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Considering Protonation as a Posttranslational Modification Regulating Protein Structure and Function

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Abstract

Posttranslational modification is an evolutionarily conserved mechanism for regulating protein activity, binding affinity, and stability. Compared with established posttranslational modifications such as phosphorylation or ubiquitination, posttranslational modification by protons within physiological pH ranges is a less recognized mechanism for regulating protein function. By changing the charge of amino acid side chains, posttranslational modification by protons can drive dynamic changes in protein conformation and function. Addition and removal of a proton is rapid and reversible and, in contrast to most other posttranslational modifications, does not require an enzyme. Signaling specificity is achieved by only a minority of sites in proteins titrating within the physiological pH range. Here, we examine the structural mechanisms and functional consequences of proton posttranslational modification of pH-sensing proteins regulating different cellular processes.

Contents

INTRODUCTION

Intracellular pH (pH_i) dynamics is conventionally viewed as a homeostatic mechanism to protect cells from alkaline and acidic loads. Consistent with this view, cytosolic pH is tightly regulated at near neutral (11). For example, increased generation of metabolic acids is generally accompanied by increased H^+ efflux by plasma membrane electroneutral ion transport proteins, such as the monocarboxylate lactate-H^+ exchangers MCT1 and MCT4, the Na^+-H^+ exchanger NHE1, and the Na^+-dependent Cl-HCO_3 transporter NBCn2. However, an emerging view is that pH_i dynamics also functions as a signaling mechanism to regulate a number of cell processes. In mammalian cells a seemingly small increase in pH_i from 0.2 to 0.3 units promotes cell proliferation and cell cycle progression (111, 121) and is now recognized to be necessary for directional cell migration, including cell polarity, actin filament assemblies, and focal adhesion remodeling (34, 35, 128, 130). Decreased pH_i contributes to apoptosis, in part by activation of cytochrome *c*, caspases (85), and the proapoptotic protein BAX (65). Dysregulated pH_i dynamics is also a hallmark of diseases, including a constitutive increased pH_i in cancers of different tissue origins and genetic backgrounds (18, 147) and a constitutive decreased pH_i in a number of neurodegenerative disorders (48, 133). In this review we present recent evidence supporting the view of pH_i dynamics as a signaling mechanism and propose that this is best understood at the molecular level by considering protonation as a reversible posttranslational modification.

Central to the view of signaling by pH_i is an understanding of how physiological changes in pH regulate the function of selective proteins, termed pH sensors, by changing activities and binding affinities (127, 139). There is abundant structure-based evidence on how pH dynamics regulates activities of ion channels (56, 78, 117), ion transport proteins and pumps (58, 96), and enzymes (43, 61, 91). The protonation state of titratable residues also regulates the electrostatic energy of interactions to change binding affinities, including protein-protein, protein-phospholipid, and macromolecular assemblies. Protein-protein binding can also be accompanied by proton uptake

or release, leading to changes in the protonation states of ionizable residues (69, 84, 89). Also, pH regulation of binding affinities is a signaling mechanism inducing dynamic changes in protein localization, including the recruitment of cytosolic proteins to the plasma membrane or to intracellular membranes by pH-dependent binding to membrane-specific phospholipids (35, 54, 72). In turn, localization puts a constraint on the pH dependence of proteins to match subcellular differences in pH_i (20). In mammalian cells, the cytoplasm, nucleus, and endoplasmic reticulum have a near-neutral pH; mitochondria are more basic; and lysosomes, endosomes, and the Golgi network are more acidic (19). As recently noted (37), the plausible adaptation of protein-protein interactions to the pH in subcellular compartments has generally escaped the attention of researchers. Although there are limited structural analyses of pH-dependent protein-DNA binding (79), given the importance of electrostatics in protein-DNA binding, the protonation state of titratable residues could markedly affect interactions with the negatively charged phosphate backbone of DNA. We propose that the molecular basis for how physiological changes in pH regulate protein activities and binding affinities can be viewed by protonation as a posttranslational modification.

PROTONS AS POSTTRANSLATIONAL MODIFICATIONS

As a mechanism for protein regulation, protons have much in common with small chemical posttranslational modifications, particularly those that change protein charge in a site-specific manner, including phosphorylation and Lys acetylation. Although the proton is a particularly small chemical modification, its addition nonetheless increases the charge by one unit and changes a hydrogen-bond acceptor to a group that can serve as a hydrogen-bond donor. Like phosphorylation, acetylation, and ubiquitination, posttranslational modification by protons can directly modulate binding to other macromolecules or small molecules, or it can drive changes in protein conformation and dynamics that then modulate some aspect of function (99).

Moreover, changes in protonation state are rapid and reversible. One major difference compared with other posttranslational modifications is that protonation or deprotonation does not need to be enzymatically catalyzed, such as transferases for adding methyl groups or kinases and phosphatases for adding and removing phosphate groups, respectively. (Although we know of no such examples, it is intriguing to speculate that protonation state changes could effectively be catalyzed if, e.g., binding of a partner protein caused conformational changes that caused the pK_a value of a titratable group to change dramatically, and the resulting change in protonation state then regulated other aspects of function.) Accordingly, an enzyme-mediated transfer cascade analogous to mitogen-activated protein kinase modules likely does not occur with pH-driven signaling.

The protonation state of any titratable residue is not in general uniquely defined. Rather, pH and pK_a values together determine the probability of being in a particular protonation state or the fraction of a population of protein molecules in a particular state. This property is similar to other posttranslational modifications such as phosphorylation in that the fraction of proteins modified on a site can vary dramatically at any given time. However, single titratable groups can also rapidly change protonation states over time, with the fractional occupancy determined by pH and pK_a values.

Virtually all proteins have titratable groups, and one major challenge of studying their regulation by pH is determining which sites could potentially serve as functionally relevant pH sensors in vivo. A similar challenge exists for other posttranslational modifications; identifying a site of modification by mass spectroscopy does not guarantee that it has any significant functional role. Recent analyses of posttranslational modifications by phosphorylation or acetylation suggest that only a fraction of identified modification sites likely have a significant biological role (9, 70). The challenge is magnified for protons because they are labile and cannot be detected directly

by mass spectroscopy or by antibodies. Hence, large-scale proteomics analyses that have been used to identify phosphorylation, methylation, or ubiquitination are not feasible for protonation state. Although bioinformatics searches based on isoelectric points have attempted to identify pH sensors, they have not detected meaningful correlations (37). However, unbiased systematic analyses of protein-protein complexes using the Protein-Protein Docking Benchmark database (**http://zlab.bu.edu/zdock/**) have generated more global predictions on binding affinities dependent on physiological pH (84, 89).

Although any titratable site is in principle a candidate pH sensor, among the many titratable sites in proteins only a minority titrates significantly within the normal cytosolic pH range. Histidine side chains are the most obvious candidates, with a nominal pK_a of ~6.5, which in many cases can be modestly upshifted owing to the protein environment. Phosphate groups have a similar pK_a value, but the pK_a values of other common side chains are mostly far from 7, meaning that they are potentially relevant to cytoplasmic regulation only when the protein environment, including desolvation or the local electrostatic potential, dramatically shifts the pK_a value.

Computational methods for predicting pK_a values, including multi-conformer continuum electrostatics (MCCE), constant pH molecular dynamics (CpHMD) (145), and the software package PROPKA (**http://propka.ki.ku.dk**), have been developed over several decades (1). Although still imperfect, these methods are nonetheless powerful tools for helping to identify potential pH-sensing residues. Other computational methods such as molecular dynamics can make predictions about how pH-driven changes in protonation states may affect the structure and dynamics of a protein (90), as has also been done for posttranslational modifications such as phosphorylation and acetylation (99). The most powerful biophysical methods for studying these effects in vitro are based on nuclear magnetic resonance (NMR), which can directly monitor titrating protons, and provide information about resulting changes in dynamics and conformation. However, advances in neutron diffraction crystallography may facilitate structural biology studies of pH sensors (156). In contrast to X-ray diffraction, which detects electron density, neutron diffraction can provide direct information on the positions of nuclei including protons and especially deuterons, which have a larger scattering cross-section.

As with other posttranslational modifications, the most straightforward route to establishing the biological role of a particular titrating site is to mutate it to a nontitrating side chain. For example, Asn and Gln are reasonable isosteres for neutral His because their sizes are approximately the same and they contain one hydrogen bond donor and one acceptor in similar positions (**Figure 1**). Mimicking the positively charged form of His is more problematic in that Lys and Arg are substantially longer. In a similar way, Glu is an imperfect isostere for phosphorylated side chains such as pSer (82); thus, the results of such mutational experiments must be interpreted cautiously.

