for arginine residues. Thus these systematic pK_a shifts are governed by the total maximum negative charge for basic residues (or positive charge for acidic residues) of the protein and are only slightly affected by the sign of the net charge. These effects are due to the mutual interactions of the titratable groups. At extreme pHs, the net charge on large proteins can become very large; shifting pK_as as observed tends to neutralize the net charge at these pHs. For example, consider a protein at pH 12. All Asp and Glu residues will be negatively charged, and electrostatic interactions with these residues will tend to disfavor abstraction of protons from basic residues. If all Lys and Arg residues are neutralized, there will be a large net negative charge and many repelling interactions, which would destabilize the protein. If the Lys and Arg residues have upwardly shifted pK_as, then the net charge will be reduced and the energy of the system will be more favorable. All of the calculations whose results are shown in Fig. 2 were performed with a high protein dielectric equal to that of the solvent, and thus these systematic pK_a shifts are likely to be underestimated compared to calculations with a lower protein dielectric constant. In addition, as the calculations were done at an

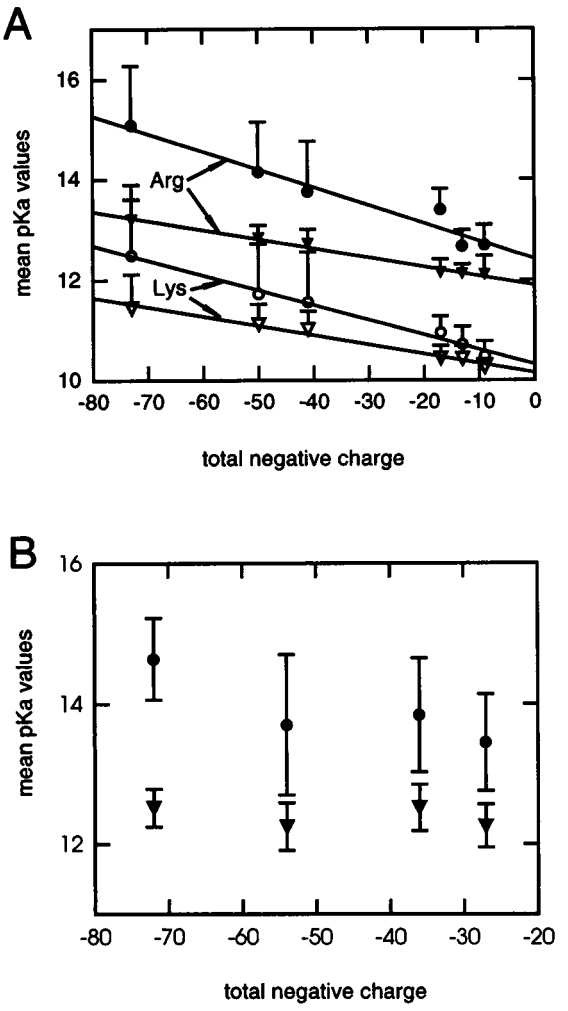

FIGURE 2 (*A*) Correlation between the mean pK_a values of Arg (●, ▼) and Lys (○, ▽) residues and the maximum negative charge (i.e., the number of acidic titration sites) of six different proteins. The circles represent the values obtained with the normal cluster method [19] and the triangles those values obtained with the modified cluster method. From left to right, these proteins are (with net charge computed at pH 7 given in parentheses) cytochrome P450cam (−16e), *Bacillus licheniformis* β-lactamase (−7e), TEM-1 β-lactamase (−7e), RNAse A (+4e), hen egg white lysozyme (+8e), and bovine pancreatic trypsin inhibitor (+6e). (*B*) Correlation between the mean pK_a values of Arg residues and the maximum negative charge for two different triose phosphate isomerase dimers and their monomers that have equal and opposite computed net charges at pH 7 (the values of the net charges from left to right are −12, +12, −6, and +6 e). The circles and triangles represent values obtained with the normal and modified cluster methods, respectively.

ionic strength of 150 mM, the systematic pK_a shifts would be expected to be larger at low ionic strength. Such systematic drifts in the pK_a shifts are artifacts of the computational model taken beyond the limits of its applicability, rather than a real property of large proteins because of the following aspects of the model:

1. The protein structure is assumed to be constant in the model at all pHs, whereas most proteins are not active and many denature at extreme pHs, losing their native structure.

2. The linearized Poisson-Boltzmann model is less appropriate for highly charged species. Some specific treatment of counterions may also become necessary.

