SHORT COMMUNICATION

Variability in the pKa of Histidine Side-Chains Correlates With Burial Within Proteins

Stephen P. Edgcomb and Kenneth P. Murphy*
Department of Biochemistry, University of Iowa, Iowa City, Iowa

ABSTRACT **Acidic pKas of histidines buried within the protein interior are frequently rationalized on the contradictory basis of either polar interactions within the protein or the effects of a hydrophobic environment. To examine these relationships, we surveyed the buried surface area, depth of burial, polar interactions, and crystallographic temperature factors of histidines of known pKa. It has been found that buried environments of histidines do not always result in acidic pKas. Instead, the variability of histidine pKas increases for residues where the majority of the side-chain is buried. Because buried histidines are always found in mixed polar/apolar environments, multiple environmental contributions to pKa values must be considered. However, the quantitative relationships between heterogeneous environments and pKa values are not immediately apparent from the available data. Proteins 2002;49:1–6.** © 2002 Wiley-Liss, Inc.

Key words: pKa; histidine; structure; solvent exposed surface area; polar interactions

INTRODUCTION

Because of the many influences of proton binding on proteins, understanding the molecular basis for proton affinities would be a boon for both the basic sciences and for the applied sciences of drug design and biotechnology. To contribute to such an understanding, many researchers have focused on site-specific titrations of ionizable residues using Nuclear Magnetic Resonance (NMR). As pointed out in the seminal review by Markley,[1] histidines are central to such studies because of their frequent inclusion in the active sites of enzymes and their contributions to protein stability. A fascinating set of histidines, unligated by cofactors, resides in the interior of proteins. Acidic pKas are frequently observed for these histidines, contributing to the growing idea that low values result from buried environments.[2,3] Two simple, but contradictory, rationales for this trend are frequently discussed: through-space interactions between the histidine and electrically polar groups[4] and effects resulting from a hydrophobic protein interior.[5]

To test the hypothesis that acidic histidine pKa values are related to burial within proteins, we have surveyed several proteins that have structural models determined by either X-ray crystallography or NMR and whose histidine pKas have been estimated by site-specific titrations using NMR. Several structural variables related to burial were calculated for each histidine. We find the premise that interior positions of histidines readily account for acidic pKa values is not valid.

For histidines where conformational changes do not accompany protonation, pKa variability correlates with burial. Thus, histidines are observed that are substantially buried but have either above average or nearly average pKas. In the set of histidines where conformational changes do accompany protonation, most of the histidines are reported to have low pKa's; however, a near average pKa has been observed within this group.

RESULTS

The survey consists of 37 histidine residues from 13 proteins. These proteins are listed with both the pdb code and the NMR reference in Table I. Of the histidines, 29 were titratable (see Methods).

The 29 titratable histidines had an average pKa value of 6.6 with a standard deviation of 1. This large standard deviation reflects intrinsic differences due to the specific environment of each residue as well as differences in the experimental conditions and analysis of each titration. For the proteins included in this study, the experimental conditions varied widely, including differences in temperature, use of a Hill coefficient in data fitting, quality of titration baselines, and ionic strength. Experimental temperatures varied from 20° to 35°C. Given the ionization enthalpy of imidazole, this temperature range would be expected to result in less than half a pH unit effect. Inclusion of a Hill coefficient or ambiguity in baselines can have large effects on observed pKa values. For groups that undergo highly cooperative proton binding, microscopic

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*Correspondence to: Kenneth P. Murphy, Department of Biochemistry, University of Iowa, 51 Newton Road, Iowa City, IA 52242-1109. E-Mail: k-murphy@uiowa.edu

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TABLE I. Proteins Included in the Survey