Progress in our understanding of the molecular basis for pH regulation faces important conceptual challenges. A very fundamental one is the meaning of pH_i in cells with small volumes. For example, in a typical prokaryotic cell with a volume of approximately 1 femtoliter (10^{-15} liters) and a pH of 7 (H^+ concentration 10^{-7} M), there are only approximately 60 free protons. Such low numbers raise the question of whether the pH can be precisely defined in such a small volume, either theoretically or practically, for example, by using pH-sensitive fluorescent dyes (BCECF, SNARF) or genetically encoded biosensors (pHluorin, pHTomato). However, the number of labile protons—those on titratable site of macromolecules or metabolites—is many orders of magnitude larger, and these will exchange rapidly with those in the bulk. Thus, a pH reporter or a pH-sensing protein is responding to the chemical potential of the overall pool of labile protons, not just those that are free in solution.

Eukaryotic cells of course typically have dramatically larger volumes, although similar issues arise for defining pH in small subcellular compartments. Somewhat more problematic is the

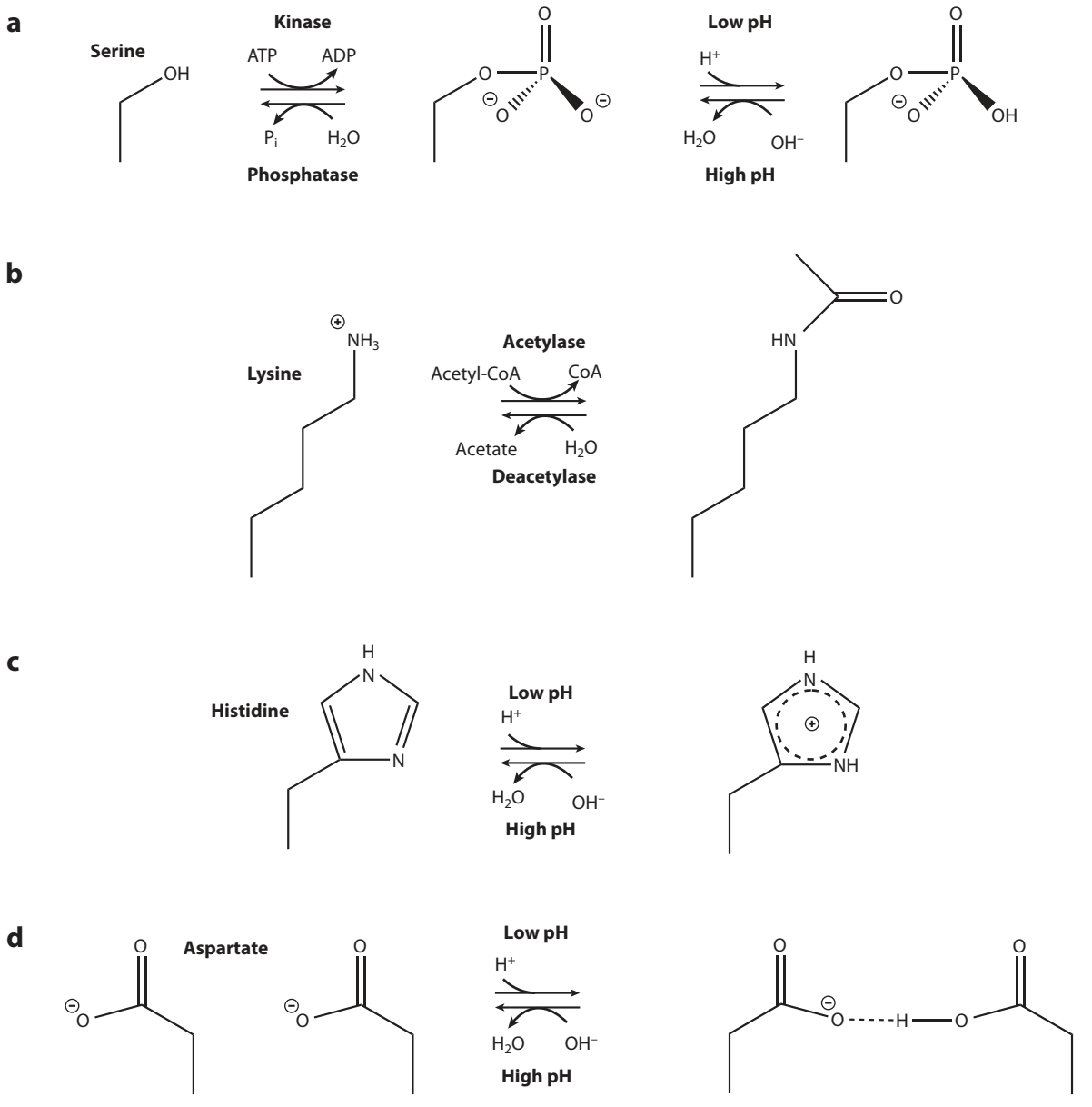


Figure 1

Examples of amino acid posttranslational modifications associated with changes in charge. (*a*) Phosphorylation of, e.g., serines (shown), threonines, tyrosines, and histidines leads to the addition of a negative charge at weakly basic conditions (151). (*b*) Lysine acetylation shields the lysine amino group, e.g., to decrease the affinity to DNA (126). (*c*) Histidines can quickly abstract protons to shuttle protons or to function as a pH sensor site, (*d*) When pK_a values are upshifted, protonation of carboxyl groups of glutamate or aspartate (shown) can lead to the formation of new hydrogen bonds important for conformational changes of proteins.

concept of pH gradients within a cellular compartment, such as local pH near a membrane, which is postulated to be modulated by electrostatic effects from lipid headgroups, or pH gradients due to large localized fluxes of protons by transporters (86). Although these are intriguing concepts, significant practical challenges are associated with measuring such effects. With respect to pH gradients, a key parameter is the effective diffusion rate of protons. In pure water, protons diffuse exceptionally rapidly, aided by the Grotthuss mechanism (27). In water containing large concentrations of titratable sites (buffer), Grotthuss-based diffusion likely still occurs, but diffusion is highly hindered in that the effective path length for a free proton is small, and the effective diffusion rate of protons is limited by the rate of diffusion of the buffering sites (132). In cells, this effective rate of diffusion would likely remain rather high (higher than the diffusion rate of macromolecules) owing to relatively high concentrations of metabolites with titrating groups, such as carnosine. As such, protons rapidly equilibrate between different portions of a cell not segregated by membranes, and maintaining a stable pH gradient requires large fluxes of protons. Hence, hydrogen ions are not generally free in the cytosol but rather complexed or with proteins and metabolites.

MODES OF PROTEIN REGULATION BY PROTON POSTTRANSLATIONAL MODIFICATION

Although changes in pH_i are pleiotropic, specificity of pH sensing is achieved by many of the mechanisms described above. Notably, among the many titratable sites in proteins only a small minority titrates significantly within the physiological pH range. Comparing structurally related proteins or protein domains reveals a number of examples of specificity in pH sensing (**Figure 2*a***). The pleckstrin homology (PH) domain of several guanine nucleotide exchange factors (GEFs), including GRP1 (general receptor for 3-phosphoinositides 1) (50) and Dbs (Dbl's big sister) (35), contains pH-sensitive His switches for phosphoinositide binding. As discussed below, specificity of pH-sensitive PH domains is determined by critical His residues at phosphoinositide-binding sites that confer pH-dependent electrostatic interactions with negatively charged phospholipids. There are also examples of members of similar protein families having opposite regulatory responses to physiological changes in pH. The related actin-severing proteins cofilin and twinfilin are activated by increased (34, 109) and decreased pH (93), respectively. The focal adhesion kinase (FAK) and the related family member proline-rich tyrosine kinase 2 (Pyk2) are structurally similar pH sensors; however, autophosphorylation of FAK increases at pHi > 7.2 (64, 137) and autophosphorylation of Pyk2 increases at pHi < 7.2 (75, 110).

