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Effect of pH on the Structure and DNA Binding of the FOXP2 Forkhead Domain

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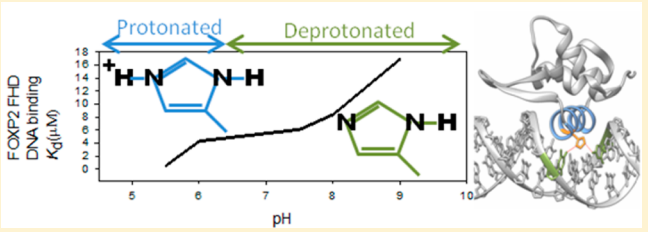
Supporting Information

ABSTRACT: Forkhead box P2 (FOXP2) is a transcription factor expressed in cardiovascular, intestinal, and neural tissues during embryonic development and is implicated in language development. FOXP2 like other FOX proteins contains a DNA binding domain known as the forkhead domain (FHD). The FHD interacts with DNA by inserting helix 3 into the major groove. One of these DNA−protein interactions is a direct hydrogen bond that is formed with His554. FOXP2 is localized in the nuclear compartment that has a pH of 7.5. Histidine contains an imidazole side chain in which the amino group typically has a pK_a of ∼6.5. It seems possible that pH fluctuations around 6.5 may result in changes in the protonation state of His554 and thus the ability of the FOXP2 FHD to bind DNA. To investigate the effect of pH on the FHD, both the structure and the binding affinity were studied in the pH range of 5−9. This was done in the presence and absence of DNA. The structure was assessed using size exclusion chromatography, far-UV circular dichroism, and intrinsic and extrinsic fluorescence. The results indicated that while pH did not affect the secondary structure in the presence or absence of DNA, the tertiary structure was pH sensitive and the protein was less compact at low pH. Furthermore, the presence of DNA caused the protein to become more compact at low pH and also had the potential to increase the dimerization propensity. Fluorescence anisotropy was used to investigate the effect of pH on the FOXP2 FHD DNA binding affinity. It was found that pH had a direct effect on binding affinity. This was attributed to the altered hydrogen bonding patterns upon protonation or deprotonation of His554. These results could implicate pH as a means of regulating transcription by the FOXP2 FHD, which may also have repercussions for the behavior of this protein in cancer cells.



The FOX (forkhead box) proteins make up a family of transcription factors that contain a highly conserved winged helix domain called the forkhead domain (FHD), which is typically involved in DNA binding.[1,2] Despite the conserved nature of the FHD, FOX proteins are functionally diverse where they are expressed in various organs and tissues and they function to either activate or repress transcription of various genes.[1] The forkhead domain is typically between 90 and 100 residues long and comprises three α-helices (H1−H3) and two less conserved winged structures (W1 and W2).[3,4] The forkhead domain binds DNA by insertion of helix H3 into the major groove,[5] and this is where specific interactions between the protein and the DNA bases occur.

The FOXP subfamily consists of four proteins (FOXP1−4) that share, in addition to the FHD, an N-terminal glutamine-rich region, a zinc finger, and a leucine zipper domain.[6] The FOXP FHD adopts the winged helix fold that is characteristic of FOX proteins;[2] however, the wing regions are shorter, and this appears to result in fewer nonspecific interactions with the DNA backbone than in other family members, resulting in a weaker interaction overall.[5] The FOXP FHD is unique among the FOX family members in that it not only exists as a monomer but also can form a domain-swapped dimer.[5] This may have interesting implications for the regulation of transcription.[7]

The main interactions formed between the FOXP2 FHD and DNA can be determined from the crystal structure.[5] These include direct H-bonds formed between Asn550 and His554 and the DNA bases as well as a water-mediated H-bond between Arg553 and the DNA. Indeed, the R553H mutation has been linked to an inherited language disorder, implicating FOXP2 in language development.[8] The protein also forms a number of H-bonds with the DNA phosphate backbone as well as extensive van der Waals interactions that stabilize the protein−DNA interaction.

