# Does protein A mirror image exist in solution?

# Outline of an experimental design aimed to detect it

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## ABSTRACT

There is abundant theoretical evidence indicating that a mirror image of *Protein A* may occur during the protein folding process. However, as to whether such mirror image exists in solution is an unsolved issue. Here we provide sound theoretical evidence indicating that the use of a mutant of protein A, namely Q10H, could be used to detect the mirror image conformation in solution. Our results indicate that the native conformation of the protein A should have a pKa, for the QH10 mutant, at ≈6.1, while the mirror-image conformation should have a pKa close to ≈7.3. Naturally, if all the population is in the native state for the Q10H mutant, the pKa should be ≈6.1, while, if all are in the mirror-image state, it would be ≈7.3, and, if it is a mixture, the pKa should be larger than 6.1, presumably in proportion to the mirror population. In addition, evidence is provided indicating the tautomeric distribution of H10 must also change between the native and mirror conformations. Although this may not be completely relevant for the purpose of determining whether the protein A mirror image exists in solution, it could provide valuable information to validate the pKa findings.

## Introduction

A mirror image conformation is one that looks approximately like the specular image of the native state. We say approximately because we do not require the amino acids to be specular images, but only the overall topology of the molecule. At least for some proteins, the mirror image will be energetically very close to the native state and thus it could also exist in solution. Among these proteins, we will focus our attention on the B-domain of staphylococcal protein A [PDB ID: 1BDD; a three–helix

bundle].[1] This protein has been the subject of extensive theoretical[2–6] and experimental[1,7–12] studies because of its biological importance and small size. In contrast to this, the mirror-image conformation has been subject of limited discussion.[2–4,6,12] The reason for this might be that the mirror image conformation of this protein has been observed only in some theoretical studies with different force fields but it has never been detected experimentally. As to whether this conformation is an artifact of the simulations or is difficult to observe the conformation experimentally, remains to be solved.

At this point, it is worth noting that the difficulties for experiments to detect the mirror-image conformation arise precisely because the secondary structures of the mirror-image and the native conformation of protein A are identical and the structural difference between these conformations are subtle.[6] Because of this, use of simple experiments such as circular dichroism, used to estimate the fraction of secondary-structure content, or more sophisticative technique, such as nuclear magnetic resonance (NMR) spectroscopy, e.g., to monitor the $^{13}C$ chemical shift changes that may occur at residue-level,[6] are useless for an accurate characterization of the mirror image conformation. A strong motivation to propose alternative methods to explore the possible coexistence in solution of the native and mirror-image conformation of protein A, comes from older evidence indicating that the mirror-image conformation could be a possible solution to the NMR-determined structure of protein A.[1] Indeed, according to Gouda et al.,[1] "*...distance-geometry calculations resulted in 41 solutions, which had correct polypeptide folds excluding 14 mirror-image substructures...*" However, the mirror-image structures were excluded from the analysis of Gouda et al.[1] without providing any reason. It seems that the decision was adopted because the "mirror-image" satisfies the NOE constraints but contain D-amino acid residues (personal communication with Ichio Shimada).

Overall, we propose here a proof-of-concept of an experimental design aimed to solve this problem. Initially we will show, by using ROSETTA,[13] that a mutant of protein A, hereafter the Q10H protein, exhibits the ability to fold into the native conformation (see Figure 1) as well as into the mirror-image conformation (see Figure 2). Later, we estimate the fraction of the native and mirror-image populations of the protein Q10H by using a recently introduced method, that take into account the protein dynamics in water by using a constant-pH MD simulation, to accurately determine the pKa values of ionizable residues, and fractions of ionized and tautomeric forms of histidine (His), in proteins at a given fixed pH.[14] Indeed, we explore the dependence of the electrostatically-calculated pKa and fractions of the imidazole ring forms of H10 as a function of pH for both the native-like and mirror-image conformations present in solution to the extent of 100%.

## Results

### Analysis of the pKa variations as a function of pH

Figure 2 shows a superposition of the lowest-energy conformations for both the native-like (green-ribbon) and the mirror-image (yellow-ribbon) of protein Q10H obtained by using ROSETTA.[13] These two structures were used to compute[15] for each ionizable residue along the sequence the value of the pKa variations ($\Delta = [pK^a_{Native} - pK^a_{Mirror}]$) at pH 7.0. The result of this analysis is shown in Figure 3 (as blue dots) where one of the largest change in $\Delta$, namely larger than $\pm 1.0$ pK units, occurs for H10. This large shift on the pKa of residue H10 appears to be a consequence of the close proximity of H10 to D38 in the mirror-image conformation of protein Q10H (see Figure 2).

