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The Role of Protonation States in Ligand-Receptor Recognition and Binding

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Abstract

In this review we discuss the role of protonation states in receptor-ligand interactions, providing experimental evidences and computational predictions that complex formation may involve titratable groups with unusual pKa's and that protonation states frequently change from unbound to bound states. These protonation changes result in proton uptake/release, which in turn causes the pH-dependence of the binding. Indeed, experimental data strongly suggests that almost any binding is pH-dependent and to be correctly modeled, the protonation states must be properly assigned prior to and after the binding. One may accurately predict the protonation states when provided with the structures of the unbound proteins and their complex; however, the modeling becomes much more complicated if the bound state has to be predicted in a docking protocol or if the structures of either bound or unbound receptor-ligand are not available. The major challenges that arise in these situations are the coupling between binding and protonation states, and the conformational changes induced by the binding and ionization states of titratable groups. In addition, any assessment of the protonation state, either before or after binding, must refer to the pH of binding, which is frequently unknown. Thus, even if the pKa's of ionizable groups can be correctly assigned for both unbound and bound state, without knowing the experimental pH one cannot assign the corresponding protonation states, and consequently one cannot calculate the resulting proton uptake/release. It is pointed out, that while experimental pH may not be the physiological pH and binding may involve proton uptake/release, there is a tendency that the native receptor-ligand complexes have evolved toward specific either subcellular or tissue characteristic pH at which the proton uptake/release is either minimal or absent.

Keywords

protonation states; receptor-ligand interactions; pKa calculations; pH-dependence; electrostatics

INTRODUCTION

Protein association/dissociation is one of the most important phenomena occurring in living cell [1, 2]. The resulting protein assemblages could be long or short living entities as permanent complexes, splicing machinery, transcription factors, and many others including dynamic functional units participating in signaling cascades or cell-cycle regulation [3]. Important examples of protein assemblages are receptor-ligand complexes in which the ligand is a small peptide or small molecule (drug). In cases such as these, one may consider

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the formation of the receptor-ligand complex to be subject to several constraints such as subunits should be co-localized in space and time; the probability of proteins binding depends on subunits concentration; and protein-protein recognition requires appropriate physiochemical environment (see reference [2] for a complete list). For the purpose of this review, which focuses on the role of protonation states in binding, we will introduce a different scheme, namely we will consider the binding process to be made of three important steps: (1) recognition: the receptor and the ligand must sense each other in the crowded cellular environment; (2) orientation: the receptor and the ligand must be properly oriented in such a way that their interfaces point toward each other; and (3) physical binding: the receptor and the ligand form 3D complex (oligomer).

During each of the above mentioned steps, the receptor and ligand will interact, and various forces will make contributions to the binding. Of these forces, the electrostatics in particular is expected to be the dominant force during steps 1 and 2; this is because there is no direct contact between the receptor and ligand in these steps, and all other forces, being short-ranged, will have negligible effect. The electrostatic force is capable of causing conformational and ionization changes in both the receptor and the ligand and can therefore alter the protonation states. These changes may result in changes in the charge distribution, which will affect the electrostatic interactions. An even more complicated balance is expected to occur during the final stage (the physical binding), since more forces will be involved and both the receptor and the ligand interfaces will be shielded from the water phase. It is very likely that the dramatic changes in the interface environment and the newly formed interactions across the interface will induce conformational and protonation changes. In this review we will outline the importance of correctly assigning protonation states for each of the three above mentioned steps.

The importance of the role of electrostatics on protein-protein interactions is well understood and was elucidated by many researchers: Nielsen demonstrated that electrostatic interactions are the major force for stability, binding characteristics, and function of proteins [4]. The electrostatics is the driving force behind electron transfer and protein-protein association [5]. The importance of surface and active center charges of acetylcholinesterase in the electrostatic attraction of its ligands, and the influence of charge on the reactive orientation of the ligand has previously been demonstrated [6]. It was shown that electrostatics plays profound role in the thrombin-γ' chain interactions [7]. The role of electrostatics on protein binding energy and its salt dependence was outlined in a series of other works [8–10]. Large scale investigations were done to assess the plausible protonation changes associated with the binding as well [11, 12].

Before proceeding with these topics, one could raise the question of the importance of protonation phenomena in receptor-ligand recognition at all [13, 14]. Is there experimental evidence that protonation states may not be standard in receptor-ligand complexes or may change during the formation of the complex? How frequently were such events experimentally documented in the past? Below, we briefly provide experimental evidence of proton uptake/release due to receptor-ligand interactions, while outlining that such data is not absolute and depends on the environmental conditions of the experiments including pH and, to a certain extent, the salt concentration.