3. Some residues do not follow standard titration curves, but are 50% ionized over a range of pHs (Yang et al., 1993).

To overcome these problems, we used the cluster method with a different protocol to derive pK_as. That is, computations were run only over an intermediate pH range (4–11 for β-lactamases to correspond to the pHs at which experimental measurements can be made) at intervals of 0.2 pH units. pK_as were computed at each pH, using Eq. 1, and then averaged over all pHs to obtain a single pK_a estimate for each titratable site, i.e.,

$$pK_a = \langle pH - (\ln([I]/[HI]))/2.303 \rangle_{pH} \quad (2)$$

This modified cluster method produced fewer extreme pK_a estimates. As shown in Fig. 2, differences for small proteins compared to the standard cluster and Monte Carlo methods were small, but became significant for larger proteins.

The TEM-1 and *B. licheniformis* β-lactamases have 77 and 88 ionizable sites, respectively. The total number of residues that can be negatively charged is 41 and 50, respectively. Thus we computed pK_as for these enzymes with the modified cluster method. From Table 1, it can be seen that the results of the pK_a calculations for the TEM-1 enzyme, using the Monte Carlo method at a single pH and the modified cluster method, are similar. The standard cluster method (results not shown) gives more extreme pK_a values for the β-lactamases.

Assignment of the protein dielectric constant

In a previous study (Demchuk and Wade, 1996), it was observed that the average root mean square deviation be-

TABLE 1 pK_a values computed for residues in the vicinity of the active site (see Fig. 3) of the TEM-1 and *B. licheniformis* β-lactamases*

Residue	ASA#	Zrf§	Model¶	78op‖	78ch	15op	15ch	3op	3ch
				(11.85)		(12.37)		(>12.7)	
K73	0	0.98	10.4	11.73	11.71	12.34	11.38	12.71	7.80
	0	*−1.59*		*11.69*	*11.67*	*12.36*	*11.43*	*10.40*	*9.72*
				(3.31)		(2.53)		(<1.5)	
D131	0	6.15	4.0	3.54	3.53	2.48	2.48	−2.57	−0.31
	0	*5.76*		*3.56*	*3.53*	*2.51*	*2.54*	*−2.20*	*−0.59*
				(>13.0)		(>13.0)		(>12.7)	
R164	13	0.83	12.0	13.17	13.17	14.01	13.94	14.00	11.07
	23	*3.16*		*13.35*	*13.34*	*14.37*	*14.24*	*16.51*	*15.10*
				(4.47)		(5.14)		(9.13)	
E166	2	−5.08	4.4	4.43	4.43	4.99	5.19	9.51	10.21
	1.7	*−4.37*		*4.36*	*4.35*	*4.90*	*5.01*	*8.73*	*10.04*
				(3.32)		(4.16)		(3.90)	
D179	0.7	−0.18	4.0	3.79	3.87	3.96	4.51	3.97	6.60
	0.3	*2.42*		*3.80*	*3.82*	*3.68*	*3.87*	*0.38*	*0.77*
				(3.77)		(5.12)		(11.86)	
D214	4	−9.91	4.0	3.88	3.85	5.30	5.45	13.79	14.37
				(12.0)		(>13.0)		(>12.7)	
R222	26	2.02	12.0	13.31	13.28	14.78	14.65	15.33	12.85
	22	*1.69*		*13.37*	*13.37*	*14.21*	*14.17*	*15.06*	*13.39*
				(3.8)		(4.69)		(2.43)	
D233	0	1.32	4.0	3.84	3.81	4.63	4.72	2.52	2.65
	0	*0.95*		*4.05*	*4.08*	*4.80*	*5.10*	*3.10*	*3.25*
				(11.2)		(10.17)		(1.44)	
K234	0	−8.68	10.4	11.05	11.04	9.98	9.15	2.61	0.78
	0	*−3.89*		*11.29*	*11.28*	*10.86*	*9.79*	*7.40*	*5.20*
				(>13.0)		(>13.0)		(10.96)	
R244	9	−1.30	12.0	12.36	12.29	11.76	11.38	11.06	9.40
	12	*−0.27*		*13.01*	*13.02*	*13.15*	*13.08*	*12.80*	*12.99*
D246	*0*	*−7.49*	*4.0*	*4.25*	*4.27*	*6.00*	*6.33*	*11.92*	*11.88*

*Values for TEM-1 are given in plain text and those for *B. licheniformis* in italics.

#ASA is the solvent-accessible surface area in $Å^2$.

§Zrf is a criterion given in Eq. 3 based on desolvation energy that indicates which protein dielectric constant assignment will give the most accurate pK_a values (Demchuk and Wade, 1996). It was computed using OPLS parameters. For Zrf < -1.6, a protein dielectric constant of ~15 should, on average, give the most accurate pK_a values, whereas for Zrf > -1.6, a protein dielectric constant of 78.5 should give pK_a values with the best agreement with experiment.