There is another change of $\Delta$ larger than $\pm 1.0$ pKa unit and it occurs for residue K8 (see blue dots in Figure 3), a residue that pertains to the flexible N-terminal region of the mutant protein Q10H, viz., ranging from residues T1 trough E9. The origin of the large computed shift in the pKa of residue K8 is the following. In the native structure of protein Q10H residue K8 is well exposed to the solvent. On the other hand, in the mirror image structure of this protein residue K8 is close to E16, making a favorable electrostatic interaction. However a close inspection of these two structures indicates that the favorable electrostatic-interaction between K8 and E16, observed in the mirror image conformation, could also occur on the native conformation, e.g., by a rearrangement of the backbone-torsional angles of the flexible N-terminal region of the protein Q10H. If this were feasible, the computed pKa shift for K8 should be $\approx 0$. Consequently, monitoring the pKa shift of K8 does not appear to be the right choice for the purpose of an accurate determination of the coexistence between the native and the mirror image states in solution. Unlike the origin of the pKa shift for K8, the interaction between H10 and D38 cannot take place in both the native and the mirror-image conformations (see Figure 2) and, hence, from here on we will focus our attention on H10 only.

At this point is worth noting the following. Consideration of the protein dynamics in water is very important for an accurate computation of conformational-dependent values, such as the pKa's. However, this effect was not tacked into account in the computation of the $\Delta$ values shown, as blue-dots, in the Figure 3. Consequently, we carried out a constant-pH MD simulation of both the native-like Q10H mutant and its mirror-image conformations.[14] During the simulations, at constant-pH 7.0, it is reasonable to consider all acid (Asp, Glu) and base (Lys, Arg) residues in the ionized state, because their respective $pK_0$'s (3.5, 4.0, and 10.5, 12.5, respectively) are shifted by more than 2.5 pK units from the pH (7.0) at which the calculations were carried out. For the same reason, the only Tyr in the sequence was consider as neutral. However, histidine residue pKa's (6.5) can vary

considerably at pH 7.0 at which the calculations are carried out and, hence, consideration of histidine ionization states for each of the imidazole ring of His forms must be considered explicitly. Consequently during the calculations the nine ionizations states of the two interacting His, namely between H10 and H19, were explicitly consider.[16] The average $\Delta$ change for H10, computed from the native-like and mirror-image conformations after 10ns MD simulations, is shown as an orange dot in Figure 3. Similarly, the computed average change for the imidazole ring forms of H10 as a function of pH for both the native like and the mirror image conformations are display in Figure 4. As shown in this Figure at a given fix pH, e.g., at pH=8.0, there are significant changes among the computed fractions of the imidazole ring forms of H10.

In general, the results shown in Figures 3 and 4 are decisive for the determination of the fraction of native and mirror image conformations in solution. Indeed, if the dominant conformation in solution is the native like then the pKa of H10 will be 6.1$\pm$0.2. On the other hand, if the dominant conformation in solution is the mirror image then the pKa will be 7.3$\pm$0.2. Any other in-between value may indicate coexistence of these two conformations in solution.

### Validation of the H10 pKa-based predictions

Small changes around the computed average pKa value for H10 (6.1) are of course possible. In such a case additional experiments are necessary to determine whether such shift is due to expected fluctuations of the native conformation (around $\pm$0.2 in pKa units) or to the presence of a small fraction of the mirror-image conformation. One such additional experiment could be the determination of the tautomers of the imidazole ring of H10. In this section we analyze this possibility by using two NMR-based methods.

First, as shown in Figures 4, there is a large change in the average fractions of H10 tautomers as a function of pH. In particular, if the population of the native conformation is dominant in solution ($\approx 100\%$) then, as shown in Figure 4, the fraction of the protonated form should be $\approx 0\%$ at pH $\approx 8.0$. In other words, only the imidazole ring of H10 tautomers will be present in solution at this pH. Therefore, their relative populations can be determined accurately by measuring the one-bond CH, ${}^1J_{CH}$, Spin-Spin Coupling Constants (SSCC) of the imidazole ring of H10.

Let us explain this in detail. Under the only condition that His is non-protonated, we have been able to show that the fraction of the $N^{\delta 1} - H$ tautomeric form ($f^{\delta 1}$) of the imidazole ring of His can be estimated by using the following equation: $f^{\delta 1} = (J^{obs} - 165.0)/15.0$,[17] where $J$ refers to ${}^1J_{C\delta 2H}$ SSCC, and here $obs$ is the observed value in solution for H10. Naturally, $f^{\varepsilon 2} = 1 - f^{\delta 1}$. Hence, if the native-like structure is the dominant topology in solution, then the following inequality should hold: $f^{\varepsilon 2} \gg f^{\delta 1}$ (see Figure 4) otherwise there would be coexistence of the native-like structure with other topology in solution.