A recent study utilized site-directed replacements of various amino acids in lactose permease of Escherichia coli (LacY) to determine the source of an observed pKa value that had the unusually high value of approximately 10.5 for sugar-binding affinity. It was concluded that the high pKa for sugar binding by wild-type LacY cannot be ascribed to any single amino acid residue, but appears to reside within a complex of residues involved in H+ translocation [15]. Another experimental study employed isothermal titration calorimetry

(ITC) in conjunction with macromolecular modeling (MM), to show that the binding of the ERα nuclear receptor to its DNA response element is coupled to proton uptake [16]. It was suggested that two ionizable residues, H196 and E203, located at the protein-DNA interface, are the ionizable residues changing their protonation states as a result of binding. It was also pointed out that H196 and E203 are predominantly conserved across approximately 50 members of the nuclear receptor family, which suggests that proton uptake may serve as a key regulatory switch for modulating protein-DNA interactions [16]. Another study utilized NMR to demonstrate that the binding of Bacillus anthracis glmS riboswitch to the α-anomer of GlcN6P binding is pH-dependent. Furthermore, the binding of glmS RNA reduces the pK(a) of the GlcN6P amine by 1.6 units in the ground state, causing proton release [17]. Proton uptake was experimentally observed in case of ion binding [18] and allosteric inhibition [19]. In general, any pH-dependent binding involves proton uptake/release and therefore causes changes in the protonation states of ionizable groups. The literature is full of examples of experimental data demonstrating pH-dependent binding from ion binding to small peptides [20] or proteins [21], and acid-organic compounds [22], to protein-small molecule [23, 24] and protein-protein binding [25–27]. All of these examples demonstrate that protonation states frequently change due to the binding.

THE ROLE OF PROTONATION STATES ON RECEPTOR AND LIGAND RECOGNITION

Within the cell, the receptor and the ligand are present and each individual molecule moves due to thermal motion, and perhaps, randomly collides with the rest of molecules in the cell. To find its partner, the receptor and the ligand must sense each other (Fig. 1 A.1 and B.1). One must recall that in order for the molecules to steer themselves towards the correct partner, they must be able to sense their partner at large distances so the driving force must be the electrostatics.

It is also useful to recall that there are two major classes of receptor-ligand complexes: hereto- and homo-complexes. The hereto-complexes are made of two different units (including cases of protein-small molecule complexes), while homo-complexes are composed of two identical monomer components. This is an important distinction when one recognizes that at large distances (during the initial stage of molecular recognition), the shape and the charge distribution within binding partners, perhaps, does not matter. The most important molecular characteristic at large distances is the net charge. Therefore, the initial co-localization in space and time for hetero-and homo-complexes should utilize different mechanisms [28]. Perhaps, in case of hetero-complexes, the electrostatics almost always plays a favorable role, since it was shown that in vast majority of cases, the monomers carry opposite net charge [8]. In contrast, in the case of homo-complexes, the net charge of the monomers has the same polarity and therefore opposes the initial co-localization of the binding partners [29], but may be interacting favorably via dipole-dipole interactions [30].

Co-localization of the binding partners will place the monomers within the same environment in terms of pH and salt concentration. Perhaps, from an evolutionary standpoint, this would have required binding partners to co-evolve in such a way that they both would be adaptable to a shared environment. Indeed recent studies have shown that binding partners' pH-dependent characteristics are similar [10]. Thus, some complexes will be formed at low, others at neutral, and still others at high pH. Therefore, in order to correctly model native binding, one should know what the physiological pH of the reaction is and be able to predict correctly the protonation states at this particular pH.

To understand the role of protonation states in the initial steering, one must be able to predict the pKa's of the ionizable groups of both the receptor and the ligand in their unbound state. Protonation states must be predicted on a theoretical basis because the use of x-ray crystallography cannot determine the position of hydrogen atoms [31]. Note that at this stage, the interactions are supposed to be quite weak and not to affect the energetics of individual molecules, including the pKa's of ionzable groups. Thus, if the structures of unbound monomers are available, one could apply standard pKa calculations [32, 33] and be able to estimate the protonation states at the desired pH. Unfortunately, it is quite likely that there will be no *a priori* information about the physiological pH at which the complex forms, but it is necessary to predict it by either using the information about sub-cellular location provided in the Protein Data bank (PDB) [34] or by carrying out additional investigations to search the literature for homologous annotated protein(s). Obviously, the problem disappears in cases of predicting protonation states for a particular experiment carried out with well-defined pH.