Posttranslational modification by protons can also allosterically regulate protein function (**Figure 2*b***). The Bohr effect of pH-driven changes for the affinity of hemoglobin for oxygen binding is the classic example of proton-induced allosteric regulation (39). The charge interaction of a His-Asp salt bridge (His146 and Asp94 in human hemoglobin) is disrupted when His146 is deprotonated with increased blood pH, which induces conformational changes that increase oxygen-binding affinity at a distant site. In the sections below, we describe how protonation of pH-sensing residues allosterically regulates talin binding to actin filaments (127) (**Figure 2*b***) and unmasks a myristoyl moiety in the HIV matrix (MA) protein (33). Although not included in examples described below, the structural basis for allosteric regulation by pH has been determined for a number of functionally distinct membrane ion transport proteins and channels. Examples include System A (SNAT2) and System N (SNAT5) amino acid transporters (7); many K^+ channels, including but not limited to Kir1.1 (112, 117), KcsA (134, 136, 161), and Kv1 (78); Ca^{2+}-ATPase (SERCA) (96); and NhaA, a Na^+-H^+ exchanger in *Escherichia coli*. The pH_i regulation of NhaA, which in turn maintains pH_i homeostasis, is a feedback mechanism

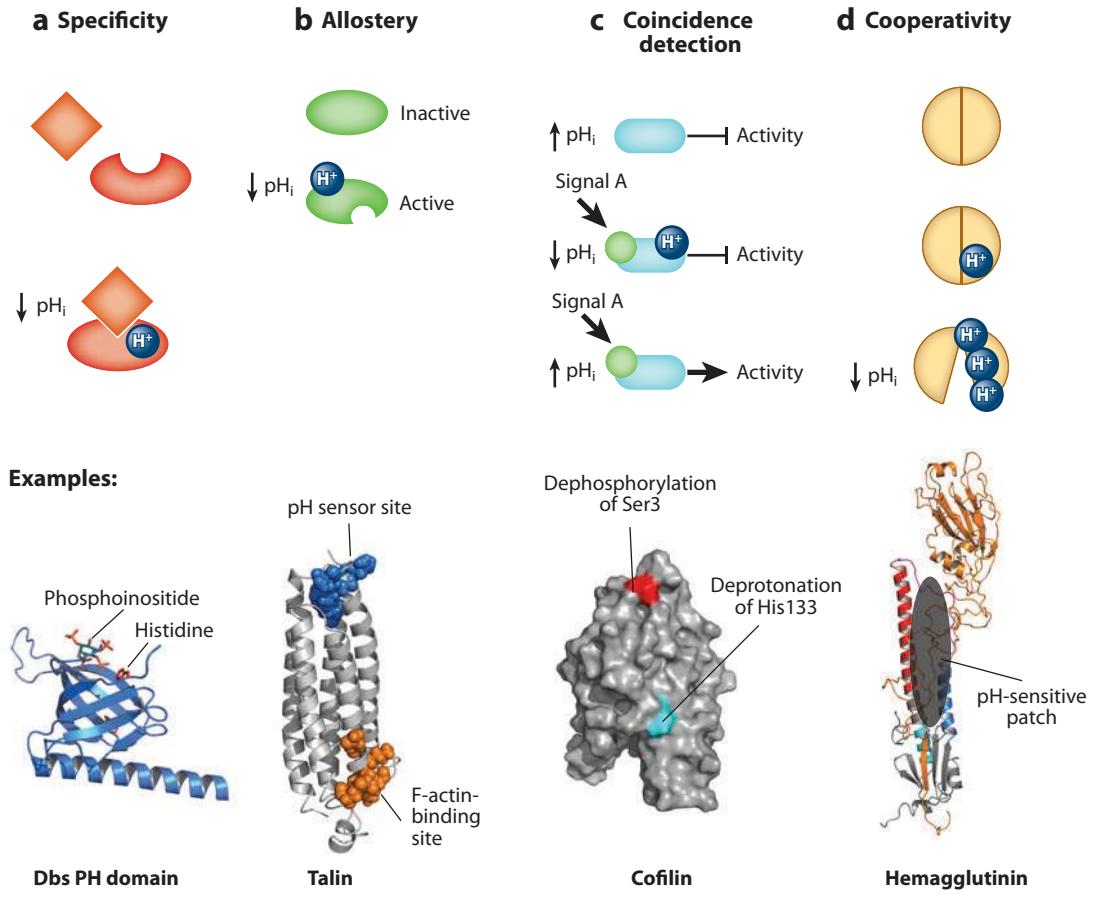


Figure 2

Signaling modes regulated by pH_i. (*a*) Protonation can regulate specificity, e.g., protonation of a histidine, in some but not all Rho guanine nucleotide exchange factors (GEFs) required for stereospecific interaction with phosphoinositides. (*b*) An allosteric regulation mode occurs when protonation of a distinct site induces a conformational change in a remote site, such as talin, that has an actin-binding side ~40 Å away from the pH sensor site (PDB ID: 2jsw). (*c*) In coincidence detection, two distinct and generally unrelated input signals are necessary for the output of protein function, as shown for cofilin, which requires dephosphorylation of an N-terminal serine and deprotonation of a C-terminal histidine for increased activity. (*d*) Cooperativity occurs when several protonation sites act together with electrostatic coupling, affecting titration and sometimes pK_a shifts, as occurs with disrupted interactions between hemagglutinin HA_1 domain and the HA_2 domain.

that is achieved by clusters of electrostatically coupled amino acids with shifted pK_a values near a negatively charged ion funnel that regulate long-range conformational changes affecting a distinct H^+ exchange site (58, 101). Similar pH-dependent allosteric regulation of mammalian Na^+-H^+ exchangers and other ion transport proteins controlling pH_i homeostasis, such as the proton-pumping V-ATPase, is predicted, although not structurally confirmed.

Another regulatory mode becoming increasingly apparent is the role of protonation in coincidence detection with other posttranslational modifications, including phosphorylation or binding of membrane phospholipids (**Figure 2*c***). Coincidence detectors require multiple inputs for a regulated output. In the section on pH-sensing actin regulatory proteins, we describe the structural mechanisms for coincidence regulation of cofilin by phosphorylation of an N-terminal

serine and protonation of a C-terminal histidine (**Figure 2*c***). Other examples include activity and trafficking of the gap junction protein connexin-43, which require changes in protonation state and ubiquitination (73), and dimer dissociation of dynein light chain LC8, which is regulated by a phosphorylation-dependent increase in the pK_a value of a critical histidine (154). Coincidence regulation by protonation allows integration of regulatory circuits, and viewed from the perspective of pH_i dynamics being pleiotropic, provides spatial discrimination by a locally restricted second regulator, such as a kinase or ubiquitinating enzyme.

In the sections below we describe examples with structural evidence for distinct modes of protein regulation by proton posttranslational modification. Several of these examples highlight two additional properties of regulation by protons. The first is cooperativity involving electrostatic coupling of multiple proton- or ligand-binding sites. For example, protonation of one amino acid can induce changes in the pK_a value or cation binding of nearby residues, as described below for the influenza virus protein hemagglutinin (**Figure 2*d***). The second is the ability to regulate multiple proteins in unison to control a complex cell behavior. This latter property is achieved by the pleiotropic nature of pH_i dynamics, independence from enzymes, signaling specificity, and coincidence regulation and is best exemplified by pH sensors that collectively mediate pH_i-dependent actin cytoskeleton remodeling during cell migration.

pH-SENSING ACTIN REGULATORY PROTEINS

The assembly of globular (G) actin to filamentous (F) actin and higher-order filament structures and the reverse process of filament disassembly drive many cell processes, such as migration, contraction, vesicle trafficking, motility of pathogens in host cells, and cancer cell invasion and metastasis. Although intrinsic actin assembly rates in vitro are faster at acidic pH (146, 162), likely due to electrostatic effects, de novo actin assembly in mammalian cells requires $pH_i > 7.2$ (147). Hence, the intrinsic pH characteristics of purified actin are overridden in cells by selective pH-sensing actin regulatory proteins, which respond to small changes (of 0.3–0.4 units) in pH_i to induce dramatic differences in actin filament assemblies and architectures.

ADF/Cofilin

Members of the actin depolymerizing factor (ADF)/cofilin (AC) family, including ADF, nonmuscle cofilin-1 and muscle cofilin-2, are pH sensors that sever and nucleate actin filaments (3). In migrating cells, the severing activity of cofilin increases filament disassembly at the rear of actin networks and generates new, free barbed ends for nucleation and assembly at the plasma membrane (10) (**Figure 3*a***). The severing activity of cofilin requires a coincidence activation mechanism of dephosphorylation of an N-terminal serine residue and deprotonation of a C-terminal histidine residue (**Figure 3*b***). These two distinct mechanisms are independently regulated (34).

For severing activity, two sites in A/C proteins bind to actin filaments. A G site includes an N-terminal region from the β1-strand, an N-terminal portion of the α1-helix, and a central region from helices α4 to α5. An F site comprises an N-terminal portion of β5 and a region spanning from the C-terminal half of α6 to β7 (**Figure 3*b***). For cofilins, a phosphate group on Ser3, which is added by LIM (Lin-11, Isl-1, Mec-3) or testis-specific serine (TES) kinase and removed by chronophin or slingshot 1L phosphatases, prevents actin binding at the G site (10). Cofilin phosphorylation was previously speculated to cause extensive perturbations that affect critical residues in the G site (42, 109). Subsequently, molecular dynamics simulations suggested a plausible mechanism (34) whereby phosphorylated Ser3 interacts with Lys126 and Lys127 in the α4 helix, which sterically blocks actin filament binding at the G site. Dephosphorylation disrupts this interaction and allows direct binding of the charged Lys126 and Lys127 to actin.