As a second, but less restrictive, validation test we can use a recently proposed NMR-based methodology aimed to determine the tautomeric forms as a function of the ionization state of the imidazole ring of histidine.[18] In this approach, the average tautomeric fraction of the $N^{\varepsilon 2}-H$ form of His ($f^{\varepsilon 2}$) can be determined by using the following equation: $f^{\varepsilon 2}=\Delta^{obs}(1-f^{H+})/\Delta^{\varepsilon}$ where $f^{H+}$ is the experimentally determined fraction for the ionized form of H10, at a given fix pH; $\Delta^{obs}=|^{13}C^{\delta 2}-^{13}C^{\gamma}|$, where $^{13}C^{\delta 2}$ and $^{13}C^{\gamma}$ are the NMR-observed chemical shifts for the imidazole ring of H10 at that pH; and $\Delta^{\varepsilon}$ is the first-order absolute shielding difference, $|^{13}C^{\delta 2}-^{13}C^{\gamma}|^{\varepsilon}$, between the $^{13}C^{\delta 2}$ and $^{13}C^{\gamma}$ nuclei for the $N^{\varepsilon 2}-H$ tautomer, i.e., present to the extent of 100%. $\Delta^{\varepsilon}$ is a parameter which must be estimated.[18] As a first approximation, a $\Delta^{\varepsilon}=27.0ppm$, obtained from the analysis of a His-rich protein,[16] namely *Loligo vulgaris* (pdb id 1E1A), a 314-residue all-$\beta$ protein,[19] should be used. Naturally, the $f^{\delta 1}$ fraction, viz., for the $N^{\delta 1}-H$ tautomer, is obtained straightforwardly as: $f^{\delta 1}=1-f^{H+}-f^{\varepsilon 2}$. Although this second approach to compute the tautomers of H10 it is more general than the previous one, i.e., by using the $^{1}J_{C\delta 2H}$ SSCC, the determination of the $^{13}C^{\gamma}$ chemical shift it is not always feasible. Indeed, only 213 $^{13}C^{\gamma}$, versus 6,984 $^{13}C^{\delta 2}$, chemical shifts of the imidazole ring of histidine have been deposited in the Biological Magnetic Resonance data Bank (BMRB).[20] Overall, if it were feasible to observe the $^{13}C^{\gamma}$ chemical shift we suggest to use both approaches to validate the pKa predictions.

Although this work is not intended to be a revision of all existing methods used to determine the tautomeric forms of the imidazole ring of His, the use of the tautomeric identification by direct observation of $^{15}N$ chemical shifts of the imidazole ring of His, which is a common practice in NMR spectroscopy,[21–23] should be mentioned. This method requires, as a necessary condition, knowledge of the *canonical* limiting values of the $^{15}N$ chemical shift of the imidazole ring of His in which each form of His is present to the extent of 100%. In this regard, there is theoretical evidence indicating that a considerable difference for the average tautomeric equilibrium constant, $K_T$, can be obtained if DFT-computed $^{15}N$ limiting values rather than canonical limiting values are used.[24] Because these results raise concerns about the magnitude of the uncertainty associated with the predictions we did not consider this method as an alternative to the above-proposed tests to validate the pKa predictions.

All in all, the estimated tautomeric forms of the imidazole ring of His are certainly not enough to accurately determine whether the coexistence of native-like and mirror-image structures occurs in solution but it could be of valuable assistance to validate the determination made by the pKa analysis.

## Discussion

We provided a proof-of-concept of an experimental design that could be used to detect the coexistence of native and mirror-image conformations for the Q10H mutant of protein A in solution. Determination of the pKa values of the ionizable residue H10 should provide a quick answer to this problem. Additionally the NMR-determination of the one-bond vicinal coupling constant or the chemical-shifts of the imidazole ring of H10 could be used to validate this finding. There are two main advantages of the proposed methodology. Firstly, there is no need for 3D structural information and, secondly, a validation test can be carried out by standard NMR-based experiments.

Whatever the output of the proposed experiments is, we will find them interesting. Indeed, if the results don't indicate the presence of the mirror image, all the theoretical predictions about the existence of the mirror image, published so far, would be *only* of Academic interest, perhaps, reduced only to show a possible intermediate conformational state in the pathway of protein folding. On the other hand, if the experiments provide evidence that there is structural coexistence, then the theoretical predictions will have a sound basis and, even more important, it may spur significant progress in the conformational analysis of proteins with mirror-images.

## Methods

In this section we will give a brief reference to existent theoretical methods aimed to predict (*i*) the 3D structure of proteins accurately;[25] or determine (*ii*) the pKa values of ionizable residues and fractions of ionized and tautomeric forms of histidine (His) and acid residues in proteins, at a given fixed pH.[14,15]

### Determination of the native and image-mirror conformations of protein Q10H

To generate the native and mirror-image conformations of protein A Q10H we used the fast-relax protocol from Rosetta,[13,26] this is an all-atom refinement protocol that basically consists of several rounds of repacking and energy minimization. The repulsive part of the Van der Waals energy function is annealed from 2% to 100%. Essentially the algorithm explores the local conformational space around the starting structure with a radius of 2 to 3 Å of rmsd (for the $C^{\alpha}$). We performed several rounds of fast-relax using a genetic-like algorithm.

1. For a given conformation of protein A mutate it by replacing Q10 with H10

2. Use the mutant as the starting point of 200 independent rounds of the fast-relaxation protocol

3. Choose 10 conformations; 2 at random and the 8 lowest-energy conformations
4. For each one of those conformations use fast-relaxation to generate 100 independent rounds (for a total of 1000 conformations)
5. repeat, from step 3, 40 times
6. keep the lowest energy conformation from all the rounds

We started from 3 different conformations. For the native conformation we used 1BDD.[1] For the mirror image we started from 2 points: a mirror-image conformation previously obtained by Vila et al,[3] an a mirror image folded *by hand*. We discarded all the conformations obtained from this last starting point as their energies were much more higher than for the other two starting points.