Another problem concerning the prediction of protonation states of unbound receptor and ligand is the common situation where their structures are unavailable. Even in cases where the structure of the complex is experimentally determined, taking the bound monomers from the complex to carry out pKa calculations for unbound state may be problematic due to the possibility that binding results in conformational changes [35]. If only the bound structures are available, one can relax them with standard molecular dynamics or normal mode modeling and then apply pKa calculations to the ensemble of generated structures [36]. If no structure is available, the 3D structures of biological macromolecules can be predicted by other means provided that there are structures of homologues. It was shown in the past that pKa calculations can be reliable if the sites are within well conserved structural regions [37]. In cases of peptides serving as a ligand to the receptor, one should account for the possibility that the ligand may be quite unstructured in the unbound state and may exist as an ensemble of structures. In this case, the protonation states may differ within the ensemble and they should be properly ensemble averaged.

Provided that the characteristic pH is known, the structures are available, and the pKa's are properly assigned to both the receptor and the ligand, one can easily determine the charge distribution within each molecule. Typically the receptor and the ligand will carry opposite net charge and will attract each other via favorable electrostatic interactions (Fig. 1 A.1 and B.1). However, for every receptor carrying a negative net charge, there will be many ligand candidates carrying a positive net charge and will also be co-localized in space and time with the receptor. Obviously the initial recognition is not very specific, but rather simply increases the concentration of plausible ligands around the target receptor. At the same time, it should not be overlooked that the co-localization occurs in specific environment in terms of pH, salt concentration, and volume constraints, and the receptor and the ligand must be able to get inside the corresponding cellular compartment and not be digested by enzymes. Taking all of these constraints into consideration, the possibility of having many candidate ligands carrying the same net charge may not be so great in many cases.

THE ROLE OF PROTONATION STATES ON RECEPTOR-LIGAND MUTUAL ORIENTATION

Once the receptor and the ligand are within close proximity to each other, they are expected to sense each other's interfaces, and restrict their rotational and translational motions in order to orient their interfaces properly (Fig. 1 A.2 and B.2). The magnitude of interactions and the loss of entropy due to the restricted mobility could cause protonation and conformational changes. The magnitude of the potential conformational and ionization changes will depend on various factors and can be grouped into two binding categories:

lock-and-key and induced fit binding. Here, we will artificially attribute this to the step 2, the mutual orientation, although much of the changes may occur at the actual physical binding. Obviously in the case of lock-and-key binding, the orientation state is not expected to cause drastic protonation changes, since the structures of unbound receptor and ligand are almost unaffected by the proximity of the partner and both interfaces are accessible from the water phase. The electrostatic interactions will be of marginal order since they will occur through the water and will therefore be significantly reduced. On the other hand, when observing the induced fit mechanism, one can expect significant conformational changes to occur during the orientation phase since the proper interfaces must be formed. These structural changes can alter the protonation states, including sites situated away from the interfaces [38].

Previous works have discussed that the binding of proteins depends on the folding of proteins themselves[39], and physicochemical properties of the interface [40]. The recent work of Honig and co-worker [64] summarized that the complementarity between surfaces of binding proteins are based on the shape [41] and electrostatic properties of binding sites [42, 43]. This is the reason why before two proteins associate to form a complex, they have to find the proper relative orientation by translational and rotational diffusion [44]. The protein association can be rate limited either by the diffusional approach of the subunits to form a transient complex, or by the subsequent conformational rearrangement to form the native complex [45].

At the stage of protein orientation, flexibility in protein structure plays a central role [46]. It was shown that even when the monomer is stable on its own, binding occurs faster through unfolded intermediates [47]. This was attributed to the hypothesis that the associating proteins will likely first form less constrained complexes, called encounter complexes, and then will slowly rearrange by translational and rotational diffusion until they reach the orientationally constrained transition state [44]. An example of protein complexes experiencing conformational changes was observed during the interaction of barnase and its intracellular inhibitor barstar; it revealed the formation of a weakly-specific complex due to long-range electrostatic interactions between protomers (the structural units of an oligomeric protein) [48, 49]. Another work observed pocket formation during protein-protein binding in a set of protein complexes [50]. Zhou and co-workers focused their investigations on predicting the protein association rate constant while putting emphasis on the role of electrostatics [51–53]. Recently they developed a web server designed for predicting protein association rate constants, called TransComp, which uses the structure of the native complex as input and generates the transient complex and the value of the association rate constant [45].

