# Origin of the pKa shift of the catalytic lysine in acetoacetate decarboxylase

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**ABSTRACT**

The pKa value of Lys115, the catalytic residue in acetoacetate decarboxylate, was calculated using atomic coordinates of the X-ray crystal structure with consideration of the protonation states of all titratable sites in the protein. The calculated pKa value of Lys115 (pKa(Lys115)) was unusually low (≈6) in agreement with the experimentally measured value. Although charged residues impact pKa(Lys115) considerably in the native protein, the significant pKa(Lys115) downshift in the protein with respect to aqueous solution was mainly due to loss of the solvation energy in the catalytic active site relative to bulk water.

**KEYWORDS:** lysine, pKa, acetoacetate decarboxylase, hydrophobic, solvation energy, protein dielectric.

**Abbreviations**

AADase, acetoacetate decarboxylase;

CaAAD, *Clostridium acetobutylicum* AADase;

CvAAD, *Chromobacterium violaceum* AADase;

LPB equation, linear Poisson-Boltzmann (LPB) equation;

MC method, Monte Carlo method;

PDB, protein data bank;

A catalytic lysine (Lys115) in the active site of the acetoacetate decarboxylase (AADase) has long been known to be deprotonated. Using a reporter group that can approach the catalytic site in the protein, the pKa value of Lys115 (pKa(Lys115)) was formerly measured to be 5.96 [1]. pKa(Lys115) in AADase was unusually low with respect to pKa(Lys) = 10.4 in an aqueous solution [2]. Since another basic group Lys116 is located next to the catalytic Lys115 in the AADase residue sequence, the reason for the Lys115 deprotonation was speculated as being due to the electrostatic repulsion with the neighboring Lys116. However, recent X-ray crystal structures revealed that the side chains of Lys115 and Lys116 are oriented away in the opposite direction (Figure 1). Thus, the origin of the significantly lowered pKa(Lys115) was revised and reattributed to a desolvation effect in the protein core [3]. (Note; some studies mention that the desolvation effect facilitates also the destabilization in the "reactant" (i.e., acetoacetate) ground state, leading to the decrease in the enzymatic activation energy barrier. However, the actual reason for the enhancement of the enzymatic reaction in the protein is that the preorganized polar environment in the protein stabilizes the reactant transition state more effectively than in water [4,5]. This also holds true for the decarboxylation reactions [6]. The present study is aimed at referring only to the issue of the decrease in the pKa(Lys115) in the protein.)

On the other hand, several charged residues are located in the neighborhood of Lys115, namely strictly conserved residues Arg29 and Glu76 (closest atom pair distance 6.6 Å and 4.3 Å, respectively) [3]. As seen in some AADase mutants, mutations of charged residues in AADase did not significantly alter the optimum pH value of the catalytic activity. Thus, the charged residues were regarded as not having an effect on pKa(Lys115) [3]. However, considering the distances between Lys115 and the charged groups, the electrostatic influence of the charged residues on pKa(Lys115) should not be marginal.

To fully understand the origin of the significantly lowered pKa(Lys115) in the protein environment, pKa(Lys115) was calculated for *Clostridium acetobutylicum* AADase (CaAAD) and *Chromobacterium violaceum* AADase (CvAAD), using atomic coordinates of the X-ray crystal structures with consideration of protonation states of all of the titratable sites in the protein. In the present study, each

titratable residue alters the protonation state in response to changes in the protonation states of the neighboring titratable residues. The influence of the protein environment on pKa(Lys115) is discussed in terms of (i) desolvation effect on pKa(Lys115) due to the protein volume, and (ii) the influence of atomic charges in the protein on pKa(Lys115) (e.g., side chains and backbones).

## COMPUTATIONAL PROCEDURES

**Atomic coordinates and charges.** For performing computations of AADase, crystal structures of the CaAAD (protein data bank (PDB); 3BH2) and CvAAD in the unliganded form (PDB: 3BGT) were used. The atomic coordinates were obtained using the same procedures used in previous studies (e.g., Refs. [7,8]). The positions of H atoms were energetically optimized with CHARMM [9] by using the CHARMM22 force field. While carrying out this procedure, the positions of all non-H atoms were fixed, and the standard charge states of all the titratable groups were maintained, i.e., basic and acidic groups were considered to be protonated and deprotonated, respectively. All of the other atoms whose coordinates were available in the crystal structure were not geometrically optimized.