¶Standard pK_a of the model compound in solution.

‖The parameters used are described by a number that refers to the value of the protein dielectric constant (3, 15, and 78.5), and letters that refer to the OPLS (op) and CHARMM (ch) parameter sets. pK_a values were computed with the modified cluster method. For comparison, values computed with the Monte Carlo method are given in superscript parentheses for the TEM-1 enzyme.

tween calculated and experimental pK_a values for three different small proteins decreased as the protein dielectric constant, $\epsilon_{interior}$, was increased. It was concluded that a high protein dielectric constant similar to that of the solvent gives, on average, the most accurate pK_a values. However, for some ionization sites, the optimum protein dielectric constant is much lower. Thus a criterion based on desolvation energy was introduced to determine whether the pK_a of an ionizable site should be calculated using a protein dielectric equal to that of the solvent or lower. A Zrf value given by

$$Zrf = [\text{sign}(\Delta pK_a^{Coulomb}(\epsilon_{interior}))][\Delta pK_a(\epsilon_{interior})] \quad (3)$$

is computed for each ionizable site from four pK_a calculations at two extreme protein dielectric constants, $\epsilon_{interior}$ (in this work, 78.5 and 3) and its value used to distinguish between the two types of ionizable site. The magnitude of Zrf is derived from $\Delta pK_a(\epsilon_{interior})$, the difference between the computed pK_as at high and low $\epsilon_{interior}$. $\Delta pK_a^{Coulomb}(\epsilon_{interior})$ is the corresponding pK_a difference when the charges are considered to be immersed in a homogeneous dielectric of zero ionic strength. Based on analysis of the protein test set (Demchuk and Wade, 1996), a criterion of $Zrf < -1.6$ was found to be robust for detection of "low dielectric constant" sites when OPLS parameters were used.

Thus, if the pK_a prediction for a particular ionizable site does not noticably depend on the value of the protein dielectric constant, then a high protein dielectric constant (equal to that of the solvent) can be applied, as it is on average the most accurate one (giving an rmsd of 0.3 and a maximum deviation of 0.7 pK_a units for the test set used; Demchuk and Wade, 1996). But if pK_a predictions are very different at the extremes of the protein dielectric range and if the pK_a shift is determined predominantly by desolvation effects ($Zrf < -1.6$), then this site can be considered as a "low dielectric constant" site with a value of 15, giving reasonable results when OPLS parameters are used (rmsd of 0.8 and maximum deviation of 1.6 pK_a units for the test set used; Demchuk and Wade, 1996). The high values of the protein dielectric constant compensate for features of the system that are not explicitly present in the model, such as dynamic motions. The optimal protein dielectric constant for titratable sites would be expected to be different when, for example, side-chain flexibility is modeled explicitly. Explicit incorporation of side-chain flexibility increases the physical realism of the model (You and Bashford, 1995; Beroza and Case, 1996), but so far has not resulted in pK_as as accurate as those computed by the method used in this work (Demchuk and Wade, 1996).

Most ionizable sites in the TEM-1 enzyme exhibit a small positive or negative Zrf value, which means that the calculated pK_a values do not depend significantly on the protein dielectric constant. For these residues, a high protein dielectric model should give the most accurate pK_a estimates. They include Lys^{73}, Arg^{164}, Glu^{179}, Glu^{233}, and Arg^{244} in the active site (see Table 1 and Fig. 3). It should be noted that the Zrf value is a more sensitive criterion than solvent accessibility, because it takes long-range interactions into account as well as the effect of the local dielectric boundary. This explains why a few residues have a small Zrf value, despite exhibiting a solvent accessibility of zero. Some ionizable sites have a large positive Zrf value. One example is Asp^{131}, which is a strictly conserved residue in class A β-lactamases. Its side chain points into the core of the protein and has no positively charged counterpart. But, as already pointed out (Swarén et al., 1995), the carboxylate group of this residue is hydrogen-bonded to the main-chain nitrogen atoms of residues 108, 109, 133, and 134, located at the N-terminii of helices H3 and H5. These interactions are strong enough to stabilize a negative charge in the core of the protein. This residue has the smallest pK_a value computed for active-site residues of the TEM-1 and *B. licheniformis* enzymes (see Table 1). The important structural role played by this residue has been demonstrated for the *Streptomyces albus G* β-lactamase (Jacob et al., 1990).