### Computation of the pKa and the tautomeric fractions of the imidazole ring of H10

The native-like and mirror-image conformations of protein Q10H, generated as describe in the previous section, were used as input files for the calculations of the pKa of *all* ionizable residues in the sequence as well as the fractions of the ionized $H^+$ and the tautomeric $N^{\varepsilon 2} - H$ and $N^{\delta 1} - H$ forms of the imidazole ring of H10. In particular, as it is well known, the tautomeric determination of the imidazole ring of His is both a very important problem in structural biology[27,28] and a challenging task.[29] For this reason, a recently introduced electrostatic-based method to determine the pKa values of ionizable residues and fractions of ionized and tautomeric forms of histidine (His) and acid residues in proteins,[14] is applied here to the analysis of protein A mutant Q10H. Protein dynamics in water, at a given pH=7.0, was taken into account by constant-pH MD simulation[16] of both the native and mirror-image conformations of the Q10H mutant.

The ionization constants pKa and the fractions of ionized and two neutral tautomers of histidine at constant pH 7.0 are modeled by MD simulations at constant pH. As mentioned in the main text, all acid (Asp, Glu) and base (Lys, Arg) residues were kept in the ionized state because their respective pKo's (3.5, 4.0, and 10.5, 12.5, respectively) are shifted by more than 2.5 pK units from the pH (7.0) at which the calculations were carried out. On the other hand, the two existent histidine residues, namely H10 and H19, were considered to be electrostatically couple residues having nine ionization states, namely, 00, 01, 02, 10, 11, 12, 20, 21, 22, where 0,1,2 represents the ionized and two neutral tautomer states, respectively.[14] Consequently, the average values and it's thermal fluctuations due to molecular dynamics in solvent are estimated along 10ns MD equilibrium trajectory for each of the nine ionization states. The average range of fluctuations of the atomic positions, i.e. in terms of the rmsd, observed along the MD simulations were 1.4 and 1.3Åfor the native-like and mirror-image structures, respectively.

## References


1. Gouda, H. *et al.* Three-Dimensional Solution Structure of the B Domain of Staphylococcal Protein A: Comparisons of the Solution and Crystal Structures. *Biochemistry* **31**, 9665–9672 (1992). DOI 10.1021/bi00155a020.

2. Olszewski, K. A., Kolinski, A. & Skolnick, J. Folding simulations and computer redesign of protein A three-helix bundle motifs. *Proteins: Struct. Funct. Genet.* **25**, 286–299 (1996). DOI 10.1002/(SICI)1097-0134(199607)25:3¡286::AID-PROT2¿3.0.CO;2-E.

3. Vila, J. A., Ripoll, D. R. & Scheraga, H. A. Atomically detailed folding simulation of the B domain of staphylococcal protein A from random structures. *Proc. Natl. Acad. Sci. United States Am.* **100**, 14812–14816 (2003). DOI 10.1073/pnas.2436463100.

4. Garcia, A. E. & Onuchic, J. N. Folding a protein in a computer: An atomic description of the folding/unfolding of protein A. *Proc. Natl. Acad. Sci.* **100**, 13898–13903 (2003). DOI 10.1073/pnas.2335541100.

5. Lee, B. M. *et al.* Induced fit and ”lock and key” recognition of 5 S RNA by zinc fingers of transcription factor IIIA. *J. Mol. Biol.* **357**, 275–291 (2006). DOI 10.1016/j.jmb.2005.12.010.

6. Kachlishvili, K. *et al.* Accounting for a mirror-image conformation as a subtle effect in protein folding. *Proc. Natl. Acad. Sci.* **111**, 8458–8463 (2014). DOI 10.1073/pnas.1407837111.

7. Deisenhofer, J. Crystallographic Refinement and Atomic Models of a Human Fc Fragment and Its Complex with Fragment B of Protein A from Staphylococcus aureus at 2.9- and 2.8-Å Resolution. *Biochemistry* **20**, 2361–2370 (1981). DOI 10.1021/bi00512a001.

8. Bai, Y., Karimi, A., Dyson, H. J. & Wright, P. E. Absence of a stable intermediate on the folding pathway of protein A. *Protein Sci.* **6**, 1449–1457 (1997). DOI 10.1002/pro.5560060709.

9. Myers, J. K. & Oas, T. G. Preorganized secondary structure as an important determinant of fast protein folding. *Nat. Struct. Biol.* **8**, 552 – 558 (2001). DOI 10.1038/88626.

10. Sato, S., Religa, T. L., Daggett, V. & Fersht, A. R. From The Cover: Testing protein-folding simulations by experiment: B domain of protein A. *Proc. Natl. Acad. Sci.* **101**, 6952–6956 (2004). DOI 10.1073/pnas.0401396101.