Atomic partial charges of the amino acids were adopted from the all-atom CHARMM22 [9] parameter set. The charges of protonated acidic oxygen atoms in Asp and Glu were both increased symmetrically by +0.5 unit charges to account implicitly for the presence of a proton. Similarly, instead of removing a proton in the deprotonated state, the charges of all protons of the basic groups of Arg and Lys were diminished symmetrically by a total unit charge.

**Modeling of the E61Q CvAAD protein structure.** To investigate influence of the E61Q mutation on the pKa(Lys115) value, the E61Q CvAAD structure was modeled by replacing one of the side chain O atoms with a N atom in the native CvAAD crystal structure. Only on the replaced Gln61 side chain, energy minimization was performed, i.e., all of the other heavy atom positions including the protein backbone at the 61st position were fixed.

**Protonation pattern and pKa.** The present computation is based on the electrostatic continuum model created by solving the linear Poisson-Boltzmann (LPB) equation with the MEAD program [10]. To facilitate a direct comparison with previous computational results, identical computational

conditions and parameters were used (e.g., Refs. [7,8]) such as atomic partial charges and dielectric constants. To obtain absolute pKa values of a target site (e.g. pKa(Lys115)), we calculated the electrostatic energy difference between the two protonation states, protonated and deprotonated, in a reference model system using a known experimentally measured pKa value (10.4 for Lys [2]). The difference in the pKa value of the protein relative to the reference system was added to the known reference pKa value. Experimentally measured pKa values employed as references are 12.0 for Arg, 4.0 for Asp, 9.5 for Cys, 4.4 for Glu, 10.4 for Lys, 9.6 for Tyr [2], and 7.0 and 6.6 for deprotonation/protonation at Nε and Nδ atoms of His, respectively [11-13]. All of the other titratable sites were fully equilibrated to the protonation state of the target site during the titration. The ensemble of the protonation patterns was sampled by the Monte Carlo (MC) method with Karlsberg [14] (Rabenstein, B. *Karlsberg online manual*, http://agknapp.chemie.fu-berlin.de/karlsberg/ (1999)). The dielectric constants were set to $\varepsilon_p = 4$ inside the protein and $\varepsilon_w = 80$ for water. All computations were performed at 300 K, pH 7.0, and an ionic strength of 100 mM. The LPB equation was solved using a 3-step grid-focusing procedure at the resolutions 2.5 Å, 1.0 Å, and 0.3 Å. The MC sampling yielded the probabilities [protonated] and [deprotonated] of the two protonation states of a molecule. The pKa value was evaluated using the Henderson-Hasselbalch equation. A bias potential was applied to obtain an equal amount of both protonation states ([protonated] = [deprotonated]), yielding the pKa value as the resulting bias potential.

**Evaluation of the protein dielectric constant $\varepsilon_p$.** The optimal $\varepsilon_p$ value depends on the protein model used. The more atomic details are included explicitly in the description of the molecular system, the lower the $\varepsilon_p$ value becomes. The $\varepsilon_P$ size is a reflection of what is not included explicitly (e.g., $\varepsilon_p = 1$ for the system with all the possible factors influencing electrostatic interactions being considered explicitly) [15,16]. As demonstrated by Warshel and coworkers [15,16] $\varepsilon_p \approx 4$ can be used when protein flexibility is taken into account explicitly. Furthermore, it is unlikely that the $\varepsilon_p$ for charge-dipole and charge-charge interactions are identical [15]. It has been suggested that $\varepsilon_p \approx 20$ and 40 should be used for interactions of charged-uncharged groups and charged-charged groups, respectively [15,17]. In this

study, using a dielectric constant of $\varepsilon_P = 4$ inside the protein and $\varepsilon_W = 80$ outside to model the polarization of bulk water, pKa(Lys115) values were calculated to be 5.73 for CaAAD and 5.96 for CvAAD in the AADase monomer form (see RESULTS AND DISCUSSION) in excellent agreement with the measured value [1]. However, by using $\varepsilon_p = 6$, the calculated pKa(Lys115) were 8.04 for CaAAD and 8.55 for CvAAD in the AADase monomer form (for further details, see Table S1 in supplementary material): this result is obviously in contrast to the measured value [1]. The same tendency was also observed in previous applications (e.g., Refs. [18,19]). Hence $\varepsilon_p = 4$ appears to be optimal in the current computational model used in the present study.