On the other hand, some ionizable sites have large negative Zrf values and should be treated with a protein dielectric constant lower than that of the solvent. In the TEM-1 enzyme, three of these, E166, D214, and K234, are located in the vicinity of the active site (Fig. 3).

Lys^{234} is a very conserved residue in class A β-lactamases. It is only substituted by arginine residues, and characterization of point mutants has clearly shown that the important feature is the presence of a positive charge at this position (Brannigan et al., 1991). Moreover, NMR and chemical modification data have demonstrated that there is no lysine residue with a pK_a value below 10 in the TEM-1 enzyme in solution (Damblon et al., 1996). The Zrf parameter for this particular residue suggests that a low protein dielectric constant should be adopted. A value of 15 gives a pK_a value of 10.0, in good agreement with experiment,

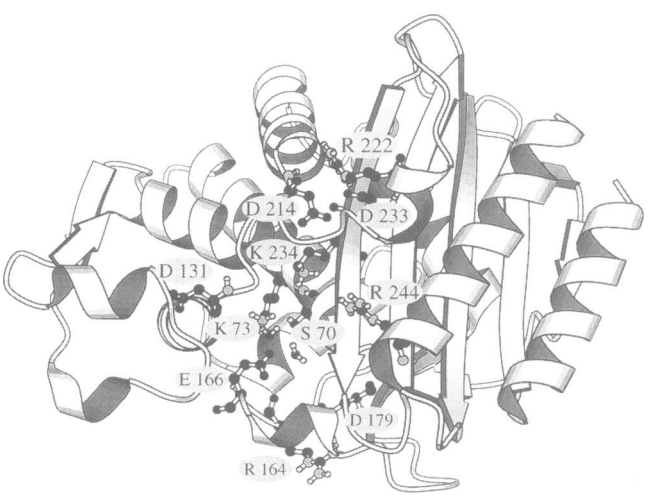

FIGURE 3 Ribbon diagram of the TEM-1 β-lactamase, showing the residues for which pK_as are given in Table 1. This figure was generated with the Molscript program [40].

whereas a dielectric constant smaller than 15 gives a lower pK_a value, which is in contradiction to the experimental observations (Table 1).

Glu^{166} is a strictly conserved residue in class A β-lactamases, where it plays the role of proton abstractor in the deacylation step and may fulfill the same role in the acylation step (see Introduction). Because of its function and its proximity to Lys^{73}, the neutral form of this residue is very unlikely to be predominant at physiological pH. Here also, the Zrf parameter predicts that a low protein dielectric constant should be adopted for this residue, and the value of 15, giving a pK_a value of 5, seems to be optimal.

Asp^{214} is a special case because it is buried, at hydrogen bond distance from both the carboxylic and backbone carbonyl groups of the strictly conserved Asp^{233}, and the free energy of charge-charge interactions between these two residues is the greatest computed in the TEM-1 enzyme. Asp^{233} is also buried, but its ionized form is stabilized by a salt bridge with Arg^{222} (see Fig. 4). Asp^{214} is not a conserved residue, and is most often substituted by an asparagine residue. For these reasons, the carboxylate group of this residue is expected to be protonated at physiological pH. The optimal protein dielectric constant value for this residue is clearly low. It should be intermediate between 3 and 15, which respectively give pK_a values of ~14 and 5. The optimum dielectric constant is probably ~8, as this gives a calculated pK_a value of 7.5 when OPLS parameters are used.

Swarén et al. (1995) calibrated the method and parameterization for their electrostatic continuum calculations on the TEM-1 β-lactamase by using Asp^{214} as a reference, because the hydrogen bond between the carboxylic groups of Asp^{214} and Asp^{233} observed in the crystal structure requires protonation of one of these two residues at pH 7.8. They assumed that the proton is born by Asp^{214}. They used CHARMM19 atomic charges and Connolly atomic radii from the Delphi program (which are very similar to the scaled CHARMM19 radii we used) and a dielectric constant of 3 for the protein interior, and rather than solving the multiple titration site problem, they neglected coupling between titratable sites and assumed that all other titratable sites have standard protonation states at pH 7.8. When we applied the same procedure, we obtained pK_a shifts for Asp^{214} and Lys^{73} similar to those of Swarén et al., i.e., +5.1 and −3.6 pK_a units, respectively. However, when we used the cluster method to solve the multiple titration problem at pH 7.8 with the same parameters, pK_as of 14.8 and 8.2 were obtained for Asp^{214} and Lys^{73}, respectively; this difference arose because of interactions between titration sites. This indicates that a protein dielectric constant of 3 is too low for use with our methodology. But a crucial point is that the effective protein dielectric constant varies at different positions in the protein (Simonson and Brooks, 1996). Thus, although the optimum protein dielectric constant might have a certain low value for a given model at the location of Asp^{214}, it cannot therefore be assumed that this protein dielectric constant is optimal for all other titratable sites in the protein. As a single reference for calibration, Asp^{214} has the disadvantage that its pK_a is very sensitive to the very short-range interaction with Asp^{233}. The Zrf criterion used in our work is, on the other hand, a statistical one that is expected to give the best results on average.