Because the imidazole side chain of histidine residues has a pK_a of ∼6.5 that lies within the physiological pH range, histidine residues can be considered to be the amino acid most sensitive to physiological pH changes. Indeed, many histidine residues have been identified as pH sensitive switches that trigger structural[9−11] or conformational stability changes within a protein,[10,12] and they have even been shown to regulate changes in the activity of proteins in response to pH changes.[13]

Given that His554 forms a conserved interaction with DNA and a change in the protonation state of this residue may affect

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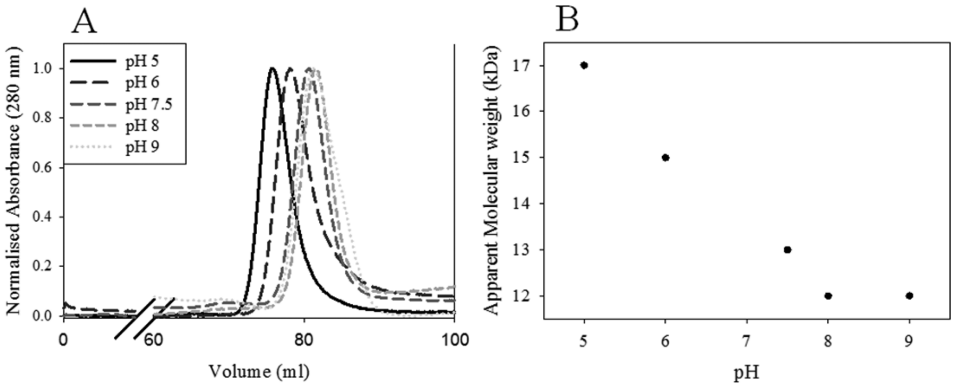

Figure 1. Size exclusion chromatography of the FOXP2 FHD. SEC was performed using a HiLoad 16/60 Superdex 75 prep grade column at pH 5, 6, 7.5, 8, and 9. (A) Size exclusion chromatography of the FOXP2 FHD at pH 5, 6, 7.5, 8, and 9. (B) Apparent molecular weight of the FOXP2 FHD at each pH.

this interaction, the focus of this study is on whether pH can influence the structure and stability of the FOXP2 FHD−DNA complex. Because FOXP2 is localized within the nuclear compartment,[14] it should typically be exposed to a pH of 7.5.[15] However, there is evidence that during the course of the cell cycle, there may be changes in the pH gradient across the cell.[16,17] The pK_a of His554 is predicted to be 6.25 on the basis of its local environment;[18−20] thus, at pH 7.5, this residue will be deprotonated. If the pH drops, the protonation of His554 can alter the H-bonding pattern with DNA. Here we investigate whether pH changes in the range of 5−9 can influence the structure and/or DNA binding properties of the FOXP2 FHD to hypothesize whether pH changes in the cell may influence how this protein regulates transcription.

EXPERIMENTAL PROCEDURES

Transformation, Expression, and Purification. Competent T7- *Escherichia coli* cells were transformed with the pET-11a plasmid containing the His-tagged FOXP2 FHD gene (Val503−Gln586). The gene was synthesized and codon-optimized for expression in *E. coli* by Genscript Corp. Soluble protein was purified using nickel affinity chromatography. Subsequently, the His tag was removed using thrombin cleavage, and the cleaved protein was further purified using nickel affinity chromatography and size exclusion chromatography.

pH Studies of the Structure of the FOXP2 FHD. To establish whether pH affects the quaternary structure or the hydrodynamic volume of the FOXP2 FHD, size exclusion chromatography was performed by loading 20 μM FHD onto a HiLoad 16/60 Superdex 75 prep grade size exclusion column at pHs ranging from 5 to 9. The buffers used in this study are listed in Table S1 of the Supporting Information. All subsequent pH-dependent studies were performed in the presence and absence of DNA. The DNA that was used for this study was a 20 bp duplex synthesized by IDT (Whitehead scientific). It has the core binding sequence 5′-TGTTTAC-3′.[21] For all protein−DNA studies, the FOXP2 FHD was mixed in a 3:1 ratio with DNA and incubated on ice for 15 min prior to the experiment. Far-UV CD was used to monitor the effect of pH on the secondary structure of the FHD in the presence and absence of DNA. Spectra were measured on 5−10 μM FOXP2 FHD at pH 5−9 using a Jasco J-1500 CD spectrometer at 20 °C. Far-UV CD spectra were recorded in the range of 250−180 nm, were averaged over three replicates, and were converted from θ (millidegrees) to θ_{MRE} (degrees per square centimeter per decimole). Changes in the tertiary structure of the FHD were monitored in the presence and absence of DNA using intrinsic fluorescence spectroscopy. This was done with 2 μM protein over a pH range of 5−9. The tyrosine and tryptophan residues were excited at 280 nm, and the emission spectra were recorded from 280 to 450 nm. Exposed hydrophobic patches were detected using the hydrophobic dye, ANS. The FHD in the presence and absence of DNA, and over the pH range of 5−9, was incubated with 200 μM ANS on ice for 30 min. The mixture was excited at 390 nm, and the fluorescence emission spectra were recorded from 390 to 600 nm. All fluorescence measurements were recorded in triplicate with a PerkinElmer LS50B fluorimeter at 20 °C.