	Residue number	pKa	PDB code	NMR reference
Titratable histidines				
Barnase	102	6.3	1A2P	Sali et al., 1988 [27]
Barnase	18	7.9		
Bovine chymotrypsinogen	57	7.3	2TGA	Markley and Ibanez, 1978[28]
Bovine chymotrypsinogen	40	4.6		
BPTP	72	9.2	1PNT	Tishmack et al., 1997[19]
BPTP	66	8.3		
Cyclophilin	70	5.8	2CPL	Yu and Fesik, 1994[3]
Cyclophilin	126	6.3		
FKBP	94	5.8	1FKS	Yu and Fesik, 1994[3]
FKBP	87	6.5		
HPr	76	6	1POH	Kalbitzer et al., 1982[29]
Lysozyme Hen Egg	15	5.5	6LYZ	Takahashi et al., 1992[7]
Lysozyme human	78	7.1	1LZ1	Takahashi et al., 1992[7]
metaquoMb equine	81	6.6	1YMB	Cocco et al., 1992[30]
metaquoMb equine	36	7.8		
metaquoMb equine	113	5.4		
metaquoMb equine	116	6.6		
metaquoMb equine	119	6.4		
PI-phospholipase c	92	5.4	1GYM	Liu et al., 1997[2]
PI-phospholipase c	82	6.9		
PI-phospholipase c	32	7.6		
PI-phospholipase c	227	6.9		
Ribonuclease A	12	5.8	7RSA	Markley, 1975a[31]
Ribonuclease A	105	6.7		
Ribonuclease A	119	6.2		
Staphylococcal Nuclease	46	5.7	1STG	Alexandrescu et al., 1988[32]
Staphylococcal Nuclease	8	6.8		
Staphylococcal Nuclease	124	6.0		
Xylanase	156	6.5	1XNB	Plesniak et al., 1996[5]
Untitratable histidines		pKa limit		
Cyclophilin	54	4.2		
Cyclophilin	92	4.2		
FKBP	25	3.6		
metaquoMb equine	24	4.8		
PI-phospholipase c	81	3		
PI-phospholipase c	61	3		
Ribonuclease A	48	6		
Xylanase	149	2.3		

pKa values will depend on the protonation state of other residues within the cooperative unit, but the effect of cooperativity is less than half a pKa unit[6] for the titratable residues 12 and 119 of ribonuclease. Changes in ionic strength have similar effects on pKa values; experimental differences greater than half a pH unit, but less than a full unit, have been observed.[7]

The protonation states of the eight untitratable residues are known with varying degrees of certainty. Heteronuclear ^{1}H-^{15}N-NMR was used to unambiguously demonstrate that His 24 of apomyoglobin[4] and His 149 of xylanase[5] are not charged at pH values as low as 2.3 and 4.2, respectively. The original authors stated the ^{1}H-^{13}C coupled spectra of cyclophilin His 54 and His 92 indicate uncharged histidines at a pH values as low as 4.2.[3] Similarly, proton-carbon coupled NMR was interpreted to indicate that His 25 of FKBP was uncharged at pH values down to 3.6.[3] His 61 and His 81 of phospholipase c were also studied by ^{1}H-^{13}C coupled spectroscopy; however, it was simply noted the chemical shifts were mostly invariant from pH 4 to 9 without speculating as to the charge state of the histidine.[2] Finally, His 48 of bovine ribonuclease A has been studied by ^{1}H NMR spectroscopy.[8,9] In the presence of acetate, His48 undergoes a smooth titration with a pKa of 6.3.[10] In the absence of acetate, the signal from the ϵ^{1}-carbon bound proton of this residue undergoes a discontinuous transition in the pH range from 8.4 to 3.55.[9] This transition is consistent with a multi-state equilibrium with an intermediate that is maximally populated at pH 5.6 and a value for the pKa of the histidine of 6.9.

We investigated possible relationships between histidine pKa and buried surface area, depth of burial, polar interactions with distances and geometries consistent

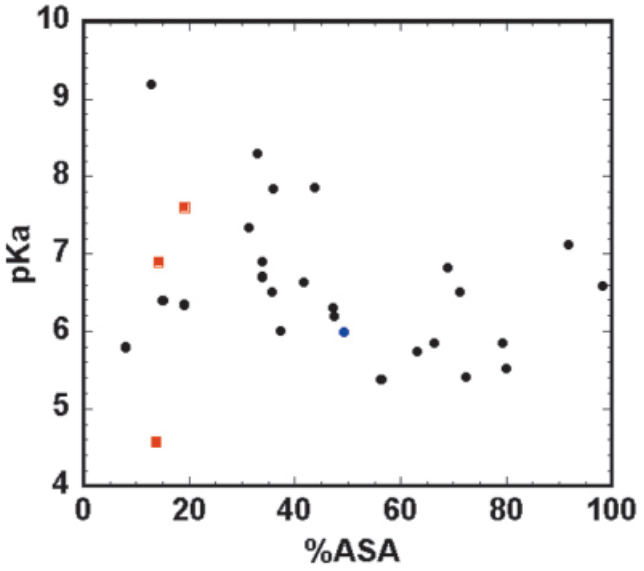

Fig. 1. pKa of titratable residues vs. %ASA: The variability of pKa for the titratable residues increases for histidines with less than 55% ASA. The untitratable residues, which are not included in the plot, all have less than 55% ASA (Table II). Blue circles indicate a histidine with side-chain nitrogens that are in hydrogen bonding range of a presumably positive group. Red squares indicate a histidine with side-chain nitrogens that are in hydrogen bonding range of a presumably negative group.