In conclusion, for the majority (74%) of ionization sites in the TEM-1 enzyme, a protein dielectric constant as high as 78.5 should be adopted in the pK_a calculations. Only three residues in the active site have a pK_a value best predicted by a protein dielectric constant lower than that of the solvent: Glu^{166} and Lys^{234} have a pK_a value best predicted by a protein dielectric constant of 15; Asp^{214} has a pK_a value best predicted by a protein dielectric constant of ~8 and is the only residue that would adopt a nonstandard protonation state at neutral pH.

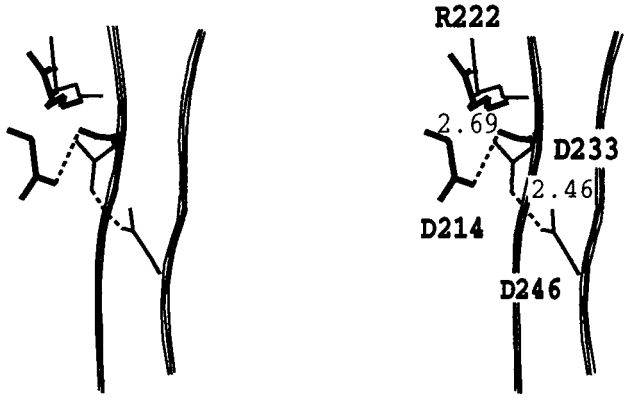

FIGURE 4 Stereo detail of Asp^{233} and the residues in its vicinity, showing the equivalent arrangement found in the D214-D233-R222 triad of the TEM-1 β-lactamase (*thick lines*) and the D246-D233-R222 triad of the *B. licheniformis* enzyme (*thin lines*). The lengths of the shortest hydrogen bond contacts to Asp^{233} are given in angstroms. The ribbons represent the protein backbone.

Parameterization of van der Waals radii and partial atomic charges

The results obtained with the two different sets of parameters, OPLS and CHARMM, are, in general, in good agreement (see Table 1). Some discrepancies appear at low protein dielectric constants. Residues Arg^{164} and Asp^{179} are involved in a salt bridge delimiting an omega loop at the entrance of the active site. The ionized form of these two residues should be stabilized by this interaction. Mutation of one of these residues leads to a destabilization of the omega loop (Herzberg et al., 1991; Raquet et al., 1995). This is correctly predicted in all cases, with the exception of the TEM-1 enzyme with CHARMm parameters and a protein dielectric constant of 3 (see Table 1), when the pK_a value of Asp^{179} is shifted upward, whereas the pK_a value of Arg^{164} is shifted downward. The same tendency is observed for the Lys^{73} and Glu^{166} pair. Lys^{73} is predicted to be shifted upward in all cases, except when CHARMm parameters are used together with a dielectric constant of 3, when the

downward shift of the Lys^{73} pK_a is compensated by an upward shift of the Glu^{166} pK_a. However, previous work (Demchuk and Wade, 1996; Antosiewicz et al., 1996) shows that pK_as computed with a protein dielectric constant of 3 are likely to be much less accurate than those computed with dielectric constants of 15 or 80. The differences observed between the results obtained with the OPLS and CHARMm parameters may originate from the larger dipoles and smaller radii assigned to polar atoms in the CHARMm parameter set. These differences increase as the protein dielectric constant is reduced and may explain why an optimal dielectric constant of 20 was found for pK_a calculations with CHARMm22 partial atomic charges (Antosiewicz et al., 1994), whereas 15 was found to be best on average for the low dielectric titratable sites when OPLS parameters were used (Demchuk and Wade, 1996). For this reason, it is not possible to use the same Zrf criterion for the CHARMm parameters as for the OPLS parameters.