pH Studies of the Binding of the FOXP2 FHD to DNA. Fluorescence anisotropy was used to measure the DNA binding affinity of the FOXP2 FHD. This was conducted over the pH range of 5−9 with 0−15 μM protein that was incubated with 0.2 μM 5-carboxy-X-rhodamine (ROX)-labeled DNA at room temperature for 15 min. The ROX-labeled DNA was selectively excited at 580 nm, and the emission was monitored at 605 nm. The 15 μM protein sample was used to calculate the G factor for each set of replicates. The G factor is used to correct the fluorimeter's bias of either vertically or horizontally polarized emissions.[22] The G factor is automatically calculated using the formula $G = (I_{hv}/I_{hh})$. Eight anisotropy readings were recorded for each sample, and the average was used to construct a DNA binding isotherm that was fit according to the model $y = (B_{max}x)/(K_d x)$.

RESULTS

There is a conserved histidine residue in the DNA binding region of FOX proteins. In FOXP2, this residue is His554, which has a predicted pK_a of 6.25 that lies within the physiological pH range. His554 is shown in the crystal structure to interact directly with Cyt11.[5] On the basis of this observation, we hypothesize that the interaction of the FOXP2 FHD with DNA via His554 may be regulated by pH. To address this, we investigated whether pH affected the structure or DNA binding affinity of the protein. The structural studies were performed in the presence and absence of DNA to determine whether pH affected the innate structure of the FHD or whether it specifically affected the structure of the FOXP2 FHD−DNA complex. If the latter is true and if the binding affinity is also dependent on pH, then we may be able to reach

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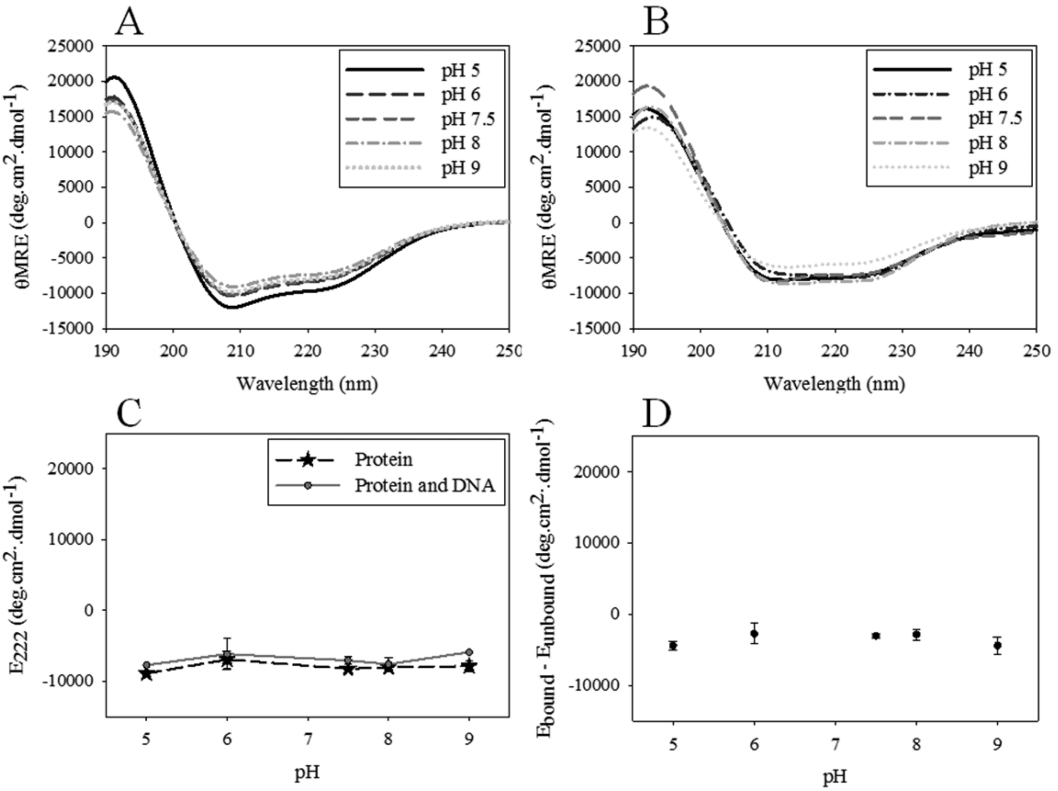