THE ROLE OF PROTONATION STATES ON THE PHYSICAL BINDING

During the final stage of the binding (the physical binding), after the partners are co-localized in space and time, and are properly oriented to each other, they should come together to form a complex (Fig. 1 A.3 and B.3). The final stage of binding is a complicated event, which may induce further conformational and ionization changes. In cases where no further conformational changes occur upon the physical binding, the protonation states of titratable residues situated at the interface can be affected by both the loss of interactions with the water phase and by newly formed interactions across the interface. The presence of the partner can also affect the pKa of distant (away from interface) groups via long range electrostatic interactions as well. In cases of further conformational changes induced by the physical binding, especially if they are propagated throughout the entire structures, the pKa changes can be scattered everywhere, including the back of the molecules far away from the binding site.

Receptor-ligand binding is driven by the difference between the free energy of the complex relative to unbound states [54, 55]. Binding free energy consists of several energy terms: including non-bonded terms such as electrostatic, van der Waals, and bonded terms such as the mechanical energy of the system, and the change of the entropy [56]. All of these energies are coupled with the protonation states and cannot be calculated separately. This coupling of various energy terms and effects is the main difficulty in modeling proton uptake/release upon the binding. Many experimental and computational studies were carried out in the past to reveal the relative role of the energy components, conformational, and ionization changes induced by the binding.

Some investigations have focused on conformational flexibility demonstrating that it may disfavor or favor the association [57]. It was shown that different sites of the proteins undergo conformational changes but at different amplitudes [58]. Analysis of the disordered and ordered proteins complexes showed that although disordered proteins adopt particular conformations in their bound form, they are still much less rigid than ordered proteins [46]. Since the interfacial area is an important characteristic of the binding, its properties were statistically assessed, and indicated that the average interface has the same non-polar character as the protein surface as a whole, but carries fewer charged groups [59]. The fewer charged group observation is attributed to the fact that charged or polar protein residues that were initially exposed to the water phase tend to be partly or fully dehydrated due to being buried inside the protein complex [39, 60]. However, the energy penalty due to the reduction of the proteins' flexibility and residues' desolvation can be compensated by newly formed interactions across the interface [61–64]. Correct assignment of protonation states would be crucial for accurate calculations of the balance of these opposing effects.

To demonstrate an analysis of the forces and energies causing receptor-ligand binding, numerous studies were designed to determine the main patterns of protein binding sites. It has often been assumed that receptor-ligand complexes associate through hydrophobic patches on their surfaces [65]. However, polar interactions between molecules are also common. It was shown that water molecules contribute to the close-packing of atoms that ensure complementarity between the two protein surfaces providing polar interactions between the proteins [59]. Further analysis showed that in some cases, interfaces have a large hydrophobic core surrounded by a ring of interfacial polar interactions, while in others, the interface consists of small hydrophobic patches with distributed polar interactions and water molecules over the entire interfacial area [65]. It was demonstrated that the weak homo-dimers have smaller contact areas between protomers and the interfaces are more planar and polar on average [66, 67]. In contrast, strong transient dimers undergo large conformational changes upon complex formation and are characterized as being larger, less planar, and sometimes possessing more hydrophobic interfaces. The interfaces of the hetero-complexes are less hydrophobic than those of homo-dimers [68, 69]. This phenomenon was explained by the possibility of protomers to be stable and function independently, while monomers in permanent complexes are found to be unstable in solution and not able to function by themselves [2,59]. Protomers from hetero-complexes are stable and are capable of proper biological functioning.

Further studies compared the influence of hydrophilic bridges between charged and polar residues on protein folding and protein-protein binding [70]. It was found that in contrast to protein folding, salt bridges across the binding interface can significantly stabilize complexes. While the hydrophobic effect in protein-protein interfaces is significant, it is not as strong as that observed in the interior of monomers. At this stage of proteins association, electrostatic interactions play an important role, but are not necessarily predominant. In an article, authors investigated how optimization of electrostatic affinity affects specificity [71]. Using charge-optimization techniques they proved that affinity-optimized electrostatic

interactions do not necessarily create specificity of ligand binding to the protein but affect the strength of binding through the formation of hydrogen bonds [72]. The effects of variable protonation states on binding affinity is the result of changes in the solvation properties of the free protomers, and also the interactions between the ligand and receptor while bound together [72]. The terminal hydrogen bonding groups are often involved in the interaction and can constrain the possible binding mode the ligand can adopt [73]. That is why the knowledge of residue protonation is crucial for protein binding affinity predictions [31, 72, 74]. Electrostatic complementarity between the ferrocene units within the ferrocene–ferrocene dimer leads to very frequent formation and constrained geometry [75]. In contrast, for binding CD2 to its ligand CD48 it was shown that charged residues contribute little to binding energy is this interaction [76]. However, the loss of these charged residues is shown to markedly reduce ligand-binding specificity. Thus, the charged residues increase the specificity of CD2 binding without increasing the affinity. It was also shown that in the CD2/CD58 interaction, the binding is accompanied by energetically significant conformational adjustments, that are not dependent on the highly charged binding interface [77]. In this particular case, long-range electrostatic forces make no net contribution to binding.