Comparative analysis of the pK_as of active-site residues in TEM-1 and B. licheniformis β-lactamases

The results obtained for the conserved active-site residues of the TEM-1 and *B. licheniformis* enzymes are compared in Table 1. These two proteins present 38% sequence similarity (Joris and Knox, 1988). All of the titratable residues in TEM-1 β-lactamase listed in Table 1 are the same in the *B. licheniformis* enzyme, except for residue 214, which is an Asp in TEM-1 and an Asn in *B. licheniformis*. The results obtained for these residues with the *B. licheniformis* protein are very similar to those obtained with the TEM-1 enzyme. This is encouraging because it demonstrates that the same pK_a shifts can be computed for the conserved residues of two proteins that have, a priori, different electrostatic environments. In the *B. licheniformis* enzyme, in addition to Glu^{166} and Lys^{234}, another residue, Asp^{246}, changes its ionization state when the protein dielectric constant is shifted from 78.5 to 3. From the crystallographic structure (see Fig. 4), it can be seen that a carboxylic oxygen of this residue makes a hydrogen bond to the carboxylic group of the strictly conserved Asp^{233}, implying the presence of a proton on one of the two carboxylic groups, just as for Asp^{214} in the TEM-1 enzyme (see above and Raquet et al., 1995). Indeed, like Asp^{214}, Asp^{246} is computed to have a raised pK_a value. Of the 20 class A β-lactamase sequences aligned (Ambler et al., 1991), all have an aspartate at position 233, 19 have an arginine at position 222 (the *Staphylococcus aureus* enzyme has a lysine), and 15 have an aspartate at position 246 (the TEM-1 enzyme has an isoleucine), and four have an equivalent aspartate at position 214. It should be interesting to investigate further the role of this triad ($Asp^{214/246}$-Asp^{233}-Arg^{222}), which has been maintained throughout the evolution of β-lactamases, despite being energetically unfavorable for protein stability.

Influence of active-site mutations

Calculations were also performed on mutants of the TEM-1 and *B. licheniformis* enzymes in which Glu^{166} is substituted by a neutral residue. We hypothesized that neutralization of this residue, which is close to Lys^{73}, could lower the pK_a of the latter residue and enable it to play the role of an alternate general base in the acylation step of the mutant. But the pK_a calculations performed on both mutants show that the downward shift relative to the wild-type enzymes is only ~0.5–1.0 pK_a units (see Table 2), suggesting that this mutation is not sufficient to induce a deprotonation of Lys^{73}. A number of mutations have been performed at position 166 of class A β-lactamases (for a review, see Matagne and Frère, 1995), and these generally indicate that the acylation step is less affected than the deacylation step. This means that these mutants could have an alternative proton abstractor, partly efficient at least, in the acylation step. Lys^{73} could be a good candidate because it is a conserved residue, at hydrogen bond distance from the active serine, whose ionization state could be influenced by the residue at position 166. Nevertheless, our results suggest that the pK_a shift is not sufficient to generate such an unprotonated lysine. Moreover, preliminary results of chemical modification on the substrate-free TEM-1 E166N mutant (Guillaume et al., 1997), performed as described by Damblon et al. (1996), suggest that there is no lysine with a pK_a below 10 for this enzyme (Guillaume and Frère, personal communication). Thus the nature of an alternative proton abstractor, if any, in these mutants is so far unknown.

Detailed analysis of contributions to the pK_a of Lys^{73}

When the free energies contributing to the pK_a shift of Lys^{73} are analyzed, it appears that the upward pK_a shift is explained by a limited number of residues (see Table 3 and Fig. 5). The strongest favorable interaction with a titratable group is made, as expected, with Glu^{166} (its closest carboxylic oxygen is 3.44 Å from the amino nitrogen of Lys^{73} but badly oriented for hydrogen bond formation), and the strongest unfavorable interaction is due to Lys^{234}. The balance of the charge-charge interactions is clearly in favor of stabilization of the ionized form, in contrast to what was postulated before (Strynadka et al., 1992; Swarén et al., 1995).

TABLE 2 Comparison of the pK_a values obtained for Lys^{73} and Lys^{234} in the TEM E166N and *B. licheniformis* E166A mutant enzymes*

Residue	Enzyme	ASA	Zrf	78op	78ch	15op	15ch	3op	3ch
K73	TEM E166N	0.2	1.24	11.18	11.17	10.67	9.93	12.42	7.82
	B. lich	3.0	2.11	11.15	11.12	10.87	10.26	13.26	10.18
K234	TEM E166N	0.1	−8.55	10.86	10.85	9.50	8.76	2.31	0.84
	B. lich E166A	0.5	−6.88	10.86	10.86	9.47	8.79	3.98	0.79

*Definitions for column headings are as for Table 1.