Figure 2. Far-UV circular dichroism of the FOXP2 FHD. (A) Representative far-UV CD spectra of the FOXP2 FHD alone at pH 5, 6, 7.5, 8, and 9. (B) Representative far-UV CD spectra of the FOXP2 FHD bound to DNA at pH 5, 6, 7.5, 8, and 9. (C) pH dependence of θ_{MRE} at 222 nm (E_{222}) of the FOXP2 FHD with and without DNA. (D) Difference in E_{222} between that of the FHD alone and that of the FHD bound to DNA over the pH range of 5−9.

Table 1. Percentage of Secondary Structure Content of the FHD and the FHD Bound to DNA As Determined Using the Dichroweb Deconvolution Tool[23,24]

	pH 5		pH 6		pH 7		pH 8		pH 9	
	FHD	FHD and DNA	FHD	FHD and DNA	FHD	FHD and DNA	FHD	FHD and DNA	FHD	FHD and DNA
helix (%)	32	27	24	22	29	29	29	30	30	20
strand (%)	18	25	25	27	21	25	22	23	20	28
turns (%)	19	21	20	20	20	19	19	19	20	21
unordered (%)	31	28	32	31	31	27	28	28	30	31

a conclusion that protonation and deprotonation of His554 influence the DNA binding properties of the FOXP2 FHD.

Quaternary Structure and Hydrodynamic Volume. Because the FHDs of the FOXP family members have the ability to form both monomers and domain-swapped dimers,[5] it was necessary to determine the quaternary structure of the protein at the protein concentrations to be used in this study and also to assess whether pH changes will influence the quaternary structure. This was done by performing size exclusion chromatography (SEC) on 20 μM FHD in the pH range of 5−9. The results are presented in Figure 1, which shows that the protein occupies a gradually increasing hydrodynamic volume as the pH decreases. The predicted size of the FOXP2 FHD based on the amino acid content is ∼12.7 kDa, and this is the approximate size predicted by SEC at pH >7.5. However, at pH <7.5, the apparent size of the protein increases to ∼17 kDa at pH 5. This size is too small to represent a dimeric form of the protein and simply implies that the acidic pH has loosened the FHD structure, resulting in it occupying a larger tumbling volume in solution. We can therefore conclude that at concentrations of <20 μM, the FOXP2 FHD is entirely monomeric in the pH range of 5−9 as can be seen by the single peaks in Figure 1A.

Secondary and Tertiary Structure. Once the quaternary structure was established to be monomeric in the concentration range and pH range of interest, we wished to determine whether pH caused any changes to the secondary or tertiary structure of the protein. The secondary structure was investigated using far-UV circular dichroism and tertiary structure using both intrinsic and extrinsic (ANS) fluorescence. These studies were completed in the presence and absence of DNA in the pH range of 5−9. Any pH-induced changes in the presence of DNA that were not detected in the absence of DNA would be an indication that the His554−DNA interaction was indeed likely to be the cause of these changes.

The results of the secondary structure investigation are shown in Figure 2. Here we see that the secondary structure of the FOXP2 FHD does not change significantly with pH in the presence or absence of DNA. Furthermore, analysis of the spectra using the spectral deconvolution tool, Dichroweb[23,24]