Conformational changes and electrostatic perturbations induced by the binding may result in change of ionization states [78]. While most changes occur within the immediate vicinity of the binding interface, it was shown that one out of five ionizable residues experience substantial changes in pKa regardless of their ligand type [14]. It is well documented that the pKa values of buried ionizable residues are usually very different from the standard pKa values of ionizable residues exposed to the water [60, 79, 80]. Onufriev and co-workers determined that 90% of examined protein-protein complexes possessed at least one ionizable group which changed its charge state due to ligand bonding at physiological conditions. They also determined that 9% of protein-protein complexes experience a substantial (greater than one unit) change in pKa values due to binding [14]. An example of significant pKa changes for histidine was shown for Herpes Simplex Virus receptor - IgG association [27], and during complex formations of trypsin and thrombin with their ligands [81] as well.

MODELING THE BINDING FREE ENERGY INCLUDING PROTONATION CHANGES AND APPLICATION TO DOCKING PROBLEM

Methods for modeling receptor-ligand binding free energy vary from structure based virtual screening methods to more sophisticated and more computationally costly free energy calculation methods. Docking methods utilize these methods or use a scoring function to predict the most stable conformation of the ligand-receptor complex using the structures of the unbound receptor and ligand. In both cases, the free energy calculations and the docking results depend on various details and assumptions, such as the choice of protonation sites on the receptor and the ligand [73, 82].

Proteins binding energy can be presented as following [83]:

$$\Delta\Delta G_{bind} = -RT\ln K_{eq} = \Delta G_{complex} - \left(\Delta G_{receptor} + \Delta G_{ligand}\right), \quad (1)$$

where K_{eq} is the equilibrium binding constant; $\Delta G_{complex}$, $\Delta G_{receptor}$, and ΔG_{ligand} are the folding free energies of the complex, receptor and ligand, respectively. These free energies include various energy contributions, and in the case of changes of protonation state from unfolded to folded state, must include the energy of ionization [38].

Many factors can influence the protonation states of the receptor and the ligand, such as their concentration and the concentration of other molecules, temperature, and mostly the

pH. Protomers differ in shape, functional groups, surface, and ability to form hydrogen bonds and therefore will experience different effects due to abovementioned factors. Only in very few cases does experimental data exist of the protonation states before and after the binding. In the vast majority of the cases, these protonation states (and changes) must be computationally predicted. However, the other frequently unknown factor is the pH of the binding. At some pH values, the binding may not involve protonation changes, while at other pH it may [10, 11]. In summary, computing the binding free energy or predicting the binding mode via *ab-initio* docking while taking into account protonation and conformational changes and having little knowledge of the physiological pH, is a challenge [84]. Below we briefly outline several works dealing with these issues.

In terms of predicting protonation states of proteins and receptor-ligand complexes, one can utilize standard pKa calculations if the structures of both the unbound and bound entities are available [32, 36]. The problem becomes more complicated if several groups change protonation state [78]. Nielsen pointed out that that this might be accomplished by calculating the titration curves of all ionized groups in a range of pH values, while taking into account the effect of the interactions between them [4]. There are now online servers that can predict the best combination of protonation states for each ionizable residue as well as the Gibbs free energy of binding for the ionization-optimized protein–ligand complex using coordinate information for the protein [85]. In terms of *in silico* screening, it was shown that the optimization of protonation states can play a significant role on selecting native-like ligand structures [86]. Another study constructed a prediction scheme with target-specific scores for estimating ligand-binding affinities to human estrogen receptor α (ERα), considering the major conformational change between agonist- and antagonist-bound forms and the change in protonation states of histidine at the ligand-binding site [87]. Different protonation was shown to result in alternative binding modes in docking simulations [88]. Another work showed the influence of variations of ligand protonation on the binding energy landscape of protein-ligand complexes using a structural consensus that was derived from multiple docking simulations [73]. Similarly, it was pointed out that the ionization state of ligand and receptor functional groups strongly affects their binding energy [89]. In Ref. [90] it was found that both the ionization state and the associated local pH play significant roles in binding free energy and are potentially valuable in protein-protein complex formation. The importance of taking protonation into account in molecular docking has been highlighted in several other articles [82, 91, 92]. Another work pointed out that one of the difficulties in the implementation of residues' protonation state in binding energy calculations is that the ionized state for each defined residue cannot be well-defined, because protons are not static, they transfer between molecules [89]. A new computation method called "computational titration" that implements the pH dependence of proteins binding and allows scoring the protonation-dependent models was reported [89]. Another group developed the combinatorial method called SPORES (structure protonation and recognition system) to generate protonation states [31].