TABLE 3 Major free energies of charge-charge interactions between Lys^{73} and the other titratable residues in the wild-type TEM-1 β-lactamase and the E166N mutant*

Residue	Wild-type	E166N
E166	−0.85	n.a.
K234	+0.62	+0.62
D131	−0.43	−0.43
D214	−0.24	−0.24
E104	−0.24	−0.24
R244	+0.22	+0.22
D179	−0.20	−0.20
D233	−0.16	−0.16
Total from above eight charge-charge interactions	−1.28	−0.43
Desolvation + protein interaction terms	[−0.29]	[−0.31]
Intrinsic pK_a	10.61	10.62
pK_a#	11.54	10.96
pK_a##	11.73	11.18

*Free energies of charge-charge interactions are given in kcal/mol, and only those exceeding 0.15 kcal/mol in magnitude are listed. They were computed with OPLS parameters and a protein dielectric constant of 78.5, as this dielectric assignment should give the most accurate computed pK_a according to the Zrf criterion. The pK_as are given from a full computation (##) and from a partial computation (#) made with only the eight major charge-charge interactions listed in this table.

In the above calculations, proton positions were assigned, assuming that titratable residues have their standard protonation state at pH 7. If Lys^{73} were to have a shifted pK_a such that it were neutral at pH 7, proton positions would be different. To evaluate whether using proton positions optimized for a neutral Lys^{73} would lead to a different pK_a shift for Lys^{73}, we recomputed pK_as with correspondingly remodeled proton positions in the vicinity of Lys^{73}. That is, the hydroxyl proton of Ser^{70} was reoriented to point to the Lys^{73} side-chain nitrogen, and the two amine hydrogens on Lys^{73} were positioned to point to the carbonyl oxygen of Ser^{130} and the side-chain oxygen of Asn^{132}. The crystallographically observed water molecule (residue W-292) was also explicitly added to the model, and Glu^{166} was assumed to be protonated. This arrangement permits a distribution of protons that favors the neutral states of Glu^{166} and Lys^{73} (see Fig. 5 *b*). However, the complete hydrogen-bond pattern in the crystal structure is not optimal. When Ser^{70} donates a hydrogen bond to Lys^{73}, it is bifurcated and also donated to Ser^{130}. However, when Glu^{166} and Lys^{73} are neutralized, the Ser^{130} hydroxyl group must also accept a hydrogen bond from the Lys^{234} amine group, which forms an ionic pair with a sulfate ion observed in the crystal, and thus the hydroxyl hydrogen of Ser^{130} is left without a hydrogen-bond partner.

With this proton arrangement, and W-292 modeled explicitly, Lys^{73} becomes a "low dielectric constant" site with a significant negative Zrf (see Table 4). However, even with a protein dielectric constant of 15, the computed pK_a is 10.2.

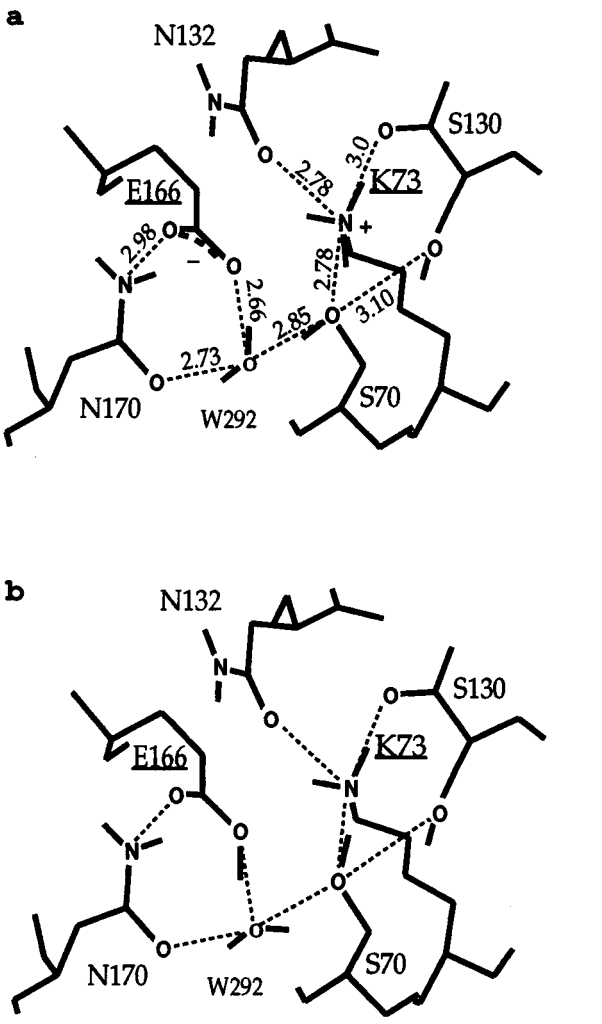

FIGURE 5 Detail of the immediate environment of the side chain of Lys^{73} in the TEM-1 β-lactamase for (*a*) charged and (*b*) neutral forms of Lys^{73} and Glu^{166}. Hydrogen bonds are indicated by dotted lines, and distances are given in angstroms.