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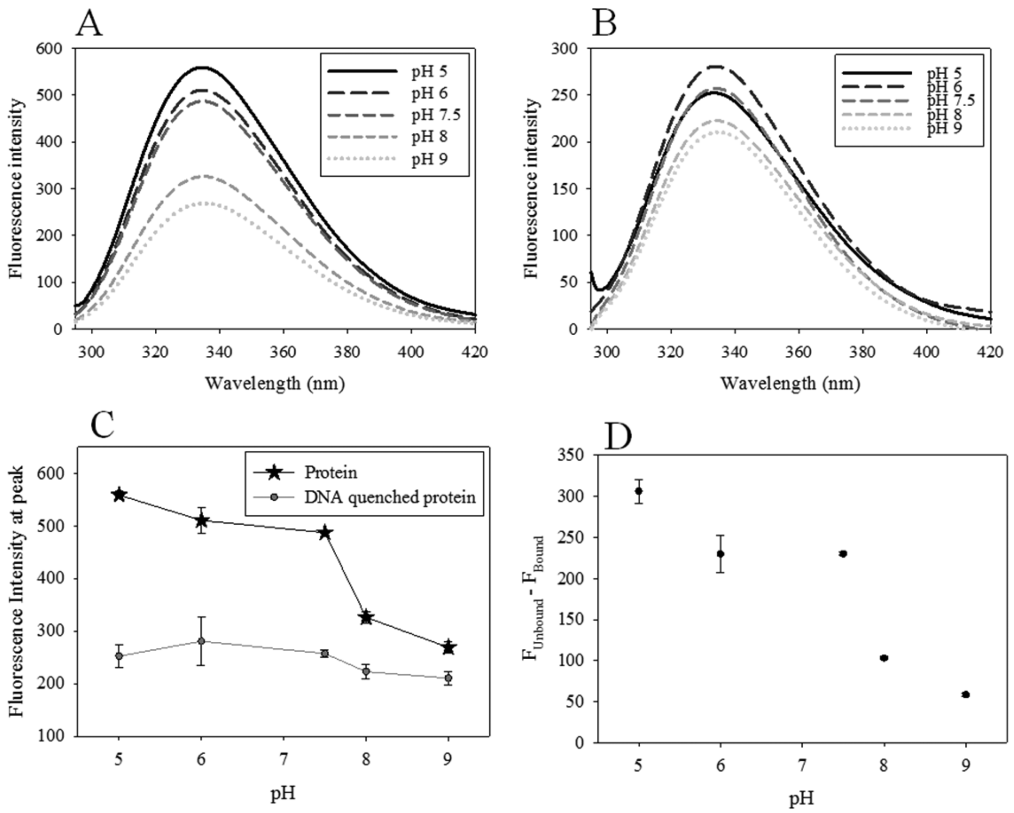

Figure 3. Intrinsic fluorescence of the FOXP2 FHD. (A) Averaged fluorescence spectra (excitation at 280 nm) of the FOXP2 FHD alone at pH 5, 6, 7.5, 8, and 9. (B) Averaged fluorescence spectra (excitation at 280 nn) of the FOXP2 FHD bound to DNA at pH 5, 6, 7.5, 8, and 9. (C) pH dependence of the average maximal fluorescence intensity of the FOXP2 FHD alone and bound to DNA. (D) pH dependence of the difference in fluorescence intensity between that of the FHD alone and that of the FHD bound to DNA.

(Table 1), shows that the percentage of secondary structural content is fairly consistent over the pH range and there is no increase in disorder at lower pH despite the detected increase in hydrodynamic volume (Figure 1).

Although the secondary structure remains unchanged, there does appear to be a pH-induced change in the tertiary structure of the FOXP2 FHD (Figure 3). Here we see that in the absence of DNA, fluorescence is enhanced at pH <8. This implies that with a change in pH, the protein undergoes a change in the local environment of the three tryptophan residues (Trp533, Trp548, and Trp573). When DNA is present, the fluorescence of the FOXP2 FHD is quenched. This quenching could be a result of either direct stacking interactions of the tryptophan residues and the DNA[25] or a conformational change in the protein induced by DNA binding. Because inspection of the crystal structure shows that none of the three tryptophan residues stack with the DNA bases, the quenching is likely a result of the change in protein tertiary structure upon DNA binding. The presence of DNA appears to cause greater quenching of tryptophan fluorescence at pH <7.5 than at the higher pH. The altered protonation state of His554 may be responsible for the altered tertiary structural environments of the tryptophan residues of the DNA-bound protein, which may in turn cause them to be closer to quenchers such as arginine, lysine, or glutamate residues.