IMPORTANCE OF PROTONATION STATES AND PH-DEPENDENCE OF BINDING

There is much research being done to understand the affect of pH on proteins and receptor-ligand complexes. This knowledge has consequences for many fields including industrial applications such as the production of pH dependent textiles and food, the production of gases from microbial processes, medical applications including tumor suppressants and drug design and many others (Fig. 2).

It is known that cells can exist and perform particular functions in complex environments within a particular range of temperature and pH conditions [93]. The pH level is different for

different parts of the body. Thus for example, in order to decompose food to basic components, the stomach maintains an acidic environment. The cells, covering inner walls of stomach, must be resistant to these extreme conditions and the proteins and receptor-ligand complexes within should be able to perform their functions. Another example are immunocompetent cells, such as monocytes and neutrophils. While being activated, they produce reactive oxygen species that acidify the environment [94]. Thus, the change in pH is used as a powerful weapon against pathogen organisms. In addition, these cells perform phagocytosis, at which monocytes and neutrophils capture the pathogens inside phagosomes inside cells. It was shown that during lysis of antigens the environment inside phagosomes/lysosomes are maintained temporary acidic, that allows to activate enzymes such as lysozyme to inactivate enemy [95]. Furthermore, the pH value is not constant throughout the cell; compartments of the cells can have widely differing pH [93], so the receptor-ligand complexes must be evolved to tolerate and sense the characteristic subcellular pH [11, 36, 96]. Besides cellular compartments, it is known that biological membranes strongly interact with titratable residues making membrane binding pH-dependent [97].

The importance of protonation states and the corresponding local pH is illustrated by the observation that tumor cells' pH is different from that of normal cells. This was used to develop different methods used for tumor suppression and treatment, as well as drug design and delivery. A recent work showed that low pH is a hallmark of tumor malignancy, and this potentially influences exosome release/uptake by cancer cells. The work was able to monitor exosome traffic as a function of different pH conditions to show an increased exosome release and uptake at low pH [98]. Another group investigated the effect of pH on p53 tumor suppressant with a mutation present. The work found a novel mechanism of tumorigenesis: a pH-dependent p53 tumor suppressor dysfunction, which led to tissue-specific tumor development. The mutated tetramer was found to be very pH dependent while the wild type was stable at all tested pH values. The mutation was shown to destablilize the p53 tetramerization domain at high pH values (6.5–8.0), so it was suggested that intracellular pH is likely to modulate the function of the mutated complex [99]. Based on differences of environmental pH of normal and tumor cells, new pH-sensitive polymeric micelles and nanogels were developed to target slightly acidic extracellular pH environments of solid tumors [100]. Another work prepared tumor-targeting peptide conjugated pH-responsive polymeric micelles to be used in cancer therapy using a pH-responsive tumor targeting delivery system. The pH-dependent drug release profile was obtained at a pH of 6.4, and efficiency in delivering anticancer drugs in mice was demonstrated. It was speculated that such a tumor-targeting peptide-conjugated and pH-responsive polymeric micelles have potential in cancer therapy [101].

The alteration in pH environment is widely used in drug design. It was explained that engineering pH dependency into the interactions of therapeutic antibodies may allow medications to be delivered less frequently or at lower doses. With this regard, a pH dependent antibody against the IL-6 receptor was incorporated to dissociate quickly from IL-6R within an acidic environment while maintaining its binding affinity to IL-6R in order to increase the number of cycles of antigen binding [102]. In order to maximize the efficiency and minimize initial drug release in the stomach, and reduce the potential gastro irritant and ulcerogenic effects of the drug, another study designed a controlled release formulation of ibuprofen with a pH dependent release profile. The work was successful in extending the release of ibuprofen from its tablet formulations [103]. Similarly, it was found that with all basic and the majority of acidic drugs, fraction unbound in plasma depended linearly on pH. Evidence was found indicating that pH-induced changes in the plasma proteins were also involved in the observed pH-dependent interaction with ligands. It was suggested that changes in fraction unbound could be clinically relevant with narrow-therapeutic-range drugs [104].