To calculate the protein dielectric effect appropriately, dipoles such as the permanent dipole and the induced dipoles should be considered appropriately. For instance, in a "semi-microscopic model" the space fixed dipoles (Langevin dipoles) contribute to reproducing the polarization effect appropriately [20,21]. The dipoles can orient in response to ionization of the titratable groups in the protein. Thus, mere removal of atomic charges of the titratable groups in the protein would cause the reorientation of the dipoles. On the other hand, in the used computational approach (so-called "macroscopic model" [22]), atomic partial charges that account for nuclear polarization effects are considered explicitly, including the induced nuclear polarization effects exerted by the change of protonation states of the protein. Mobile salt ions of the solution are described by an ionic strength. In the present study, flexibility of protein structure was not considered explicitly. However, protonation states of all titratable sites in the whole protein were considered explicitly, i.e., the flexibility of protein charges where all the titratable sites are simultaneously equilibrated. It is important to note that, in general the protonation states of the titratable groups are modulated in response to protonation state of the titration target Lys115. Here, the calculated pKa(Lys115) is not an "intrinsic pKa value ($pK_a^{intr}$)". Note that ($pK_a^{intr}$) can be described as:

$$pK_a^{intr} = pK_a^{model} - \frac{1}{RT \ln 10} (\Delta\Delta G^{Born} + \Delta\Delta G^{back}) \qquad \text{(eq. 1)}$$

where $pK_a^{model}$ is a pKa value of the reference model system, $\Delta\Delta G^{Born}$ is a Born energy term, and $\Delta\Delta G^{back}$ is an energy term associated with the background charge (i.e., charges of non-titratable residues and protein backbone) [22]). Hence reorientation of dipoles in the semi-microscopic model could correspond to the protonation pattern change in the macroscopic model. Thus, to estimate influence of the residue atomic charges on pKa(Lys115), mere removal of atomic charges of the titratable groups in the protein would cause changes in the protonation state of titratable residues. In this case, the resulting shift in pKa(Lys115) does not correctly refer to the "direct" contribution of the residue atomic charges to pKa(Lys115) since the resulting pKa(Lys115) shift contains an artifact as the protonation pattern change induced by elimination of the residue atomic charges. Therefore, to calculate the influence of the atomic charges on pKa(Lys115), the following procedure was specifically taken in the present study: first, the atomic charge values of all titratable groups in the protein were accordingly fixed to the protonation probabilities of all titratable groups in the pKa(Lys115) calculation (i.e., at [protonated Lys115] = [deprotonated Lys115] sampled by the MC method with Karlsberg [14]). Then, without allowing changes of the protein protonation pattern, the residue influence on pKa(Lys115) was calculated by turning off the atomic charges of the focusing residue. That is, the residue contribution to the pKa(Lys115) shift was obtained by calculating the $pK_a^{intr}$(Lys115) shift for both the presence and absence of the focusing residue charges. As a consequence, the residue contribution to pKa(Lys115) originates solely from the $\Delta\Delta G^{back}$ term difference (eq. 1) between the presence and absence of the focusing residue charges.

## RESULTS AND DISCUSSION

**Calculated pKa(Lys115) in the monomer form.** By using the crystal structures, pKa(Lys115) values were calculated to be 5.73 for CaAAD and 5.96 for CvAAD in the AADase monomer form. Calculated values of pKa(Lys115) ≈6 are in excellent agreement with the value of 5.96 measured previously for CaAAD using a reporter molecule [1] (Table 1). With respect to pKa(Lys) = 10.4 in aqueous solution [2], the calculated (and measured) pKa(Lys115) are significantly downshifted by >4.

The calculated pKa(Lys115) ≈2 in the uncharged CaAAD and CvAAD proteins indicate that the protein volume, rather than charge-charge interaction, is the dominant factor that lowers pKa(Lys115) by ≈8 in AADase. In contrast, "protein atomic charges" promote protonation at Lys115, upshifting pKa(Lys115) by ≈3.6 (Figure 2, Table 1). The upshift is due to the solvation effect by protein The magnitude of the pKa(Lys115) upshift due to the protein atomic charge (≈3.6) is smaller than the magnitude of the pKa(Lys115) downshift due to the protein volume (≈8). As a consequence pKa(Lys115) is considerably low in the protein.