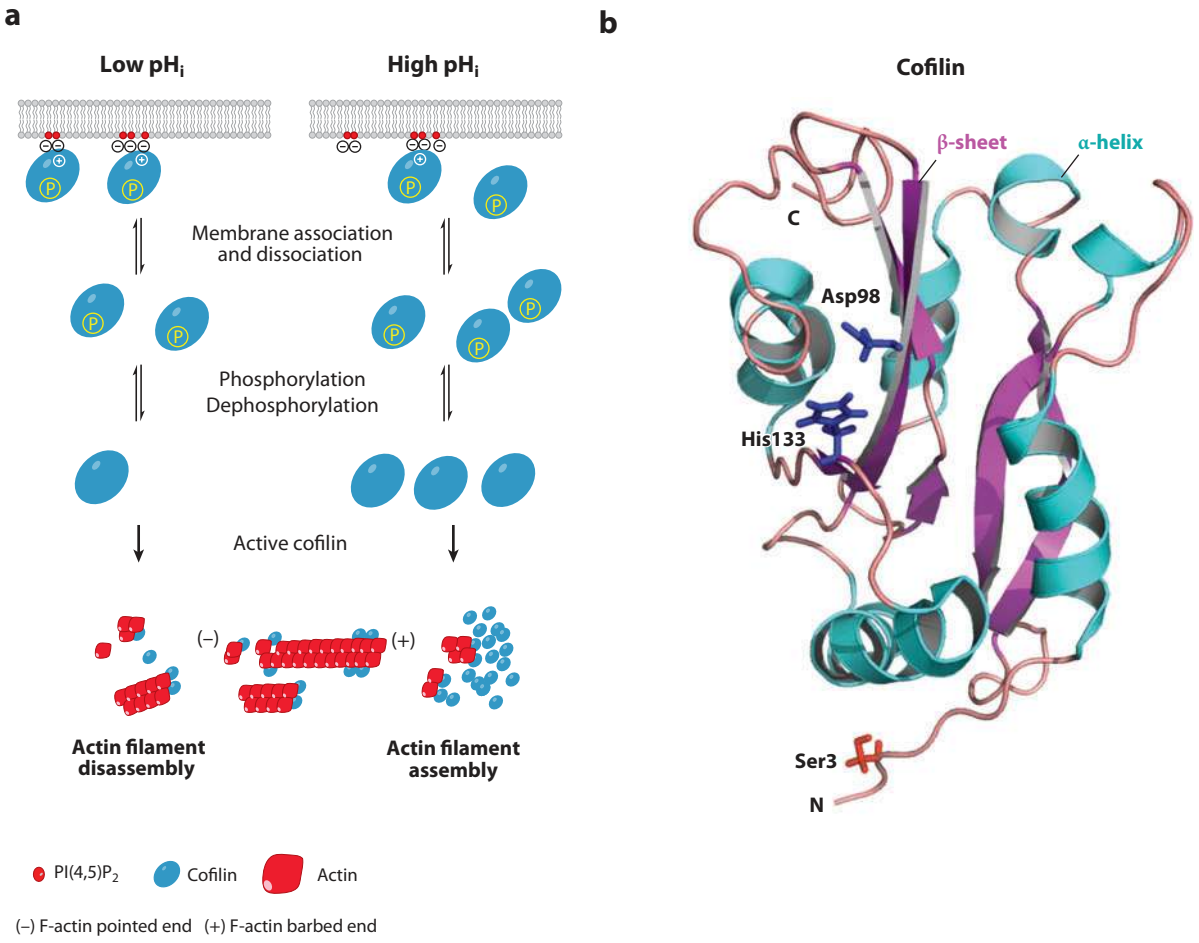


Figure 3

Coincidence regulation of cofilin. (*a*) Model for coincidence detection of cofilin near the plasma membrane. At pH_i less than 7.2, the affinity of cofilin is higher, increasing binding to plasma membrane $PI(4,5)P_2$ (*red*). At pH_i greater than 7.2, less cofilin is bound to $PI(4,5)P_2$, increasing the cytosolic pool of active cofilin if Ser3 is dephosphorylated. At higher pH_i, actin assembly is increased due partly to higher cofilin concentration. (*b*) Schematic representation structure of human cofilin structure (PDB ID: 1q8x) (109). The five α-helices are colored cyan and the six β-sheets are colored purple. Side chains of Ser3, Asp98, and His133 are shown. N-terminal Ser3 is modified by phosphorylation. A salt bridge between Asp98 and His133 is formed under slight acidic conditions. His133 closely interacts with $PI(4,5)P_2$ when doubly protonated (34).

Severing activity also requires an alkaline pH (10). However, pH dependence differs among family members and acts through different mechanisms. Of the three mammalian isoforms, ADF is the most pH sensitive (143), and severing activity of yeast cofilin and the *Acanthamoeba* cofilin homolog actophorin is relatively pH insensitive (80). A structural basis for these differences is indicated by an NMR study of human cofilin (109), which suggests a pH-dependent salt bridge at the F site between His133 and Asp98 in the β4-strand (**Figure 3*a***). The salt bridge is stable at slightly acidic conditions but likely disrupted at higher pH values, leading to partial unfolding of the F site and presumably increased actin binding. The relatively pH-independent actophorin and yeast cofilin lack the spatially conserved histidine found in mammalian A/C proteins. Differences

in pH sensitivity of ADF and cofilin are due likely to cofilin being more stable because of tighter packing of its C terminus to the central β-sheet (49, 109). Hence, because cofilin has more noncovalent interactions than ADF, disruption of the salt bridge by increasing pH has a more destabilizing effect on the ADF structure.

However, NMR analysis of cofilin at pH 7.5 compared with pH 6.5 shows minimal chemical shift differences in residues within the F site (34), suggesting an alternative mechanism independent of conformational changes for pH-dependent activity. Two recently reported alternative mechanisms include binding to $PI(4,5)P_2$ in the plasma membrane (34) and binding to cortactin in invadopodia (i.e., invasive plasma membrane protrusions of cancer cells) (81). Binding to $PI(4,5)P_2$ maintains cofilin inactive at the plasma membrane but locally available for the rapid assembly of actin filaments (74, 141) (**Figure 3*a***). How cofilin is inactive when bound to $PI(4,5)P_2$ was resolved by an NMR study (42) showing that the $PI(4,5)P_2$-binding site includes His133 and basic residues at the F site. Hence, $PI(4,5)P_2$ and actin binding are mutually exclusive. We showed that cofilin binding to $PI(4,5)P_2$ is pH dependent, with increased binding at lower pH (34). Computational docking simulations suggest that doubly protonated His133 but not neutral His133 interacts with terminal phosphates of $PI(4,5)P_2$. However, both phosphates in $PI(4,5)P_2$ have pK_a values close to neutral (68), and cofilin includes alternative $PI(4,5)P_2$-binding sites at basic residues near His133 (34, 160), which could contribute to fine-tuning binding avidity and maintaining a membrane-sequestered pool of cofilin (**Figure 3*a***). Binding to cortactin also sequesters inactive cofilin at the distal membrane of invadopodia. The binding affinity of cofilin for cortactin decreases at higher pH (81). Whether a pH_i gradient occurs, higher at the distal margin of membrane protrusions as suggested (81), to locally disrupt cortactin-cofilin binding and increase cofilin activity is unclear; however, we propose that with coincidence activation by locally regulated dephosphorylation of Ser3, a uniform increase in cytosolic pH may be sufficient.

Talin

Changes in pH_i also regulate dynamic remodeling of actin filaments at cell substrate (focal) adhesion sites. This regulatory mechanism is particularly critical at the leading edge of migrating cells where increased pH_i is necessary for cycles of focal adhesion disassembly (128, 130). The effect of pH_i on focal adhesion remodeling is mediated in part by talin binding to F-actin, which tethers actin filaments to focal adhesions. The N-terminal FERM domain of talin binds the cytosolic domain of β-subunits of integrin receptors and contains a pH-insensitive F-actin-binding site (26). The C-terminal I/LWEQ module, which is conserved in huntingtin interacting protein-1 (Hip1), Hip1-related proteins (Hip1R/Hip12), and yeast protein Sla2, also binds F-actin but with established pH dependence (26) (**Figure 4*a***).