The tertiary structural change that occurs at low pH is further confirmed with an ANS binding study (Figure 4). By incubation of the protein with ANS and measurement of ANS fluorescence, any changes to the tertiary structure via the exposed hydrophobic surface area can be detected. ANS fluorescence shows a blue-shift and an increase in intensity when it binds to hydrophobic patches on the surface of a protein.[26] Here we see that the FOXP2 FHD does not bind to ANS at any pH when in the absence of DNA, nor does it bind to ANS when in the presence of DNA at pH 6−9. However, at pH 5, ANS binding was detected in the presence of DNA. This result indicates that at low pH, DNA binding alone is able to induce a tertiary structural change in the FOXP2 FHD that results in exposed hydrophobic surface. This effect may be a result of changes to the DNA binding pattern that occur upon His554 protonation.

DNA Binding Affinity. The effect of pH on DNA binding affinity was determined by using fluorescence anisotropy to create a DNA binding isotherm for the FOXP2 FHD in the pH range of 5.5−8 (Figure 5). The results from this study show a clear trend in which the DNA binding affinity increases with a decrease in pH. This indicates that the protonation state of His554 may indeed affect DNA binding affinity.

■ DISCUSSION

There is evidence to suggest that the intracellular pH is not entirely constant. pH fluctuations are evident, particularly during periods of change in the cell cycle, where the pH has been shown to drop most dramatically during mid interphase.[27] Transcription by FOXP2 is regulated by changing the way it binds DNA. There is potential that any changes in pH that occur along with the changing phases of the cell cycle may affect DNA binding. Cellular pH changes could therefore be a means of regulation of transcription by this protein. Furthermore, cancer cells have been shown to have a different

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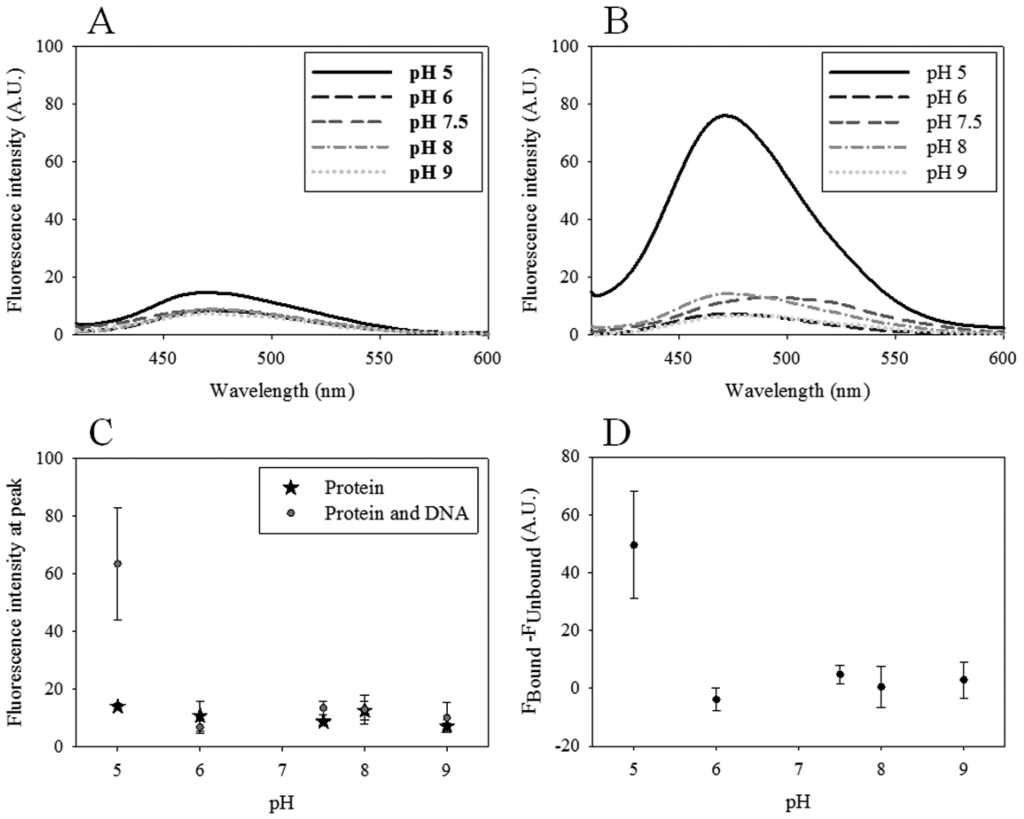