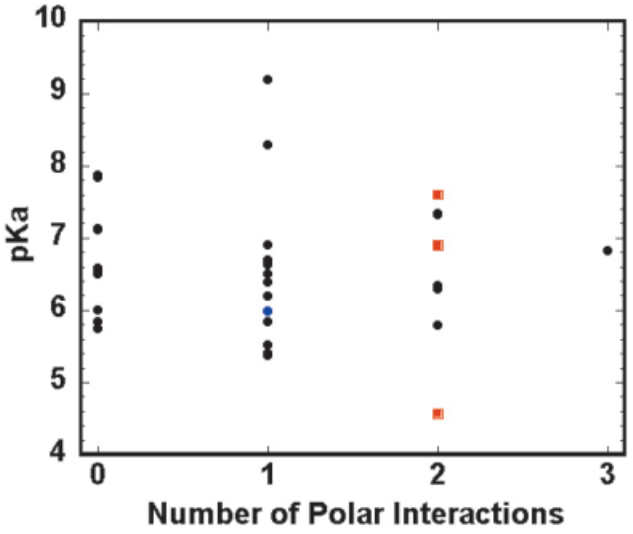

Fig. 2. pKa of titrable residues vs. number of atoms within hydrogen-bonding range of the histidine nitrogen residues: The plot contains only the titratable residues. All but one of the untitratable residues has at least two such interactions (Table II). Blue circles indicate a histidine with side-chain nitrogens that are in hydrogen bonding range of a presumably positive group. Red squares indicate a histidine with side-chain nitrogens that are in hydrogen bonding range of a presumably negative group.

with hydrogen bonds, and inclusion within ordered regions of proteins. The first step was to survey the inter-relationships between these variables. The histidines have an average %ASA (% accessible surface area, see Methods) of 46% with a standard deviation of 25%. The %ASA decreases exponentially with depth and approaches 0% near 5 Å. There is a clear correlation between burial and polar interactions with the histidine nitrogen. Nearly all of histidines with less than 30% ASA are involved in a polar interaction with each nitrogen, and this category contains the histidines involved in potential salt bridges with negatively charged groups. In contrast, the histidine side-chains, having no polar interactions with their nitrogen atoms, are spread between 60 and 100% ASA with a small cluster near 40%. Finally, there is a correlation between burial and crystallographic order as reflected by calculated Z-score of the temperature factor. The average Z-score of the histidine residues is 0.0 with a standard deviation of 1. The average %ASA for the more highly ordered histidines (i.e., those with negative Z-scores) is 38% with a standard deviation of 22%. For the more disordered histidines (positive Z-score), the average is 49% with a standard deviation of 27%.

The greatest variability in pKa values is observed in histidine residues with less than 55% ASA. Figure 1, a plot relating pKa of the titratable residues to %ASA, can be divided into two regions. For the histidines with greater than 55% ASA, the average pKa of the titratable histidines is 6.0, with a standard deviation of 0.6. The average pKa of the titratable residues with less than 55% ASA is higher with a value of 6.8 and a higher standard deviation of 1. A correlation between pKa and both depth of burial and Z-score was also observed for the titratable histidines. However, this correlation was not greater than what was observed between these variables and %ASA.

Figure 2 contains a plot relating pKa of the titratable histidines to polar interactions with hydrogen bonding distances and geometries. The pKa values of histidines with zero or one polar interaction with their side chain nitrogens can be described as relatively tight clusters with a few extreme values. In the case of histidines with zero or one polar interaction, the extreme pKa values belong to residues that have less than 55% ASA. This is consistent with the observation of increased pKa variability with increased burial. In the case of histidines with multiple polar interactions, the pKa values are scattered over a broad range from 4.5 to 7.3. Again, this is consistent with the correlation between variability and burial.