11. Dimitriadis, G. *et al.* Microsecond folding dynamics of the F13W G29A mutant of the B domain of staphylococcal protein A by laser-induced temperature jump. *Proc. Natl. Acad. Sci.* **101**, 3809–3814 (2004). DOI 10.1073/pnas.0306433101.

**12.** Noel, J. K. *et al.* Mirror images as naturally competing conformations in protein folding. *J. Phys. Chem. B* **116**, 6880–6888 (2012). DOI 10.1021/jp212623d.

**13.** Bradley, P., Misura, K. M. S. & Baker, D. Toward High-Resolution de Novo Structure Prediction for Small Proteins. *Science* **309**, 1868–1871 (2005). DOI 10.1126/science.1113801.

**14.** Vorobjev, Y. N., Scheraga, H. A. & Vila, J. A. Coupled molecular dynamics and continuum electrostatic method to compute the ionization pKa's of proteins as a function of pH. Test on a large set of proteins. *J. Biomol. Struct. Dyn.* **36**, 561–574 (2018). DOI 10.1080/07391102.2017.1288169.

**15.** Vorobjev, Y. N., Vila, J. A. & Scheraga, H. A. FAMBE-pH: A fast and accurate method to compute the total solvation free energies of proteins. *J. Phys. Chem. B* **112**, 11122–11136 (2008). DOI 10.1021/jp709969n.

**16.** Vorobjev, Y. N., Scheraga, H. A. & Vila, J. A. A comprehensive analysis of the computed tautomer fractions of the imidazole ring of histidines in Loligo vulgaris. *J. Biomol. Struct. Dyn.* **1102**, 1–12 (2017). DOI 10.1080/07391102.2017.1377636.

**17.** Vila, J. A. & Scheraga, H. A. Limiting values of the one-bond C-H spin-spin coupling constants of the imidazole ring of histidine at high-pH. *J. Mol. Struct.* **1134**, 576–581 (2017). DOI 10.1016/j.molstruc.2017.01.022.

**18.** Vila, J. A., Arnautova, Y. A., Vorobjev, Y. & Scheraga, H. A. Assessing the fractions of tautomeric forms of the imidazole ring of histidine in proteins as a function of pH. *Proc. Natl. Acad. Sci.* **108**, 5602–5607 (2011). DOI 10.1073/pnas.1102373108.

**19.** Scharff, E. I., Koepke, J., Fritzsch, G., Lücke, C. & Rüterjans, H. Crystal structure of diisopropylfluorophosphatase from Loligo vulgaris. *Structure* **9**, 493–502 (2001). DOI 10.1016/S0969-2126(01)00610-4.

**20.** Ulrich, E. L. *et al.* BioMagResBank. *Nucleic Acids Res.* **36**, 402–408 (2008). DOI 10.1093/nar/gkm957.

**21.** Pelton, J. G., Torchia, D. A., Meadow, N. D. & Roseman, S. Tautomeric states of the active-site histidines of phosphorylated and unphosphorylated IIIGlc, a signal-transducing protein from escherichia coli, using two-dimensional heteronuclear NMR techniques. *Protein Sci.* **2**, 543–558 (1993). DOI 10.1002/pro.5560020406.

**22.** Shimahara, H. *et al.* Tautomerism of histidine 64 associated with proton transfer in catalysis of carbonic anhydrase. *J. Biol. Chem.* **282**, 9646–9656 (2007). DOI 10.1074/jbc.M609679200.

**23.** Hass, M. A. S., Hansen, D. F., Christensen, H. E. M., Led, J. J. & Kay, L. E. Characterization of conformational exchange of a histidine side chain: Protonation, rotamerization, and tautomerization of His61 in plastocyanin from Anabaena variabilis. *J. Am. Chem. Soc.* **130**, 8460–8470 (2008). DOI 10.1021/ja801330h.

**24.** Vila, J. A. Limiting Values of the Histidine at High pH. *J. Phys. Chem. B* **116**, 6665–6669 (2012).

**25.** Bradley, P. *et al.* Rosetta Predictions in CASP5: Successes, Failures, and Prospects for Complete Automation. *Proteins: Struct. Funct. Genet.* **53**, 457–468 (2003). DOI 10.1002/prot.10552.

**26.** Chaudhury, S., Lyskov, S. & Gray, J. J. PyRosetta: A Script-Based Interface for Implementing Molecular Modeling Algorithms Using Rosetta. *Bioinformatics* **26**, 689–691 (2010). DOI 10.1093/bioinformatics/btq007.

**27.** Schnell, J. R. & Chou, J. J. Structure and mechanism of the M2 proton channel of influenza A virus. *Nature* **451**, 591–595 (2008). DOI 10.1038/nature06531.

**28.** Bermúdez, C., Mata, S., Cabezas, C. & Alonso, J. L. Tautomerism in Neutral Histidine. *Angewandte Chemie - Int. Ed.* **53**, 11015–11018 (2014). DOI 10.1002/anie.201405347.