In addition to medical applications, there are many fields that also utilize pH including many industries that produce materials that can have pH dependent qualities and food processing companies who must track pH in order to ensure food safety. The possibility of using pH control to enhance separation and to clean up the ionic liquid solvent has been suggested by the work of many groups [105]. It was explained that room temperature ionic liquids are emerging as solvent replacements for volatile organic compounds traditionally used in liquid/liquid separations. Experimentally, using thymol blue indicator dye, the group was able to demonstrate a reversible pH-dependent liquid/liquid partitioning [106].

pH plays a central role in the production and fermentation of dairy products including milk and cheeses. A recent study investigated the effect of pH on the turbidity of reconstituted skim milk powder, and showed a relationship between the barostability of casein micelles and pH [107]. In another study, it was found that the activity of cell-envelope proteinases and aminopeptidases was a function of pH and reached a maximum at about pH 6 and decreased at lower pH values [108]. In addition to milk, pH has a great effect on the production of cheese. With this regard, the effect of pH on the composition, microstructural, and functional properties of Mozzarella cheese was investigated and it was found that cheeses with a high pH with a reduced calcium content resulted in a significant decrease in the protein level and increases in the moisture content of the cheese [109]. PH is often used as a control method in the production of hydrogen and methane from cheese whey. Thus, an automatic pH controller was used to maintain the pH culture in order to control the amount of gas produced [110]. Many other examples can be pointed out illustrating the importance of the pH and protonation states in food industry [111–114].

In addition to the use of pH in materials and food processing, it is also used as a method for controlling the production of gases such as hydrogen and methane in organic processes. Hydrogen gas can result from the microbial fermentation of organic substrates. A technique for limiting methanogenesis in mixed cultures is maintaining a low pH environment during culture growth [115]. Another work determined that the production rate of hydrogen gas from the conversion of wastewaters is also a function of pH, and the optimal pH range for hydrogen production was at a pH range of 5.0–6.0 [116]. The pH of soil is often used to control the formation of methane in soil from microbial processes [117].

CONCLUSION

It was outlined that protonation states of the receptor and the ligand before and after the binding are a very important factor for the binding as indicated by both the experimental data and computational modeling reported in the literature. It was shown that in many cases, the formation of the complex is accompanied by proton uptake/release. At the same time, the reviewed works suggested that each receptor-ligand complex has evolved toward a specific pH, termed pH-optimum, at which the proton uptake/release upon the binding is zero. Perhaps, the experimental observations and numerical studies indicating frequent proton uptake/release upon complex formations were carried at pHs different from the pH-optimum of the corresponding complexes and this was the reason for such a conclusion. In any case, the computational methods should be able to model receptor-ligand binding at any pH, including pH at which the binding induces proton transfer and is pH-dependent. However, it was demonstrated that predicting the absolute binding energy, calculating the pH dependence and scoring small molecule binding to the target protein taking into consideration plausible protonation changes is still a challenge.

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GLOSSARY

Protomer	the structural unit of an oligomeric protein
Receptor	the protein (typically the larger partner) to with the other protomer binds
Ligand	the protein, the peptide or small molecule that binds to the receptor
Recognition	the receptor and the ligand sense each other in the crowded cellular environment
Orientation	the receptor and the ligand are oriented in such a way that their interfaces point toward each other
Physical binding	the receptor and the ligand form 3D complex (oligomer)
Hetero-complex (hereto-dimer)	complex made of two different protomers
Homo-complex (homo-dimer)	complex made of two identical units