As a pH sensor, talin highlights three of the properties described above on signaling modes, including cooperativity, allostery, and the ability of pH_i dynamics to regulate multiple proteins in unison to control a complex cell behavior. As an example of cooperativity, the I/LWEQ module includes a five-helix bundle (40) that contains a cluster of residues, Glu2337, Glu2342, His2418, Glu2481, and Asp2482, with markedly upshifted pK_a values (128) (**Figure 4*a***). As an example of allostery, NMR and CpHMD simulations indicate that protonation of residues in the pH sensor induces significant changes in the structure and dynamics of a remote actin-binding site to increase actin binding (128) (**Figure 4*a***). A mutant talin-His2418Phe has reduced and pH-insensitive actin binding, indicating a critical role of His2418 in pH-dependent allosteric regulation (128). As an example of coordinated actions of pH sensors, in addition to talin-actin binding dynamics, focal adhesion remodeling also requires FAK activity, which increases at pH values greater than 7.2 (64, 137).

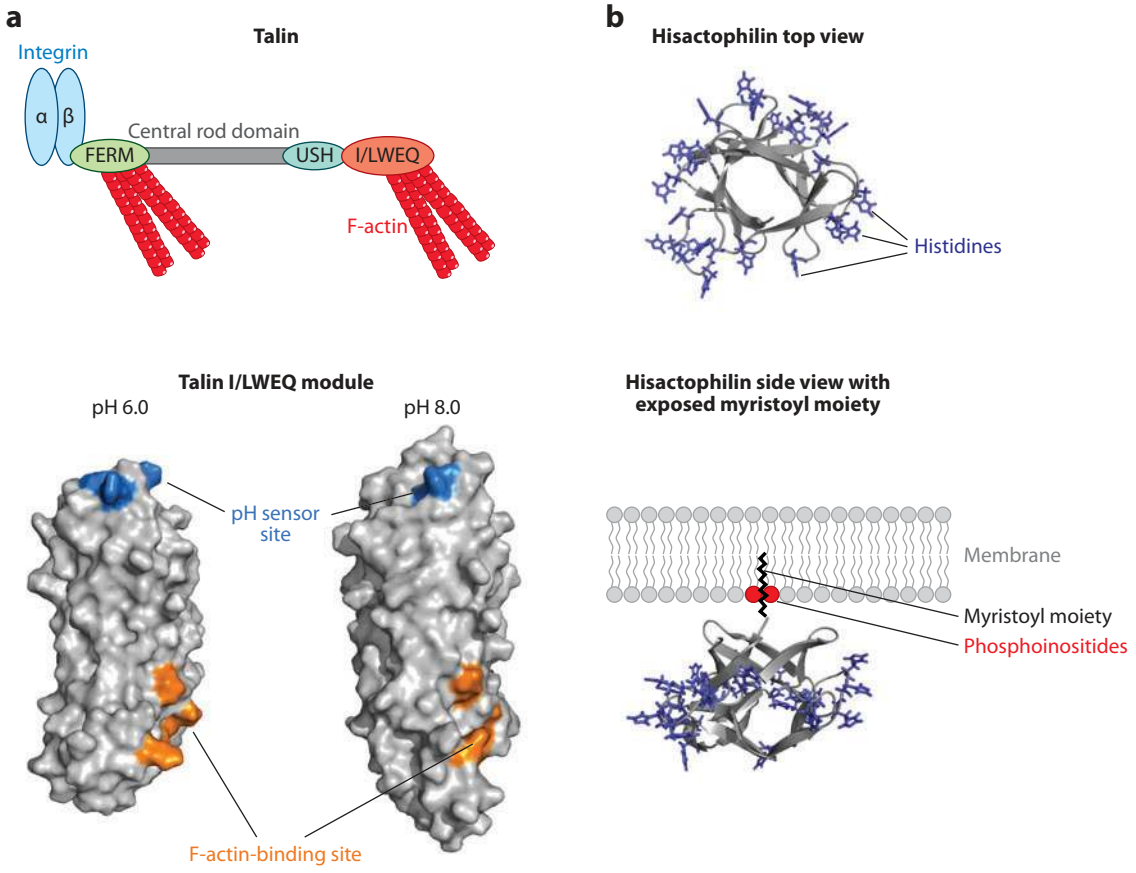


Figure 4

Allosteric regulation of talin binding to actin by pH. (*a*) Domain organization of talin (*upper panel*), including an N-terminal FERM domain (which binds the β-subunit of integrin receptors and F-actin), a central rod domain (*gray*), and a C-terminal I/LWEQ (*red*) actin-binding module that binds F-actin. F-actin binding by the I/LWEQ module but not by the FERM domain is pH sensitive, with more binding occurring at lower pH. (*Lower panel*) Surface representation of the C-terminal actin-binding domain of talin. Residues Glu2337, Glu2342, His2418, and Asp2482 form a pH sensor that induces pH-dependent conformations to allow actin binding only at lower pH. (*b*) Schematic representation of the hisactophilin structure and model of membrane attachment of hisactophilin (PDB ID: 1hcd). (*Upper panel*) Histidines (*shown as sticks*) in loops and turns are equally distributed around the protein. (*Lower panel*) However, these histidines point toward the cytoplasm and the β-sheets point toward the membrane when the N-terminal myristoyl moiety is exposed partly by $PI(4,5)P_2$.

Hisactophilin

The slime mold *Dictyostelium discoideum* expresses the unique, remarkably pH-sensitive actin-binding proteins hisactophilin I and hisactophilin II. Both isoforms contain 118 amino acids and nearly one-third of the residues are histidines. Hisactophilins bind to actin only at pH values below 7.2, suggesting that protonation of histidines promotes binding to actin in a switch-like manner (119). Hisactophilins function in osmoprotection. In response to hyperosmolarity, the *D. discoideum* cytosol acidifies and acid-increased hisactophilin-actin binding generates a rigid osmoprotective actin cytoskeletal network (108). The structure of hisactophilin has a β-trefoil fold consisting of 12 β-strands connected by turns and loops (44) (**Figure 4*b***). The β-trefoil

fold is shared by a number of diverse proteins with unrelated amino acid sequence, including interleukin-1β (32) and the mammalian actin-bundling protein fascin, which consists of four β-trefoil domains (60). In hisactophilin almost all the histidines are located in loops and are at one side of the asymmetric protein, and the other side of the protein forms a tight β-barrel structure inserted into the plasma membrane by myristoylation (8), a common posttranslational modification with a C_{14} fatty acyl chain at the N-terminal glycine residue. Myristoylation is required but energetically not sufficient for membrane targeting (104). Hisactophilin oscillates between a cytosolic form at pH 7.5 and a membrane-bound form at pH 6.5 (46), regulated by a cluster of charged amino acids adjacent to the myristoyl moiety (47). These two forms differ in the pH-dependent orientation of the myristoyl moiety, which can be either bound to a hydrophobic pocket or exposed to bind membranes (125). A similar pH-dependent myristoyl switch regulates multimerization and membrane targeting of the HIV Gag and MA proteins (33) and is described in more detail below.

Guanine Nucleotide Exchange Factors

Several GEFs show specificity of pH-dependent binding of their PH domains to membrane phosphoinositides. One example is Dbs, a Dbl family Rho GEF that activates the Rho GTPase Cdc42 at the leading edge of migrating cells to control polarized movement. Dbs contains a PH domain that binds $PI(4,5)P_2$ with higher affinity at lower pH (35). However, other Dbl family Rho GEFs, such as intersectin, contain a similar PH domain that has pH-independent binding to $PI(4,5)P_2$. The PH domain in Dbl family Rho GEFs is adjacent and C-terminal to the catalytic Dbl homology (DH) domain (115), and $PI(4,5)P_2$ binding can allosterically inhibit GEF activity (116). Dbs but not intersectin has a histidine (His843) at the $PI(4,5)P_2$-binding site of the PH domain, which determines pH-sensitive binding (35), similar to pH-dependent binding of cofilin to $PI(4,5)P_2$ described above. Although pH-dependent phosphoinositide binding has been shown for only a limited number of Rho GEFs, the shared feature of a binding site histidine could be used to predict pH sensing and hence pH-dependent GEF localization and activity.

The PH domain of Grp1, a GEF for Arf GTPases, also has pH-dependent binding to phosphoinositides. In contrast to Dbs, Grp1 binds $PI(3,4,5)P_3$ at endosomal membranes, with higher affinity binding at lower pH (50), and phosphoinositide binding increases Grp1 activity (29). Increased binding affinity is directly determined by protonation of a histidine (His355) located in a 20-residue insertion within the loop between β6 and β7 of Grp1 that contacts the phosphate group 4 of inositol 1,3,4,5-tetrakisphosphate (IP_4), a soluble analog of $PI(3,4,5)P_3$ (50, 77). Arf GTPases localize at acidic endosomes and regulate endosomal trafficking and actin dynamics (30). A predicted lower pH at the cytoplasmic side of endosomes (100) possibly regulates Grb1 localization and activity by increasing affinity for $PI(3,4,5)P_3$.