**29.** Machuqueiro, M. & Baptista, A. M. Is the prediction of pKa values by constant-pH molecular dynamics being hindered by inherited problems? *Proteins: Struct. Funct. Bioinforma.* **79**, 3437–3447 (2011). DOI 10.1002/prot.23115.

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## Author contributions statement

J.A.V. conceptualize the project, A.O.M. and J.V. design the experiments; A.O.M. and Y.V. conducted the experiments, A.O.M., Y.V. and J.A.V. analyze the results, A.O.M, H.A.S. and J.A.V. write the manuscript. All authors reviewed the manuscript.

## Additional information

The authors declare no competing interests.

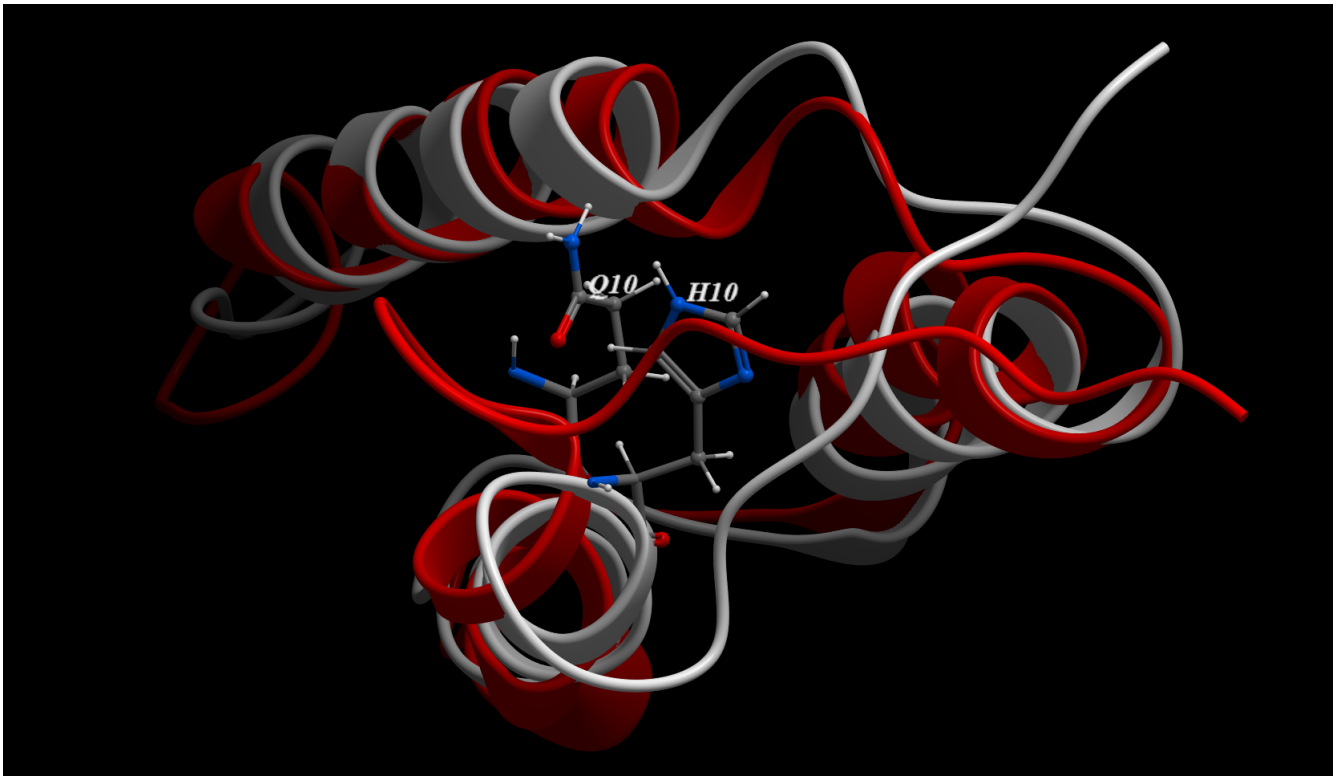


**Figure 1.** Red- and white-ribbon diagrams for the native structures of protein A (PDB ID 1BDD[1]) and the equivalent for protein Q10H, respectively. The position of the side-chain of Q10 and H10 for protein A and protein Q10H are highlighted. The $C^{\alpha}$ rmsd between the two native structures is 1.4 Å.