**Influence of charged residues on pKa(Lys115).** Since Lys115 and Lys116 are neighbors in the sequence, Westheimer hypothesized that the low pKa(Lys115) value was due to energetically unfavorable charge-charge repulsion between the two basic residues [23]. However, recent X-ray crystal structure revealed that the side chains of Lys115 and Lys116 are oriented in the opposite direction ($N\zeta_{115}$-$N\zeta_{116}$ distances: 14.8 Å in CaAAD and 14.1 Å in CvAAD, Figure 1) [3]. Indeed, the influence of Lys116 on pKa(Lys115) is with –0.2 marginal (Table 2). Note that Lys116 is fully protonated (i.e. positively charged) and exposed to the bulk solvent. Thus, the weak influence of Lys116 on pKa(Lys115) is due to the large distance from Lys115: the electrostatic influence of Lys116 is fully shielded by bulk water ($\varepsilon_w$ = 80) before it reaches the Lys115 site.

In the present study, among all titratable sites the influences of Arg29, Glu61, and Glu76 are remarkably large in the native AADase (Table 2). Arg29 downshifts pKa(Lys115) by ≈3-4 due to charge-charge repulsion but the downshift is compensated by negatively charged residues such as Glu61 and Glu76. Ho et al. proposed that Arg29, Glu61, and Glu76 do not cause the pKa perturbation of Lys115 based on the experimental results that the optimum pH values of the enzymatic activities were not altered significantly in the R29Q, E61Q, and E76Q CvAAD proteins [3]. However, this does not always mean that these charged residues are not impacting the pKa(Lys115) in the native CvAAD. The following examples imply that mutational studies do not always *directly* point to the nature of the residues in question in the "*native*" protein;

**a) E76Q mutant.** Under physiological conditions (e.g., at pH 7 in bulk water), Glu residues are deprotonated and negatively charged. On the other hand, the calculated pKa(Glu76) is 8 in CvAAD (Table 3), i.e., Glu76 is protonated at pH 7, implying that the protonated Glu76 in the native CvAAD will actually behave like Gln. Indeed, experimental measurements by Ho et al. demonstrated that the E76Q mutation did not alter the optimum pH value for the enzymatic activity [3]. Thus, from the present results, Glu76 is revealed to be already protonated even in the native CvAAD. This can explain why the optimum pH value for the enzymatic activity remains essentially unchanged in the E76Q mutation.

**b) E61Q mutant.** The calculated pKa(Glu61) is 4.83 in CvAAD, implying that this residue is ionized in the native CvAAD (Table 3). The ionized Glu61 should stabilize protonated Lys115, thus promoting the pKa(Lys115) upshift by 2.5 in the native CvAAD (Tables 2 and 4). The elimination of the ionized Glu61 in the E61Q mutation, in turn, would result in the decrease in pKa(Lys115) by 2.5 if changes in the protonation pattern of the titratable residues in CvAAD did not occur. However, calculated pKa(Lys115) are almost equal (5.96 and 5.60 for the native structure and the E61Q mutant structure, respectively (not shown)). Thus, there must be associated changes in the protonation state of some titiratable residues in response to the E61Q mutation.

Indeed, the E61Q mutation is accompanied by further deprotonation of Glu76 (Table 4). The more ionized Glu76 in the E61Q mutant can stabilize protonated Lys115 more strongly. As a consequence, the loss of Glu61 in the E61Q mutation can be compensated by further deprotonation of Glu76. In addition, the total influences of residues 61 and 76 on pKa(Lys115) are 5.42 for the native CvAAD and 5.36 for the E61Q mutant (Table 4). These results imply the strong coupling of protonation states of the titratable residues, where changes in the protonation pattern of these residues can buffer the pKa(Lys115) change and maintain the deprotonated state of Lys115 required for the enzymatic activity.

Hence mutant studies of E61Q by Ho et al. should be interpreted that the unaltered pKa(Lys115) in the E61Q mutant is due to charge compensation by the change of the Glu76 protonation state, and not that Glu61 has no electrostatic effect on pKa(Lys115) in the native CvAAD.