Figure 4. Fluorescence of the FOXP2 FHD and the FHD bound to DNA in the presence of ANS. (A) Representative fluorescence spectra (excitation at 390 nm) of the FOXP2 FHD alone in the presence of ANS at pH 5, 6, 7.5, 8, and 9. (B) Representative fluorescence spectra (excitation at 390 nm) of the FHD bound to DNA in the presence of ANS at pH 5, 6, 7.5, 8, and 9. (C) Fluorescence intensity at the peak of the spectra of the FHD alone and the FHD bound to DNA in the presence of ANS at varying pH values. (D) pH dependence of the difference in fluorescence intensity between that of the FOXP2 FHD bound to DNA and that of the FHD alone in the presence of ANS.

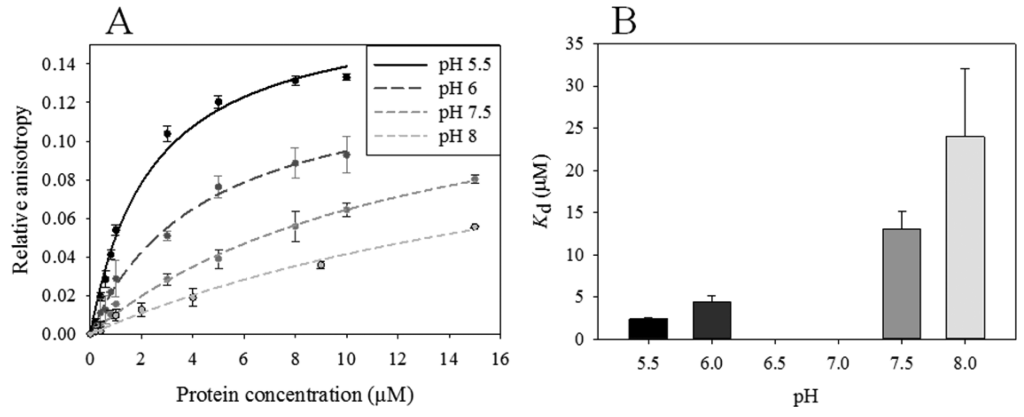

Figure 5. (A) FOXP2 FHD DNA binding isotherm obtained from fluorescence anisotropy assays at pH 5.5, 6, 7.5, and 8. DNA (0.2 μM) labeled with ROX was used with protein concentrations ranging from 0 to 15 μM. (B) pH dependence of the K_d of the FHD bound to DNA.

acid to base balance compared to that of normal cells,[28] and the pH range within tumor cells is greater than in normal cells.[29] Because FOXP2 has been implicated in cancer,[30] it is certainly relevant to investigate the effect that pH changes have on the structure of the FOXP2–DNA complex as well as on the affinity of the protein for DNA.

In this study, we identified the interaction between FOXP2 His554 and DNA Cyt11 and hypothesized that because the predicted pK_a of the imidazole side chain of His554 is 6.25, there is potential for the protonation state to change under physiological conditions. This change in protonation state will result in altered H-bonding patterns with the DNA. We sought to investigate whether these altered H-bonding patterns could result in structural changes to the protein–DNA complex and also whether they would result in changes in the affinity of the FOXP2 FHD for the DNA.

Given the propensity of the FOXP2 FHD to form domain-swapped dimers, it was necessary to consider the quaternary structure of the protein and, furthermore, the effect that pH changes may have on the quaternary structure. The protein remains monomeric from pH 5 to 9 at the protein concentration used (Figure 1). This is not all that surprising

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because the dimer interface is hydrophobic[5] and should therefore not be affected by electrostatic interactions. pH does appear to have some general influence on the structure of the FOXP2 FHD in the absence of DNA, however. This can be seen by the increased hydrodynamic volume (Figure 1) and enhanced fluorescence (Figure 3) at low pH. Importantly, though, the secondary structural content remains intact (Figure 2), and the percentage of disordered structure does not increase with a decrease in pH (Table 1). Therefore, although a lower pH causes the protein to become less compact, which alters the local environment of the tryptophan residues, the protein maintains its structural topology, and hence most likely its function, in the pH range of 5−9.