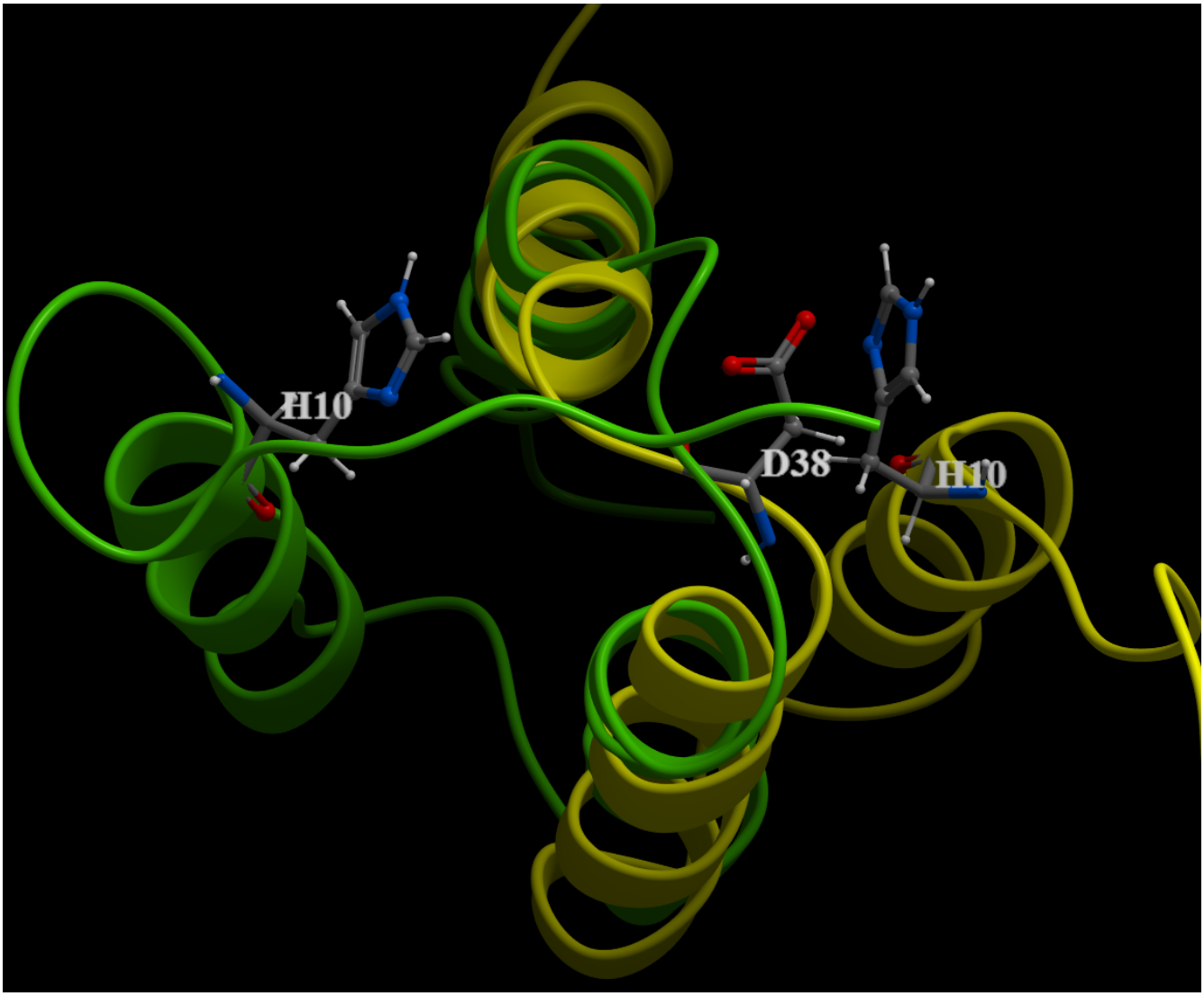


**Figure 2.** Green- and yellow-ribbon diagrams for the native and "mirror" image conformations of Protein A, respectively. The position of the side-chain of H10 is highlighted for each of these conformations. Moreover, the side-chain of D38 is also displayed to point out the close proximity between D38 and H10 in the "mirror" image conformation. The favorable electrostatic interaction between D38 and H10 may be responsible for the large ($\Delta \approx -1.2$) change in the computed pKa between the native-like and the "mirror-image conformations.