Recent findings indicate physiological pH sensing by a number of other common phosphoinositide-binding domains. The FYVE (Fab1p, YOTB, Vac1p, and EEA1) domain (72), the ENTH and ANTH (Epsin and AP180 N-terminal) domains (54), and the PH domain of FAPP1 (four-phosphate-adaptor protein 1) (51) have increased phosphoinositide binding affinities at physiological pH values below neutral. A common feature of these domains is at least one histidine is essential for stereospecific phosphoinositide recognition. Although not experimentally confirmed, proteins containing these domains likely have pH-dependent membrane localization and activity and, if associated with the actin cytoskeleton, could coordinately regulate the pH-sensitive actin filament dynamics at membranes.

AMYLOIDOSIS

Although extreme pH alterations affect the folding and stability of most proteins, physiological pH alterations induce partial or full unfolding in selective proteins due to changes in electrostatic interactions. Protein misfolding plays a role in pathological conditions such as amyloidosis, a group of more than 20 disparate human diseases including Alzheimer's disease, Parkinson's disease, and type II diabetes (45, 105). Amyloid fibril formation is due to the folding of the secondary structure of an endogenous protein or protein fragment into a cross-β-sheet quaternary structure that oligomerizes into unbranched filaments. As folding is dependent on the sequence of the protein, misfolding induced by physiological changes in pH is highly specific. For example, low pH can enhance, in the case of prion protein, or inhibit, in the case of islet amyloid polypeptide protein (IAPP), amyloid formation through the protonation of specific histidine residues. In contrast to pathological amyloid formation, organisms also use highly regulated amyloid assembly for a diverse set of normal biological functions, including proteins involved in the production of mammalian melanosomes, *E. coli* biofilms, malarial coat proteins, and silk fibrils from some spider species (45).

Prion Protein and Spongiform Encephalopathies

Spongiform encephalopathies, including scrapie, mad cow, Kuru, and Creutzfeldt-Jacob disease (CJD), are characterized by aggregation of the misfolded prion protein (PrP), a 209-amino-acid protein on the surface of neuronal cells (for review, see Reference 94). The normal PrP^{C} form (for common or cellular) of the protein misfolds into an aggregation-prone, protease-resistant, and infectious PrP^{Sc} form (for scrapie) that accumulates in the brain as amyloid plaques. The structure of PrP^{C} includes a disordered, unfolded N terminus and a well-structured C terminus, which is composed of three α-helices (A–C) and a short antiparallel β-sheet (113, 159). Although the atomic structure of PrP^{Sc} has not been resolved, low-resolution structural analyses indicate a significant change in folding between PrP^{C} and PrP^{Sc}, specifically a marked increase in β-sheet content and a decrease in α-helix content (87, 102). What triggers misfolding of PrP^{C} is not known; however, because (*a*) low pH induces the formation of alternatively folded variants of PrP and (*b*) both PrP^{C} and PrP^{Sc} are cycled through the endocytic pathway, the conversion of PrP^{C} to PrP^{Sc} is suggested to occur in the acidic environment of endosomes (17, 28, 144). Molecular dynamics simulations indicate that protonation of a critical His residue, His187, is important for the stability of PrP^{C}; deprotonation at neutral pH increases β-sheet content and conformational mobility within PrP^{C} (55, 140). His187, positioned between the three helixes, is partially buried and has a significantly downshifted pK_a of ∼4 (6, 55). At neutral pH, His187 is hydrogen-bonded to the backbone carbonyl of Arg156 (4, 66, 140). This salt bridge is not seen upon protonation of His187 or in a mutant PrP-H187R found in familial CJD. The positively charged Arg substitution results in a β-sheet-rich, aggregation-prone molecule. Because stabilizing the His187-Arg156 salt bridge at low pH would prevent misfolding of PrP^{C}, it could be an effective therapeutic strategy to decrease infectivity of PrP^{Sc}.

Islet Amyloid Polypeptide Protein and Type II Diabetes

IAPP (or amylin), a protein secreted from pancreatic β cells, is the predominant component of amyloid fibrils found in a majority of patients with type II diabetes (150). Human IAPP (hIAPP) is a 37-amino-acid peptide that is stored in the insulin secretory granule (pH ∼6) and cosecreted into the extracellular space (pH ∼7.4) with insulin (24, 53, 150). The progression of soluble, monomeric hIAPP to a misfolded amyloid fibril is pH dependent—inhibited at the acidic pH of

the secretory granule and enhanced at the higher extracellular pH (13, 67, 88). Although the precise mechanism of pH-dependent aggregation is unknown, one hint comes from rat IAPP (rIAPP), which in contrast to hIAPP does not form fibrils in solution. However, a single point mutant in rIAPP, Arg18His (**Figure 5*a***), is necessary and sufficient to induce fibrillogenesis (153). At pH values above the pK_a value of His18, peptides encoding residues 10 to 19 of hIAPP aggregate to form amyloid, whereas aggregation is slower at pH values below the pK_a value. Substitution of His18 to Ala abolishes pH-dependent aggregation (138). Further, insulin binding, which maintains an α-helical conformation of hIAPP and inhibits fibril formation, is dependent on the charged state of the amino acid residue at position 18. Introducing a charge either by protonation of His18 in hIAPP or by an Arg18 substitution in rIAPP induces the formation of hydrogen bonds and salt bridges with the insulin β-chain (62, 148). Deprotonation of His18 at neutral pH reduces the polar interactions, destabilizes the α-helical motif of insulin-bound hIAPP, and decreases the affinity of hIAPP for insulin (148). Similarly, the hIAPP-membrane interaction, which facilitates hIAPP aggregation and amyloid toxicity, is regulated by pH. Using a truncated hIAPP construct (residues 1–19), investigators have demonstrated that introduction of a charge at His18 by either protonation or mutation to Arg changes the hIAPP-membrane topology from buried to a surface-associated conformation (12, 98). The structure of membrane-bound hIAPP has a kinked helix motif, with a neutral pH having a much more pronounced interhelical angle (30° at pH ∼4.6 compared with 85° at pH ∼7.3) (97, 103) (**Figure 5*b***). The change in membrane topology and conformation is linked to a reduced ability to disrupt phospholipid vesicles and cell membranes (12, 98). Taken together, these data suggest that when hIAPP is released from the acidic insulin secretory granule into the neutral pH of the cytoplasm or extracellular space, His18 becomes deprotonated, which decreases affinity for insulin and enhances misfolding to promote amyloid fibril formation and cytotoxicity.

pH SENSORS IN PATHOGENS

The pH-dependent dynamic structure of many bacterial and viral proteins is well characterized, and examples highlight many principles of posttranslational modification by protons, including allostery, specificity, and cooperativity. A mechanism shared by bacterial toxins and enveloped and nonenveloped viruses is spatial and temporal disassembly to transit host cells membranes. Most common is disassembly at endosomal membranes, which is triggered by acidic pH. One example is the anthrax toxin component PA63, which undergoes a dramatic acidic pH-induced conformational change in the endosomal compartment. Protonation in the loop between β2 and β3 of PA63 causes structural reorganization and formation of a pore required for translocation of anthrax toxin subunits to the cytosol (157). Other toxins with confirmed pH-induced conformational changes include diphtheria toxin (106, 114), botulinum toxin (124), and cholera toxin B (31). Here, we describe well-characterized pH-regulated structural changes necessary for the function of three viral proteins, hemagglutinin and the M2 pump in influenza virus and the Gag MA proteins in HIV.

Hemagglutinin

The influenza glycoprotein hemagglutinin (HA) mediates receptor binding and membrane fusion and is an example of cooperativity in pH sensing. Pathogenesis of the enveloped influenza virus that causes seasonal flu requires dramatic structural changes of HA driven by transition from neutral to acidic pH (15). The precursor form HA_0 contains protease cleavage sites to generate the subunits HA_1 and HA_2 that form protein complexes on the surface of the influenza virus.