**pKa(Lys115) in the dodecamer form.** AADase is known to exist as a homododecameric enzyme in solution [24]. By using atomic coordinates of the crystallographic dodecamer form, pKa(Lys115) was calculated to be 5.37 for the CaAAD dodecamer and 6.30 for the CvAAD dodecamer (Table 1). The influence of atomic charges on pKa(Lys115) remains essentially unchanged in the monomeric and dodecameric forms of CaAAD. A slightly different pKa(Lys115) for the CvAAD monomer and dodecameric forms (≈0.5, a very marginal difference, Table 1) may be attributed to different electrostatic characters in the subunit interface in CaAAD (hydrophobic) and CvAAD (ionic) as pointed out in Ref. [3]. In conclusion, pKa(Lys115) does not differ in the monomeric and dodecameric forms. The unusually lowered pKa(Lys115) is determined predominantly by the protein environment of the AADase monomer unit.

## CONCLUSION

Although charged residues impact pKa(Lys115) considerably in the native protein, the significant pKa(Lys115) downshift in AADase is mainly due to the absence of solvation energy in the protein inner core. The significantly lowered pKa(Lys115) value is determined by the AADase monomer unit: pKa(Lys115) is essentially the same in the dodecamer form.

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**FIGURE LEGENDS**

**Figure 1**. Location of the charged residues in the CaAAD catalytic site.

**Figure 2**. pKa(Lys) shift from aqueous solution to the CaAAD catalytic site. The meshed arrow indicates the pKa(Lys115) shift due to the protein volume and the open arrow indicates pKa(Lys115) shift due to the protein atomic charges.

**Table 1**: Influence of the protein environment on pKa(Lys115) of CaAAD and CvAAD in terms of pKa units.

| | **monomer** | | **dodecamer** | |
|---|---|---|---|---|
| | **CaAAD** | **CvAAD** | **CaAAD** | **CvAAD** |
| **pKa values** | | | | |
| pKa(Lys115), calculated | 5.73 | 5.96 | 5.37 | 6.30 |
| pKa(Lys115), experimental | (5.96 [a]) | | | |
| pKa(Lys) in water (as reference) | (10.4 [b]) | (10.4) | (10.4) | (10.4) |
| pKa(Lys115), uncharged AADase protein | 2.11 | 2.29 | 1.90 | 2.08 |
| **pKa shifting components** | **[amount of shift, Δ(p*K*a(Lys115))]** | | | |
| protein volume (uncharged protein) [c] | –8.29 | –8.11 | –8.50 | –8.32 |
| atomic charges [d] | 3.62 | 3.67 | 3.47 | 4.22 |

[a] See Ref. [1].

[b] See Ref. [2].

[c] Contribution of protein volume to pKa(Lys115): (pKa(Lys115), uncharged AAD protein) – (reference).

[d] Contribution of protein atomic charges to pKa(Lys115): (pKa(Lys115), calculated) – (uncharged protein).

**Table 2**: Contributions of atomic charges of key residues to the pKa(Lys115) value (in pKa units) in the CaAAD and CvAAD monomer. All atoms of Gly and Pro residues are treated as belonging to the protein backbone for this table.

| | CaAAD | | | CvAAD | | |
|---|---|---|---|---|---|---|
| **residues** | **side.**[a] | **b.b.**[b] | **total** | **side.**[a] | **b.b.**[b] | **total** |
| Arg29 | –4.27 | –0.11 | –4.38 | –3.45 | –0.09 | –3.54 |
| Arg59 | –1.34 | 0.09 | –1.25 | –1.01 | 0.07 | –0.94 |
| Glu61 | 1.09 | –0.05 | 1.04 | 2.54 | –0.02 | 2.52 |
| Glu76 | 6.66 | –0.16 | 6.50 | 3.07 | –0.17 | 2.90 |
| Asp91 | 1.77 | 0.04 | 1.81 | –[d] | –[d] | –[d] |
| Ala103/Pro103 [c] | 0.02 | 1.84 | 1.86 | –[d] | 3.04 | 3.04 |
| Gly107 | –[d] | –1.38 | –1.38 | –[d] | –1.85 | –1.85 |
| Lys116 | –0.21 | 0.35 | 0.14 | –0.22 | 0.32 | 0.10 |

[a] Side chain.