Investigation of the structure in the presence of DNA indicates that as in the absence of DNA, there is no major secondary structural change to the protein in the pH range of 5−9. The FHD therefore does not undergo a major structural change upon DNA binding, and at any pH in the range under investigation, the structures of the DNA-bound and unbound protein are similar. Despite causing very little change to the secondary structure, DNA does change the tertiary environment of the protein. This is seen at low pH where the protein fluorescence is more prone to quenching than in the presence of DNA (Figure 3). On the basis of the fact that the protein is more compact in the absence of DNA when at high pH (Figure 1) and that the intrinsic fluorescence is quenched under the same conditions (Figure 3), the observation that quenching is more pronounced at low pH when DNA is present could lead to the conclusion that DNA may help to keep the structure more compact at low pH.

The protein is capable of binding ANS only in the presence of DNA and only at pH 5. The protein does not bind ANS at all in the absence of DNA (Figure 4). This again implies that there is a change to the tertiary structure of the protein at low pH in the presence of DNA. This change is subtle between pH 7.5 and 6, but at pH 5, it becomes more dramatic where the hydrophobic surface becomes exposed despite the fact that the secondary structure remains unaltered. This result has interesting implications because an increase in hydrophobicity may increase the probability of domain swapping due to the fact that the dimer interface is hydrophobic. Because dimerization may be an important means of transcriptional regulation by providing FOXP2 with the ability to bring two distal strands of DNA into the proximity of each other,[31,7] this result implies that pH changes may also be significant for regulation.

Despite the change in tertiary structure with a change in pH, the protein is capable of binding DNA across the pH range of 5.5−9 and, in fact, binds more strongly at lower pH than at higher pH (Figure 5). This result clearly demonstrates that pH has an effect on DNA binding affinity. This result may or may not be a direct result of the His554−DNA interaction. One could argue that as the pH drops, the protein will become more positively charged, which will enhance electrostatic attraction with the negatively charged DNA, and that the increased affinity observed at low pH could simply be due to such a nonspecific event. The pH range of 5−9 used in this study spans the pK_a of histidine residues only. The second closest pK_a is that of the glutamate side chain, which is ∼4.1, and so should lie outside the range. However, it has been shown that on the basis of their tertiary environments, the pK_a values of residues in proteins could differ quite substantially.[32] To address this, we investigated the predicted pK_a of residues in FOXP2 using the prediction tool PROPKA.[18−20] Here we see that none of the glutamate or aspartate residues in the FOXP2 FHD have predicted pK_a values of >5, with the closest being Glu579 with a predicted pK_a of 4.51. The predicted pK_a values of all the lysine and arginine residues are above 9. This means that in the pH range of 5−9, the charge on the protein will change only on the basis of the protonation state of the histidine residues. Besides His554, the FOXP2 FHD has one other histidine residue, His559. Interestingly, the predicted pK_a of this residue is 3.34. Thus, it certainly seems likely that the observed pH dependence of the DNA affinity is directly related to the His554−DNA interaction. According to the crystal structure, His554 forms an H-bond with the N4 amino group of Cyt11. The pK_a of this group is >11, and the base should remain protonated in the pH range of 5−9. Deprotonated His554 is an H-bond acceptor; when it becomes protonated, the H-bonding pattern could change via water-mediated or other H-bond acceptors on the DNA or protein. This could change the orientation at which the protein and DNA interact, which can influence the strength of other bonds hence affecting the affinity.

■ CONCLUSION

FOXP2 does not undergo a significant change in secondary structure in the pH range of 5−9 in the presence or absence of DNA, implying that the protein does not undergo a major structural transition upon DNA binding in the pH range. There are, however, changes in the tertiary environment of the tryptophan residues with a change in pH. The addition of DNA causes the protein to become more compact and likely increases the propensity to dimerize at low pH. The affinity for DNA also increases at low pH, suggesting that pH changes could affect transcriptional regulation. This implies that at different stages of the cell cycle, transcription by FOXP2 may be different and also that the protein may not behave correctly in cancer cells. Future work should study transcription reporters at various stages of the cell cycle and in different cell lines. This work may have long-term implications for cancer therapy.

■ ASSOCIATED CONTENT

Supporting Information

Buffers used in all experiments. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00155.

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Notes

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■ ABBREVIATIONS

ANS, 8-anilino-1-naphthalenesulfonate; FOX, forkhead box; FHD, forkhead domain; ROX, 5-carboxy-X-rhodamine; far-UV CD, far-ultraviolet circular dichroism; SEC, size exclusion chromatography.

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