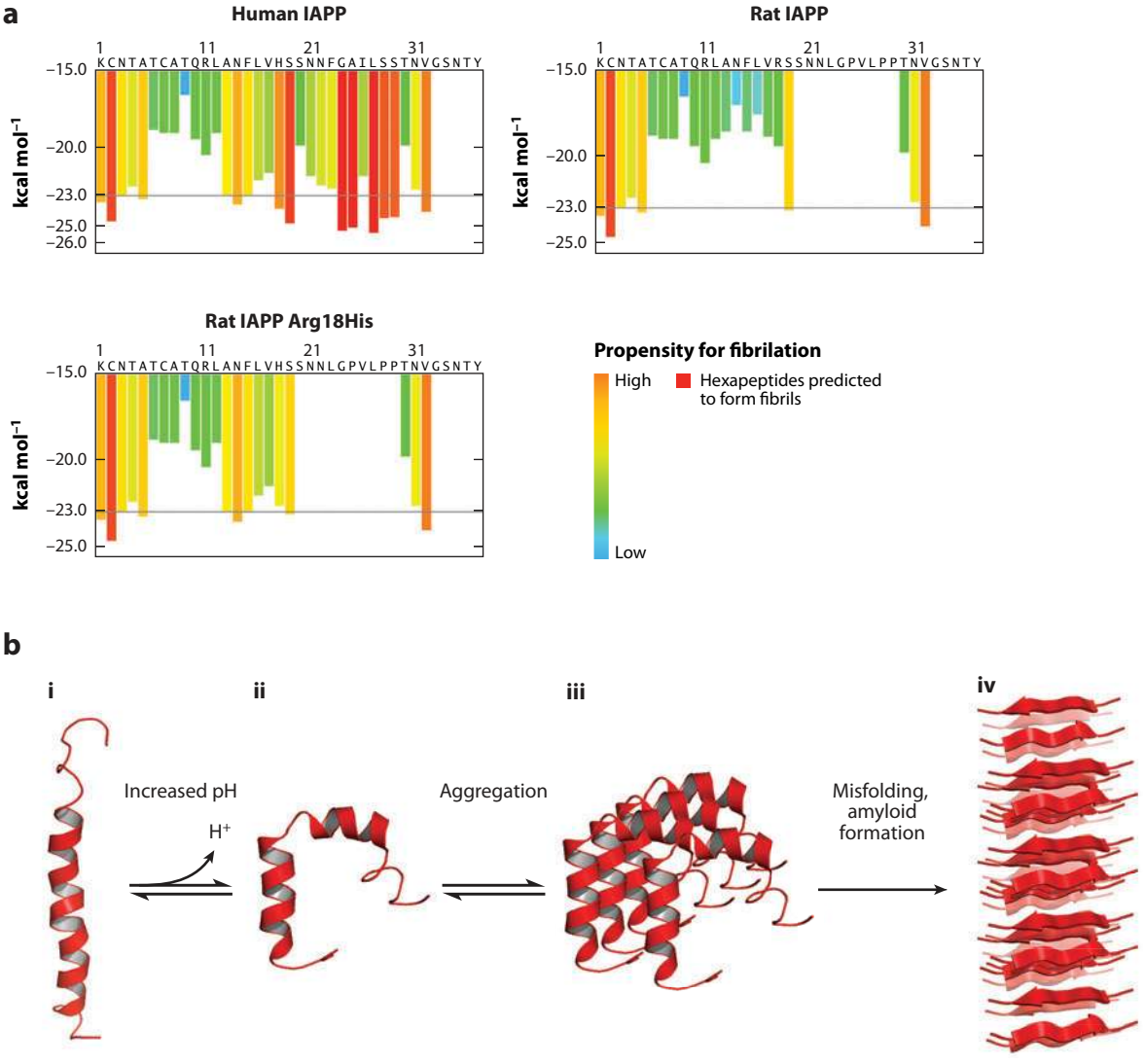


Figure 5

Structural regulation of human islet amyloid polypeptide protein (IAPP) by pH. (*a*) IAPP sequences and segment propensity for fibril formation. The predicted energy for fibrillation of every six-residue segment of human IAPP, rat IAPP, and rat IAPP Arg18His is shown. Warmer colors represent a greater propensity for fibrillation, with red histogram bars representing hexapeptides that are predicted to form fibrils. Owing to variations in the sequence, human IAPP has a much higher propensity to form fibrils than mouse IAPP does. Mutation of Arg18 to His increases the propensity of rat IAPP to form fibrils. Graphs were generated using ZipperDB (**http://services.mbi.ucla.edu/zipperdb/**) (41) and modified from Reference 153. (*b*) Potential model of pH-dependent amyloid formation by IAPP. (*i*) Human IAPP, located in the insulin secretory granules, is bound to insulin β-chain (not shown) or to the membrane surface in an extended kinked helix conformation. (*ii*) As IAPP is released from the acidic environment of the vesicle to the neutral pH of the cytoplasm or extracellular space, His18 becomes deprotonated, weakening the insulin–human IAPP interaction and promoting insertion of human IAPP into the membrane by inducing a change in conformation. Membrane-associated human IAPP (*iii*) aggregates and (*iv*) undergoes further conformational changes leading to the formation of β-sheet-rich amyloid fibrils. Low pH structure: 2kb8 (103). Neutral pH structure: 2l86 (97). In both structures, the N terminus is located at the bottom of the figure. Figure adapted from References 97 and 150.

HA_1 is important for recognition of sialic acid receptors but also keeps HA_2 in a prestressed conformation until exposure to acidic pH, which induces a large conformational change in HA_2 to drive membrane fusion (21).

Native HA is a trimer, with HA_1 and HA_2 linked by a disulfide bond. The C terminus of HA_2 is anchored in the viral membrane, and a triple coiled-coil formation of the helical segments C and D confers formation of a homotrimer of HA_2. At neutral pH, helix A packs against segments C and D connected by a 20-residue loop B (149) and the hydrophobic N terminus of helix A, which contains the fusion peptide, is buried within the overall structure (**Figure 6*a***). However, at acidic pH, loop B adopts a helical structure that induces a change in the orientation of helix A from antiparallel to the end of a now elongated helix comprising A, B, and C (15). With this structural rearrangement, the fusion peptide moves 100 Å and drives insertion of the fusion peptide into the host cell membrane (**Figure 6*a***). Another consequence is that helical segment D now packs against the triple-stranded coiled coil, producing a new hydrophobic core that stabilizes this conformation. The HA_2 C-terminal helix H close to the viral membrane transitions becomes unstructured, which gives the HA complex the necessary flexibility for membrane fusion (15). These conformational changes require cooperativity in pH regulation by histidine-rich patches throughout HA_1 and HA_2 (patch 1 includes HA_1 residues His18 and His38 and HA_2 residue His111; patch 2 includes HA_1 residues His47, His275, His/Lys285, His298, Lys46, and Lys50) that are highly conserved despite a high viral mutagenesis rate (129). Protonation of these histidines disrupts a number of contacts between HA_1 and HA_2, as well as an interaction of HA_1 histidine residues with a basic region at the base of the globular domain. Although the fusion conformation of HA_2 is thermodynamically favored, interactions with HA_1 maintain HA_2 in a metastable prefusion conformation that is relieved at low pH. In the absence of HA_1, the HA_2 domain spontaneously adopts the fusion conformation at neutral pH (22, 23).

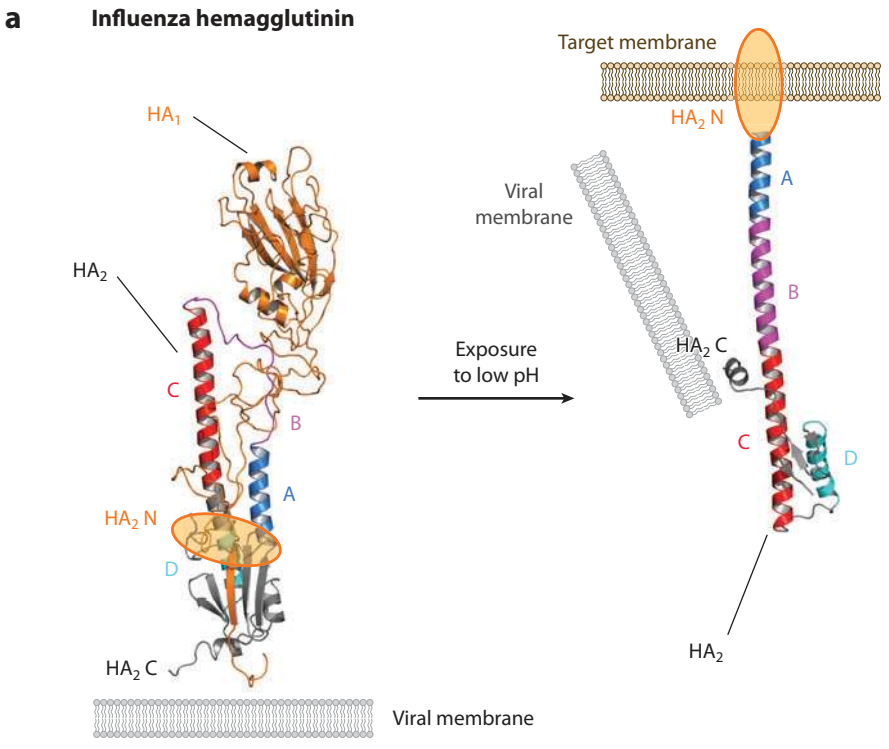
M2 Pump

The influenza virus also expresses a pH-sensitive proton channel—the M2 pump, which is a splice variant of mRNA encoding the matrix (M) protein. Formation of a homotetramer of the 96-amino-acid polypeptide M2 generates a minimalistic proton-selective channel activated by cooperative proton posttranslational modifications in the host cell endosome to acidify the virion for virus release (120, 131). Assembly and activity are regulated by the protonation state of