[b] Backbone.

[c] Ala103 for CaAAD and Pro103 for CvAAD.

[d] not applicable.

**Table 3**: Calculated pKa values of key titratable residues in the CaAAD and CvAAD monomers in pKa units.

| | **CaAAD** | **CvAAD** |
|---|---|---|
| Arg29 | 11.38 | 12.35 |
| Arg59 | 12.50 | 11.33 |
| Glu61 | 8.20 | 4.83 |
| Glu76 | 5.56 | 8.03 |
| Lys115 | 5.73 | 5.96 |
| Lys116 | 11.07 | 9.90 |

**Table 4**: Changes in the influence of Glu61 and Glu76 on pKa(Lys115) in the E61Q mutation of CvAAD: protonation probability (in $H^+$ unit) and the atomic charge contribution to the pKa(Lys115) value (in pKa units). Values were obtained in the presence of 50 % protonated Lys115, i.e., when pKa(Lys115) is obtained.

| **native** | | | | | **E61Q** | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **$[H^+]$** | **side.[a]** | **b.b.[b]** | **total** | | **$[H^+]$** | **side.[a]** | **b.b.[b]** | **total** |
| Glu61 | 0.02 | 2.54 | –0.02 | 2.52 | Gln61 | –[c] | –0.10 | –0.02 | –0.12 |
| Glu76 | 0.51 | 3.07 | –0.17 | 2.90 | Glu76 | 0.02 | 5.65 | –0.17 | 5.48 |
| (61+76) | | 5.61 | –0.19 | **5.42** | (61+76) | | 5.55 | –0.19 | **5.36** |

[a] Side chain.

[b] Backbone.

[c] not applicable.