Glu^{166} is also a "low dielectric constant" site, but for a protein dielectric constant of 15 it has a pK_a of 5.8. As the protein dielectric constant is reduced below 15, the pK_a of Lys^{73} drops significantly, and that of Glu^{166} rises in a complementary fashion. Note that all of these results are rather insensitive to the charge assignment on the Lys amine group (i.e., whether charges for the neutral form are distributed equally over three proton sites (as in the standard procedure) or, more realistically, are placed on only two proton sites).

However, the sensitivity to the orientation of the Ser^{70} hydroxyl proton and polarization is not negligible. The effect of the hydroxyl proton orientation in our calculations is twofold. First, it lowers the pK_a of Lys^{73} by ~2 units, and second, it inverts the sign of the Zrf value, indicating that a low protein dielectric should be used. In addition, the effect of the hydroxyl proton polarization by the amine nitrogen at H-bonding distance could also result in a further decrease in the pK_a of Lys^{73} by ~1 pK_a unit, to bring it to a minimum

TABLE 4 pK_a values computed for Lys^{73} and Glu^{166} in TEM-1 β-lactamase, with the proton distribution modified to favor neutral forms of Lys^{73} and Glu^{166} (shown in Fig. 5 *b*)*

Site	Charges#	Zrf	78op	15op	3op
K73	s	−6.98	11.34	10.20	4.36
	e	−7.31	11.34	10.19	4.03
D166	s	−11.04	4.51	5.78	15.55
	e	−11.06	4.49	5.87	15.55

*Definitions for column headings are as for Table 1.

#Both standard (s) and enhanced (e) partial charges were assigned to the amine moiety of Lys^{73} (see text). The enhanced charges give a more realistic representation of the charge distribution of the neutral residue: charges of $+0.33e$ are assigned to two proton sites, and $-0.66e$ is assigned to the nitrogen. The standard assignment is charges of $+0.1e$ on three proton sites and $-0.2e$ on the nitrogen. The CD, OE1, OE2, and HE2 atoms of the protonated carboxylic group of Glu^{166} were assigned charges of +0.8, −0.7, −0.5, and $0.4e$, respectively, in both cases. Water molecule W-292 was represented by the TIP3P water model (Jorgensen et al., 1983).

value of 9.3 (i.e., estimating that polarization could maximally increase the partial charge on the Ser^{70} hydroxyl hydrogen from $+0.435e$ to $0.7e$ results in a pK_a value of 9.35 for Lys^{73} with a protein dielectric constant of 15).

We conclude that, within the limits of the methodology used, employing a more realistic description of protons favoring a neutral Lys^{73} under the catalytic conditions does not alter our conclusion that the pK_a of Lys^{73} is not sufficiently downward-shifted for it to act as the general base.

CONCLUSION

In conclusion, it is likely that, in both the substrate-free TEM-1 and *B. licheniformis* enzymes, only one residue adopts a nonstandard protonation state at neutral pH: Asp^{214} in TEM-1 and Asp^{246} in *B. licheniformis*. These residues seem to play similar roles in triads of ionizable residues not far from the site of catalysis. The calculated pK_a values for Lys^{73} in both class A enzymes are in good agreement with the experimentally determined value in the TEM-1 protein in solution (Damblon et al., 1996) and are above 10. But this contradicts a previous pK_a calculation made on this enzyme (Swarén et al., 1995), as well as a proposition made on a structural basis (Strynadka et al., 1992). More recently, a complex mechanism of proton transfer was proposed (Ishiguro and Imajo, 1996), in which the carboxylate group of the substrate could abstract the proton of the Ser^{130} residue, which in turn would be reprotonated by Lys^{73}, leaving the latter residue unprotonated and ready to abstract the proton of the active serine. This would imply an unusually low pK_a for the Ser^{130} residue, which was not observed in our calculations (data not shown). Our results support a mechanism whereby the proton of the active Ser^{70} is transferred to the carboxylate group of Glu^{166} via a water molecule. Even in the mutant proteins where Glu^{166} is replaced by a neutral residue, the downward shift of the pK_a of Lys^{73} is not sufficient for this residue to be uncharged at pH 7. The nature of an alternative proton abstractor, if any, in these mutants is still enigmatic.

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