Figure 6

Low pH induces a large conformational change of the influenza HA_2 protein (only one monomer is shown). (*a*) The structure of prefusion complex (*left*) of HA_1 (*gold*, PDB ID: 2hmg) and HA_2 (*multicolored*, PDB ID: 1htm) and HA_2 at the fusion pH (*right*) structure is shown as schematic representations. In the prefusion complex, HA_2 has a metastable conformation and HA_1 acts as a clamp to keep HA_2 in this conformation. The hydrophobic fusion peptide (*highlighted in orange*) is buried in a hydrophobic core distant from the tip of the protein complex. Low pH leads to protonation of several charged residues throughout HA_1 and HA_2, and HA_1 dissociates partly. This leads to a spontaneous conformational change of region B (*magenta*) that becomes an α-helix to form a new continuous helix comprising A (*blue*), B, and C (*red*). The fusion peptide moves ~100 Å from the core to the tip for insertion into the target membrane. Helix D (*cyan*) now packs against helix A, and a new hydrophobic core is formed. The C terminus moves more toward the new N terminus and has more flexibility necessary for membrane fusion. (*b*) Schematic representation showing multimerization events of HIV Gag and MA proteins as a function of pH. Decreasing pH promotes myristoyl exposure membrane targeting and formation of multimers of both Gag and MA proteins, critical mediators for HIV assembly. Figure reprinted with permission from Reference 33. Abbreviations: MA, matrix; CA, capsid; NC, nucleocapsid.

a single histidine residue (His37) in the transmembrane (TM) domain of each M2 monomer (16). When the M2 pump is assembled in a lipid bilayer, the pK_a values of these four histidines change to 8.2, 8.2, 6.3, and <5.0, with the third imidazole ring crucial for channel activation (57). Recent structural studies using solid-state NMR spectroscopy identified low pH (pH 4.5) and high pH (pH 8.5) states of the M2 pump (56). At high pH, the four histidines are neutral (57) and their imidazole rings pack in an edge-face stacked fashion, creating an electron-rich region



that prevents formation of a hydrogen-bonded water chain and disrupts Grotthuss hopping (56). At low pH, three of the four histidines are protonated and the imidazolium rings repel each other, causing backbone conformational changes that result in a wider pore. In this model, water molecules in the C-terminal part of the pore are protonated after proper alignment with charged imidazoliums, which then flip back to bind another proton. Microsecond reorientations of the histidine actively transport protons into the viral interior. Here, cooperative protonation of histidines leads to activation of the M2 pump by requiring a proton threshold determined by the pK_a value of the third histidine present in the assembled pump.

Gag and Matrix Proteins

HIV expresses a Gag protein that has protonation-induced allosteric unmasking of an N-terminal myristoyl moiety (33). Assembly of the polyprotein HIV Gag at punctate sites on the plasma or endosomal membrane is necessary for the formation of immature virions. Gag is cleaved by viral proteases to generate the proteins matrix (MA), capsid (CA), nucleocapsid (NC), spacer peptides 1 and 2, and P6 during or soon after budding. Subsequently, these proteins reorganize and intrinsically form virus-like particles. Myristoylation is required but not sufficient for targeting Gag and MA to the membrane (14, 25) and requires a cluster of conserved basic residues (158). Gag and MA proteins can expose or sequester the myristoyl moiety, similar to hisactophilin as described above. The pH-dependent myristoyl switch requires the conserved histidine residue His89, which forms a salt bridge with Glu12 when protonated. Deprotonation destabilizes the salt bridge, increases sequestration of the myristoyl moiety by MA, and leads to monomer formation of MA (**Figure 6*b***). Importantly, mutation of His89 impairs correct targeting of Gag and MA and significantly reduces virus production (36). As a critical mediator of HIV-1 virus assembly (52), Gag and MA could be considered a coincidence detector because, in addition to pH-dependent myristoyl exposure, their function is also regulated by binding to plasma membrane $PI(4,5)P_2$, oligomerization of MA, the presence of CA, and calmodulin binding (38).

ENGINEERING pH-SENSITIVE SWITCHES

Structural design principles we learn from understanding the biology of endogenous pH sensors can be applied to engineering pH-sensitive switches. Engineered protein or small-molecule switches can use environmental triggers to generate a predicted behavior (95), including ligand binding, membrane permeability, and changes in protein stability and lifetime. One important application is drug delivery. A strategy currently under intensive investigation is exploiting pH changes during the process of endocytosis to facilitate the escape of biological therapeutics from the endosome into the cytosol (142).

To exploit the trigger of pH_i dynamics or changes in pH between subcellular compartments, engineering changes in the ionization state of surface residues is a feasible approach that has been used for many applications. More difficult is designing changes in the pK_a value and ionization state of buried residues, although recent evidence with the *E. coli* protein thioredoxin (107) shows this can also be achieved. However, because the pK_a value of residues is highly dependent on the structural environment, predicting the sensitivity of ionizable groups to physiological pH is challenging, even with sufficient structural or simulation data. One solution to this challenge is using a combinatorial histidine library, as recently described for sampling every possible combination of histidine and wild-type residues in a model anti-RNase A single-domain VHH antibody (95).

The majority of validated pH-sensitive engineered switches involve histidines, although targeting modification of phosphorylated residues such as Tyr (83, 92, 135) can be achieved, particularly

because the pK_a value of phosphorylated serine, threonine, and tyrosine is generally near neutral. Switches using residues that are normally uncharged at physiological pH have also been generated, such as amphiphilic peptides rich in Glu and Leu designed to confer pH-dependent transit in different membrane compartments (71). A few examples of generated histidine switches include a modified cytokine granulocyte colony-stimulating factor to alter its endosomal trafficking and increase its half-life and efficacy (118), an interleukin receptor antibody to retain antigen binding in acidic compartments (59), and a Lys → His iso-1-cytochrome *c* mutant to increase the electron transfer gate (5).

Switches engineered to respond to the dysregulated pH in disease have been developed for direct therapeutic efficacy or for therapeutic targeting and stability. Most common are switches designed for the more acidic extracellular pH and more alkaline pH_i of cancer cells. A number of peptides have been developed to exploit the low extracellular pH in tumors for site-specific drug delivery. Examples include soluble pH (low) insertion peptides (pHLIPs), which fold and insert across a membrane to form a stable transmembrane α-helix under acidic conditions (2, 152), and GALA, a pH-responsive peptide that converts from a random coil to an amphipathic α-helix at acidic pH to bind bilayer membranes (76). Engineered pH-responsive substrates such as polymers (122, 123, 155) and lipid micelles (63) have also been used as a strategy to release carried drugs at sites of tumor or intracellular vesicle acidity. Although therapeutic approaches to alter pH sensing by endogenous proteins have not been reported, one example would be a strategy to inhibit the pH-dependent activation of cofilin in alkaline cancer cells to limit metastatic progression. Albeit technically challenging, approaches such as this may be feasible as we attain an increased understanding of how protonation acts as a posttranslational modification to regulate protein function.

SUMMARY POINTS

1. How physiological changes in intracellular and extracellular pH regulate protein functions is best understood at the molecular level by considering protonation as a reversible posttranslational modification, analogous to phosphorylation, methylation, and ubiquitination.
2. Signaling specificity by proton posttranslational modification is achieved by only a minority of sites in selective proteins termed pH sensors that titrate within the physiological pH range. In addition to specificity, modes of regulation by posttranslational modification include allostery, coincidence detection, and cooperativity.
3. Structure-based evidence is emerging for how proton posttranslational modification affects dynamic protein conformations to regulate activity, binding affinity, stability, and localization.
4. With dysregulated pH being an enabling feature of many diseases and pathologies, engineered pH-sensitive switches that target proton posttranslational modification offer therapeutic promise.

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Errata

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