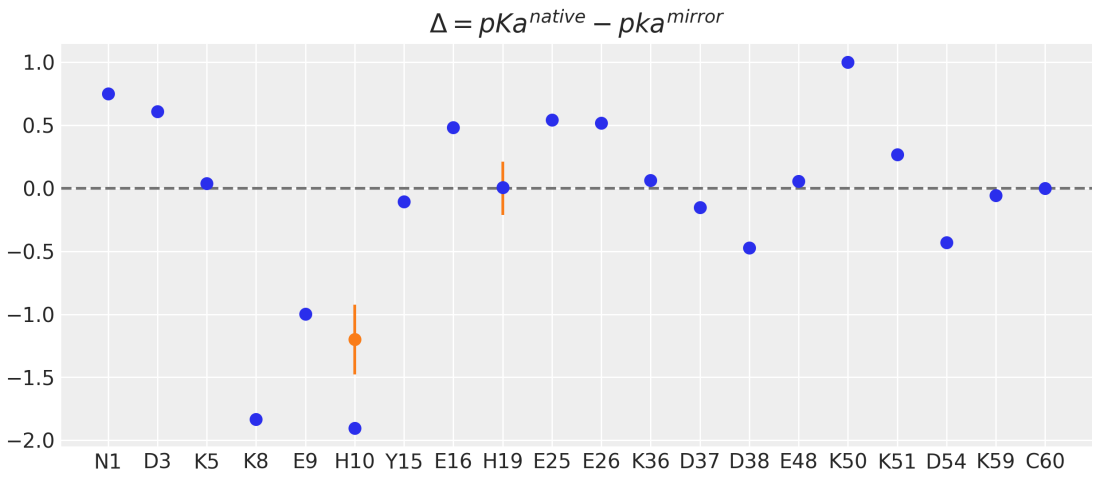


**Figure 3.** Dots indicate the pKa change (Δ), computed at pH 7.0, for each ionizable residue along the protein Q10H sequence. The blue-dots were computed from the single lowest-energy generated conformations of both the native-like and mirror-image topology, respectively. The orange-dots were computed for the two histidines in the sequence, namely H10 and H19, as an average over 10ns MD simulations for both the native-like and mirror-image conformations; vertical orange-lines denotes the standard deviations of the computed average Δ values

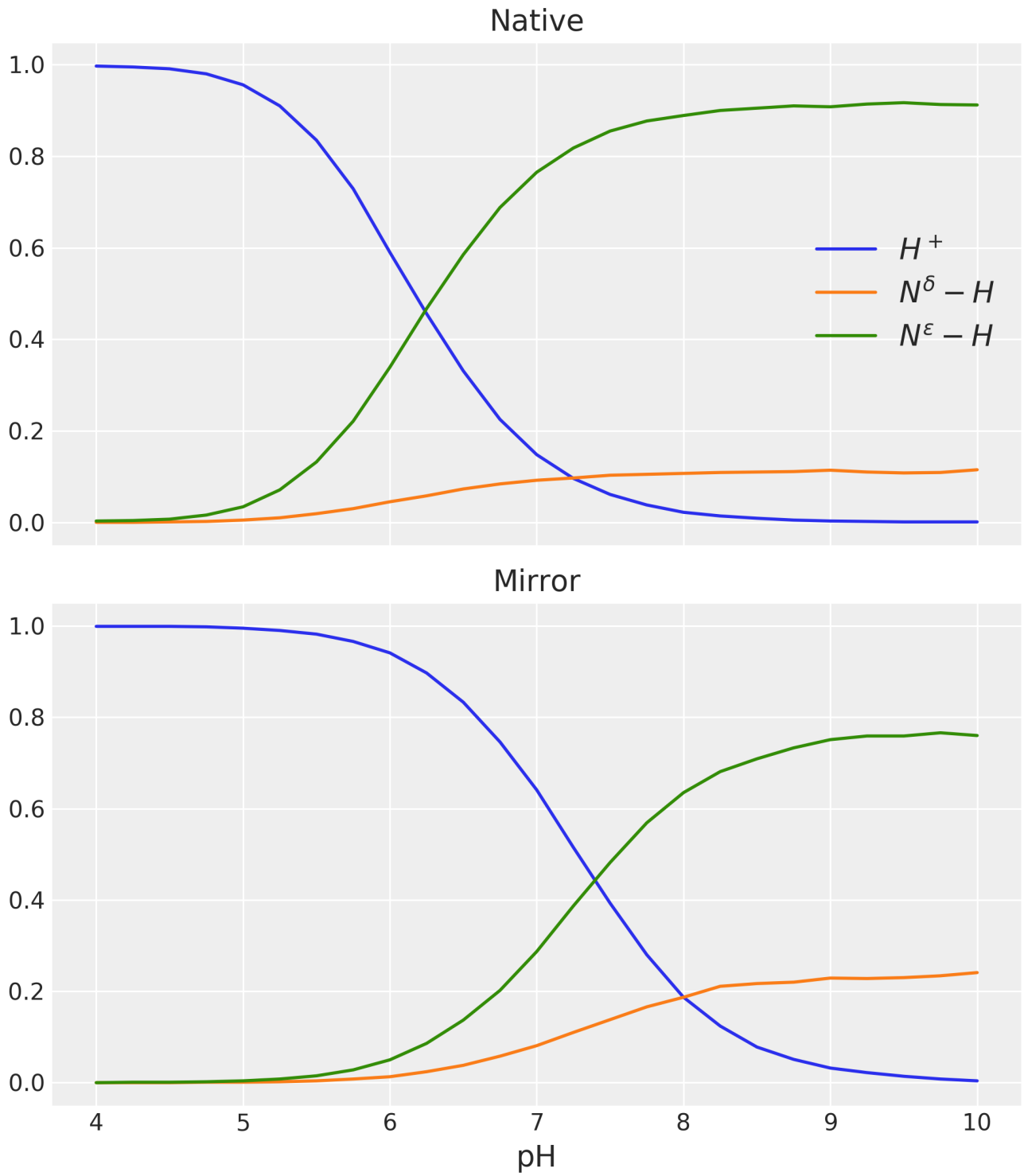


**Figure 4.** Fractions of the imidazol ring forms of H10 as a function of pH, for the ”Native” and ”Mirror” topologies of the Q10H mutant of protein A. The values, for each topology, are estimated along 10ns MD equilibrium trajectory for each of nine ionization states of two electrostatically-coupled histidines residues, namely H10 and H19