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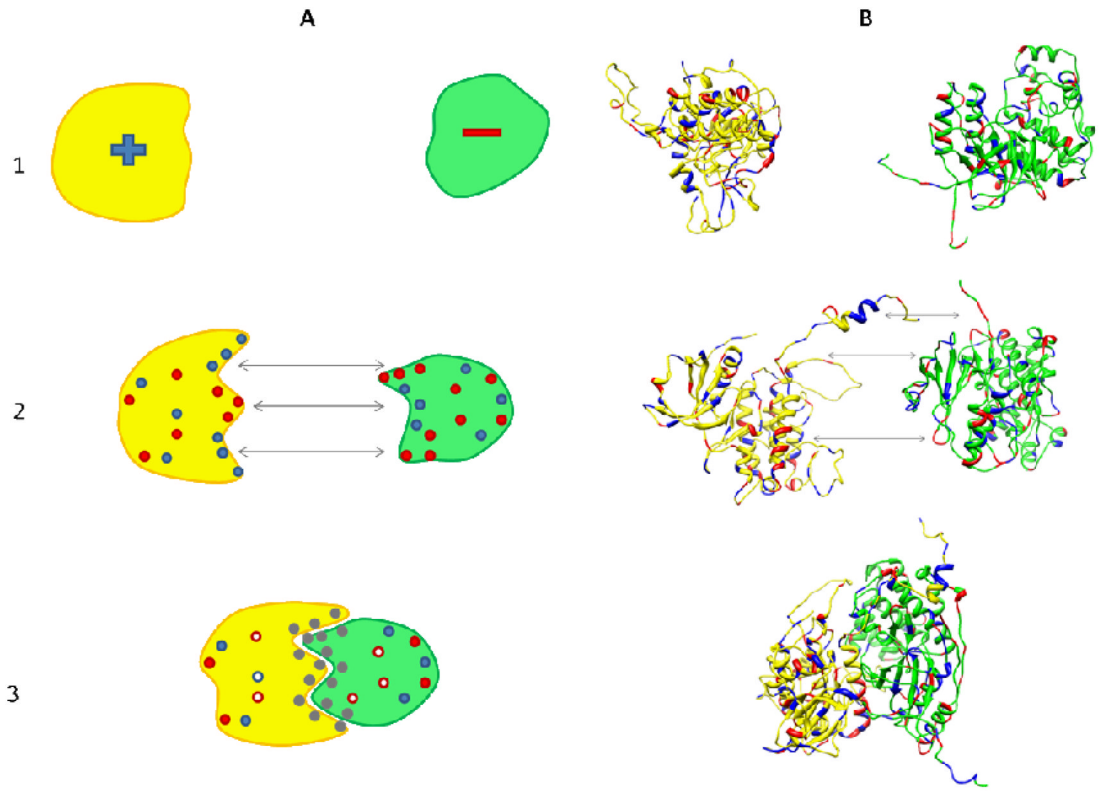

Figure 1.

Cartoon (A) and real case (B) representation of protein-protein complex formation, where 1 – recognition stage (proteins sense each other while being large distance separated due to electrostatic force); 2 – orientation stage (each protein experiences conformational changes and electrostatic perturbations due to electrostatic repulsion and attraction of charged groups); and 3 – physical binding stage occurs due to electrostatic, van der Waals and interactions with water molecules and the change of the system entropy.

Protein1 (MAPK2) and protein2 (MAPK14) are shown in yellow and green. Red and blue represents negatively and positively charged residues respectfully. In grey are residues which protonation state changes after complex formation. Red/blue open circles show residues which protonation state might be changed after complex formation. For real case visualization was made with Chimera software [118]

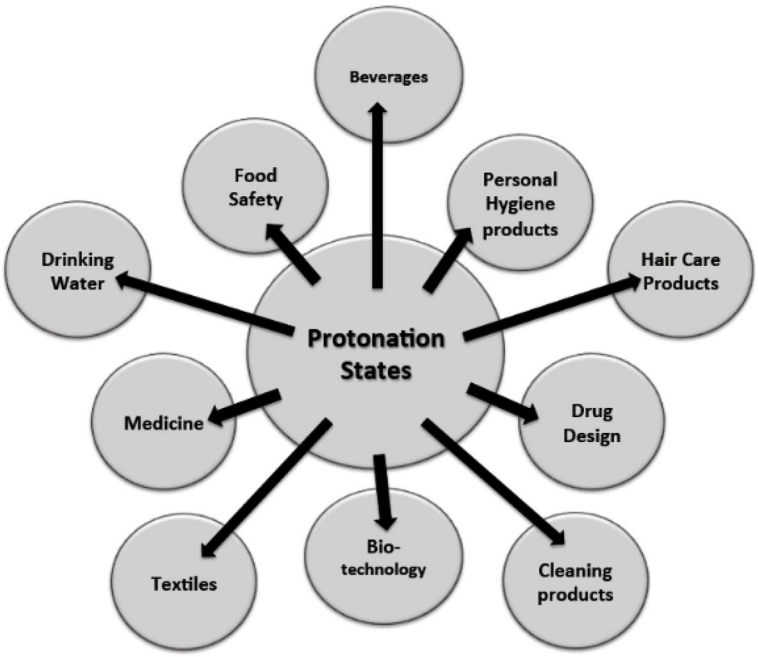

Figure 2.
Importance of protonation states for various science and industry areas.