The untitratable residues represent histidines with the most extreme proton affinities, and they are among the least solvent exposed histidines. Table II summarizes their environment. The average %ASA of the untitratable histidines is 10.2%, with a standard deviation of 17.6%. These values are skewed upward by the relatively high %ASA of His 25 of FKBP, but they are still significantly lower than observed for the titratable histidines. On average, the untitratable residues are also more deeply buried than the titratable histidines. The average depth of the untitratable residues is 5.5 Å, with a standard deviation of 1.4 Å. Consistent with their buried position, all of the calculable Z-scores of untitratable histidines are negative and they all have multiple polar interactions. His 24 of myoglobin is the only untitratable histidine that must be a hydrogen bond acceptor. Finally, there is no obvious relationship between the local charge environment and a histidine being untitratable. The local proximal charges,

TABLE II. Untitratable Histidines

Protein	His	pKa limit[a]	%ASA	Depth[b]	Z-score	Polar interactions[c]	Charged residues[d]	Proximal charge[e]
2CPL	54	4.2	7.4	4.6	−1.5	H_2O, H_2O	1K, 1R, 1H, 1D	0.18
2CPL	92	4.2	0.3	7.3	−2.4	D-δ^1O, S-γO,	1H, 3K, 1D, 1E	0.04
1FKS	25	3.6	52.6	3.6	NA[f]	S γO	3R, 3D, 1E	−0.06
1YBM	24	4.8	6.6	4.9	−0.3	D-MC O, H-ε^2N	1H, 2K, 1D, 2E	0.57
1GYM	61	3	11.8	4.6	−0.3	Y-MC O, E-ε^2O	1K, 2R, 2D, 1E	−0.33
1GYM	81	3	2.9	5.3	−0.4	G-MC O, H_2O, H_2O,	1R, 1H, 1D, 1E	0.11
7RSA	48	6	0	6.3	−1.0	D-MC O, T-MC O	2D, 1E	−0.50
1XNB	149	2.3	0	7.6	−1.1	T-γ^1O, S-γO, H_2O	1H, 2D	−0.27

[a]Upper bound pKa limit.
[b]Depth in angstroms.
[c]Groups forming hydrogen bonds with the nitrogens of the histidine side chain; group identity (one letter code for amino acids)-atom; H_2O indicates group is a structured water; MC is main chain.
[d]Number(of residue type),identity (one letter code) of charged residues within 10 angstroms of the histidine side chain.
[e]Estimated from charged residues within 10 Å of the histidine side chain.
[f]Structure was from an NMR minimized model.

observed in Table II, are evenly divided between positive and negative values.

DISCUSSION

We find that, contrary to the hypothesis that pKa values of histidines decrease with burial, pKa variability correlates with burial within a protein and the average pKa of titratable residues actually increases. What then can we conclude about the effects of a hydrophobic protein interior and through space electrostatic interactions? In a result consistent with several studies of protein structure,[11–13] we found no polar groups buried in completely hydrophobic environments of wild type proteins. Even in the analogous cases of valine to charged residue mutations of the hydrophobic core of staphylococcal nuclease, a partially polar environment has been proposed.[14,15] This is most notable in the valine to aspartic acid mutation where ordered water molecules are associated with the mutated side-chain.[15] It appears that protein interiors must always be considered heterogeneous polar/apolar environments.

For heterogeneous protein interiors, coulombic arguments suggest the apolar component favors uncharged residues in conflict with polar interactions from within the protein or buried water, which are more favorable for charged residues. Continuum electrostatic techniques, that are based on Coulomb's Law and utilize numerical solutions to the Poisson-Boltzmann equation, have been applied to several proteins within our current survey.[7,16–20] At best, the agreement between calculated and predicted pKas has been qualitative. The lack of a uniform value for the dielectric constant contributes to the qualitative nature of the predictions.

Simple inspection of the residues within this survey provides a number of cases that are difficult to reconcile with simple electrostatic arguments. For example, His 40 of bovine chymotrypsinogen has the lowest measurable pKa of all the histidines examined; and although it has only 24%ASA, it is within a comparatively polar environment. One nitrogen atom of the side-chain is hydrogen bonded to a serine hydroxyl and the other is hydrogen bonded to Asp194. Additionally, within a 10 Å sphere around this histidine, two aspartic acids and one glutamic acid are opposed only by a comparatively distal arginine.

It could be argued that polar interactions are insufficient to compensate for the low dielectric of the protein interior. The fact that the untitratable residues are all buried within protein interiors and most have been interpreted to have very low pKas are consistent with such a position. However, His 48 of bovine ribonuclease provides an exception. In several structural studies where ribonuclease has been crystallized in buffers with pH values below pH 5.5, His 48 appears completely buried (ref. 21 and references therein). Although its titration is accompanied by a conformational change of the protein, Markley[9] has proposed it has a pKa value near 6.9 based on chemical shift changes of its ϵ-carbon bound proton. It appears the protonated form of His 48 can be accommodated within the protein without direct interaction with a negatively charged residue and within an environment that does not carry a proximal negative charge of greater magnitude than observed for histidine 40 of chymotrypsinogen.