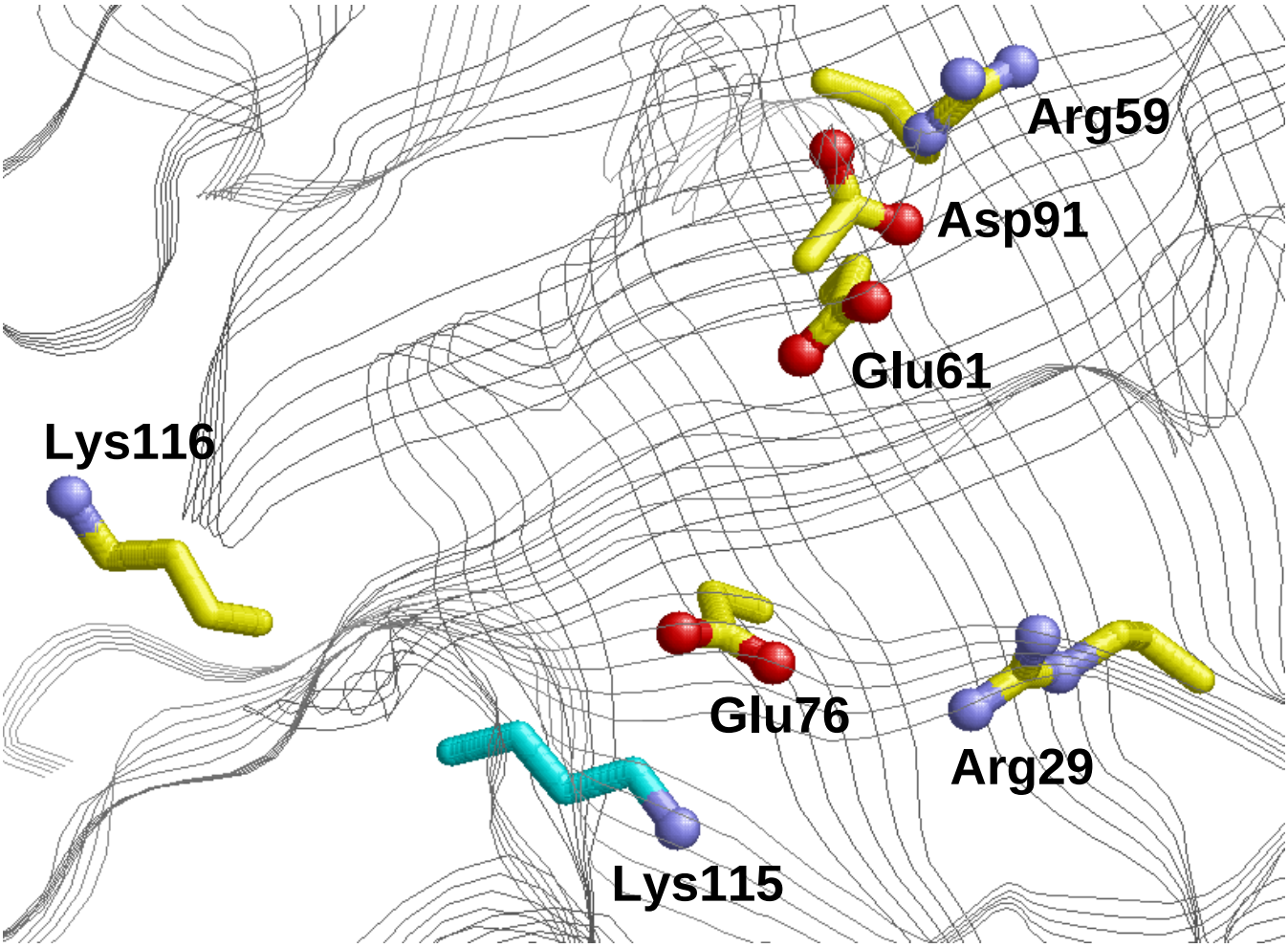


Figure 1.

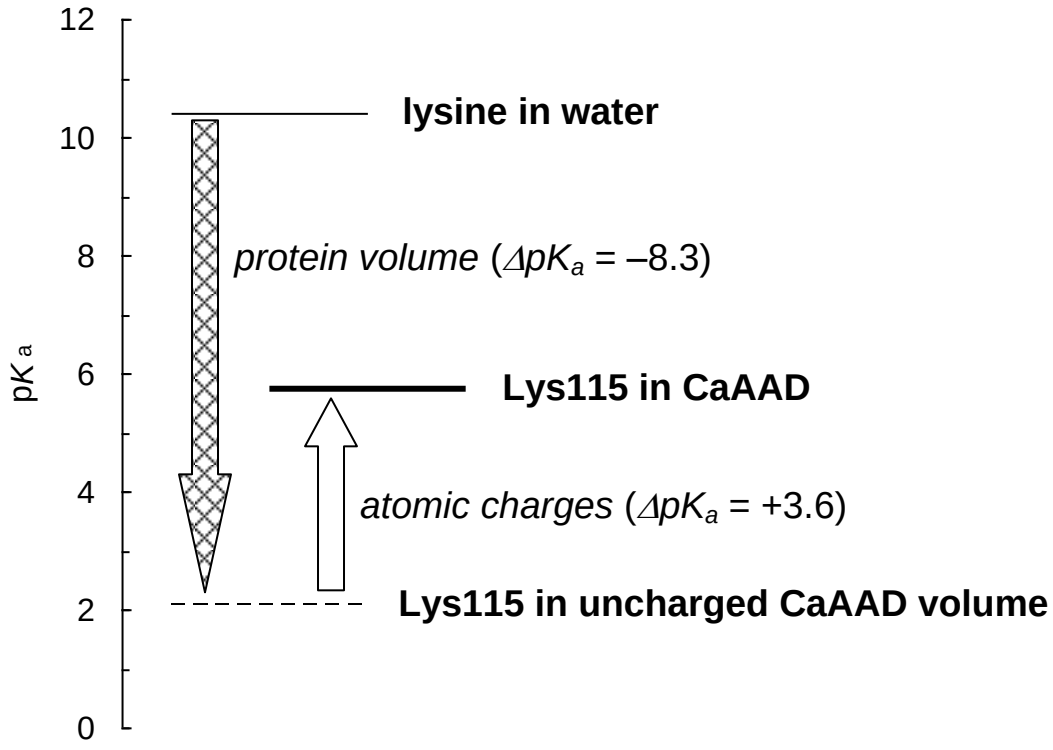
12
10
8
6
4
2
0
pK a
lysine in water
protein volume (ΔpK_a = –8.3)
Lys115 in CaAAD
atomic charges (ΔpK_a = +3.6)
Lys115 in uncharged CaAAD volume

Figure 2.