In summary, we observe a correlation between histidine burial and variability in pKa values. To our knowledge, this is the first time this relationship has been documented. We propose reproduction of this variability as a criterion for evaluating the effectiveness of future theoretical calculations of pKa values. As has recently been suggested by a mutational study of OMTKY3,[22] Possion-Boltzmann techniques can overestimate long-range electrostatic effects while underestimating contributions from closely associated polar groups. The current survey demonstrates regions where short-range interactions will be the most difficult to calculate for histidine residues. Histidines that have between 55 and 10% ASA provide a challenge because of the variety of pKa values that are possible. A more difficult test may be the accurate predictions of histidines that are completely buried yet have pKa values near neutral.

METHODS

The protein structures include twelve models based on X-ray diffraction data and one minimized, average, NMR structure. The inclusion of human and chicken lysozyme does not result in redundancy because the histidines are not conserved in the two proteins. The phosphate-free structure of bovine ribonuclease A[23] was included in the survey because it most closely approximated the solution conditions used in collecting the NMR data. In the presence of inhibitors, histidine 119 of ribonuclease is observed in two conformations.[21] This is not the case in ion-free ribonuclease,[23] so only one position for the histidine was considered in the calculations for the survey. The diffraction models all have a crystallographic resolution < 2.3 Å and an R-value ≤ 0.2. Histidine residues 64, 82, 93, and 97 of metaquomyoglobin are not included in the survey due to their proximity to the heme cofactor of the protein. His 121 of staphylococcal nuclease was excluded because of proximity to a bound inhibitor. His 15 of HPr and His 48 of metaquomyoglobin are not included due to their proximity to bound sulfate groups. The majority of the published NMR spectra of the histidine residues surveyed contained signals from either the ϵ^1 or δ^2-carbon bound protons although in some cases multinuclear NMR was used.

The histidine conformations of all residues studied in this report were investigated using the WhatIf web interface[24] and the results are reported here for the sake of completeness. Based on the analysis of hydrogen bond networks, the algorithm suggested the following histidine residues would be more correctly positioned if their side-chain was rotated by 180°: His 66 of BPTP, His 76 of HPr, His 61 and 82 of PI-phospholipase c, and His 105 of ribonuclease A. However, the histidine conformations submitted by the original authors were used throughout our analysis of the relationships between histidine pKa and structural environment.

The most obvious grouping of the histidine residues includes two mutually exclusive sets: titratable and untitratable. The first group consists of residues with NMR signals that underwent pH dependent titrations on the fast NMR time scale. The original investigators used nonlinear least squares analysis to fit these titrations to either the Hendersen-Hasselbalch or the modified Hill equation.[1] In most cases, a Hill coefficient was not included in the fit. For the cases when it was included, the majority of the values were within 0.2 units of 1.

The second group of histidines either did not experience a significant chemical shift change over the pH range of the experiment or they experienced a change in chemical shift that was consistent with a conformational change within the protein.

The percent of solvent exposed surface area, %ASA, is calculated relative to a histidine residue in a model Ala-His-Ala tripeptide. The measurements of exposed surface area were preformed using the program ACCESS (Scott R. Presnell, Zymogenetics, Seattle, WA), an implementation of the Lee and Richards[25] solvent-accessibility algorithm, using a probe radius of 1.4 Å and a slice width of 0.25 Å.

Depth of histidine burial and polar interactions consistent with hydrogen bonds to the histidine side chains were calculated using previously published methods. Depth measurements were made using the program rundepS, an implementation of the Chakravarty and Varadarajan algorithm.[26] Hydrogen bonds were identified using the program H-bond plus, an implementation of the Stickle et al. algorithm.[13] The charged residues within a 10 Å sphere of a histidine residue were identified using the molecular modeling program SYBYL (Tripos). Each ionizable group was assumed to be fully charged, the charge was normalized by distance to the histidine, and the values were summed.

To assess the certainty of the positions of the histidine residues in the diffraction models, an average Z-score was calculated for the side-chain thermal factors.

$$Z = (\bar{X} - \bar{x})/s$$

$\bar{X}$ is the average temperature factor for the side chain atoms of the histidine, $\bar{x}$ is the average temperature factor for all of the side chains in the protein, and s is the standard deviation for all of the side chain atoms of the protein. A positive Z-score represents a histidine residue that is more disordered than the average side chain atoms, while a negative Z-score represents a more ordered histidine.

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