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International Journal of Pharmaceutical Research & Allied Sciences, 2025, 14(2):189-199

https://doi.org/10.51847/KC6oEHQCmP



Original Article

ISSN : 2277-3657
CODEN(USA) : IJPRPM

Protonation as a Hidden Thermodynamic Variable in Molecular Dynamics of Drug Binding: A Conceptual Framework for Reconciling Simulation Conditions, Assay pH, and Ligand Optimization

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ABSTRACT

Molecular dynamics simulations of drug binding frequently assign fixed protonation states even though the measured affinity reflects an ensemble of ligand, receptor, and complex states under defined experimental conditions. This discrepancy can complicate free-energy interpretation, particularly when proton exchange is coupled to hydration, electrostatic polarization, or conformational rearrangement. This theoretical study develops a mechanism-based account of how microscopic protonation equilibria contribute to observed binding behavior and why ostensibly protonation-aware calculations may still disagree with assay measurements. Recent evidence from multistate free-energy methods, constant-pH molecular dynamics, ionization prediction, and protein–ligand investigations is integrated with a critical experimental–computational counterexample. The analysis separates chemical-state populations, state-conditioned association, and assay-level observability, while retaining alternative explanations involving solvent, force fields, and molecular accessibility. It proposes that concordance requires explicit specification of the states and thermodynamic conditions to which each calculated or measured quantity refers. Discriminating comparisons are identified for evaluating proton linkage against competing mechanisms. The account is nonempirical and does not establish a universally predictive method; its contribution is a falsifiable interpretation of when molecular simulations and binding assays can legitimately be compared.

Key words: *Protonation-coupled binding, Ligand microstates, Constant-pH molecular dynamics, Binding free energy, Microscopic pKa, Assay pH*

Received: 07 July 2025; **Revised:** 10 August 2025; **Accepted:** 14 August 2025

INTRODUCTION

The protonation state assigned to a drug during molecular simulation determines its formal charge, electrostatic interactions, hydrogen-bonding possibilities, and often the conformational preferences of its receptor complex. Experimental affinity, however, is usually measured in a solution that permits proton exchange and distributes molecular populations among chemically distinguishable states. This creates a central interpretive problem: a precisely calculated binding free energy for one molecular state may not represent the equilibrium quantity reported by a binding assay. In a study of donepezil and acetylcholinesterase, computational approaches favored binding as the ligand became protonated, whereas calorimetry showed reduced affinity at pH 6.0 [1]. That disagreement provides a particularly useful test of what protonation-aware predictions can explain.

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Methods for incorporating alternative ligand protonation and tautomeric states demonstrate that chemical-state multiplicity can be included within alchemical free-energy calculations [2]. Nevertheless, successful implementation does not resolve every question about which chemical states are experimentally relevant. Aqueous ionization terminology itself can conceal differences between microscopic site-specific equilibria and macroscopic ionization measurements [3]. An apparently straightforward input pKa may therefore fail to identify the microstate probabilities required for a binding calculation.

Electrostatic modeling adds another source of ambiguity. Protonation and electronic polarization can both influence the energetic consequences of transferring a charged ligand from solution into a protein environment [4]. Agreement between a single simulated charge state and a measured affinity may accordingly be coincidental, while disagreement may involve several distinct physical processes.

The question addressed here is whether the simulated and experimental quantities refer to thermodynamically comparable ensembles and, when they do not, which mechanism explains the divergence. Mechanism-based reasoning is appropriate because it links explicitly specified molecular states to conditional physical processes and observable consequences. The analysis develops a nonempirical theoretical interpretation and evaluates its limits against existing molecular and methodological evidence.

Protonation-dependent binding as a theoretical and computational problem

A drug molecule with several accessible protonation or tautomeric states cannot, in general, be represented by one chemically fixed structure across every pH and environment. Each microstate possesses a different statistical weight in aqueous solution, and the distribution may change when the ligand enters a protein-binding pocket. Multistate free-energy perturbation formalism provides a basis for accounting for the relative contributions of alternative states [5]. Consequently, a conditional association free energy calculated for a specified ligand–receptor state pair and an apparent association free energy measured over exchanging populations are different thermodynamic quantities.

The receptor also contributes ionization equilibria. A protein residue may possess an environmentally shifted pKa, with protonation probabilities affected by electrostatic neighbors, solvent exposure, and local conformation. Nonequilibrium alchemical methods for protein pKa calculations illustrate that these distributions must themselves be estimated within a physical model [6]. A drug–target complex can therefore change proton occupancy through the ligand, receptor, or both.

Microscopic pKa values describe specified ionization relationships among molecular microstates; a macroscopic pKa aggregates experimentally distinguishable charge transitions. Computational approaches such as QupKake make microscopic prediction tractable, although their predictions remain dependent on molecular representation and benchmark data [7]. Thermodynamically informed machine learning further demonstrates the value of consistency among predicted ionization relationships [8]. The distinction is consequential when the measured macroscopic transition cannot identify which tautomer or protonation site participates in binding.

Joint prediction of pKa and protonation states reinforces the need to separate an equilibrium constant from an assignment of molecular identity [9]. Benchmarking against curated experimental values likewise requires careful identification of the charge transitions to which those values apply [10]. An aqueous pKa measurement constrains solution ionization behavior; it does not directly measure the microscopic protonation distribution inside a bound protein complex.

A useful statistical-mechanical representation introduces protonation-dependent partition functions for the free protein, free ligand, and protein–ligand complex. Under a consistent standard-state convention, the apparent association constant may be expressed as

$$K_a,obs(pH) = K_a,ref \times Q_PL(a_H) / [Q_P(a_H)\, Q_L(a_H)] \quad (1)$$

where the three binding polynomials describe the relative statistical weights of their accessible protonation states, a_H is proton activity, and the reference constant and polynomials use the same reference-state convention. These expressions are thermodynamic identities under their specified equilibrium assumptions; they do not independently establish which microstates are populated in a particular system.

The derivative of the observed association constant with respect to proton activity is related to the difference between the mean number of bound protons in the complex and in the separated binding partners. This proton-linkage relationship supplies a testable equilibrium implication. It does not identify a unique protonation site,

conformational mechanism, or rate-determining event. Those additional claims require independent chemical-state evidence and adequate sampling of the relevant molecular ensemble.

Current evidence on protonation states and drug–target interactions

Constant-pH molecular dynamics has revealed circumstances in which ligand protonation is coupled to solvent behavior. Simulations of related β-secretase inhibitor complexes found different pyrimidine protonation states in BACE1 and BACE2, associated with altered water occupancy, pocket rigidity, and hydrogen-bonding patterns [11]. The findings suggest a physically specific route by which a chemical-state transition can influence molecular recognition. Because the evidence is system-specific and simulation-based, its broader relevance requires corroboration.

Structural and thermodynamic studies of ligands binding to trypsin and thrombin provide a complementary experimental perspective. Chemically related ligands were observed in different protonation states despite similarities in their bound poses; differences in local electrostatics and hydration offered plausible explanations [12]. This evidence establishes that receptor environment can materially affect the state favored during binding. It also cautions against assigning selectivity exclusively to a single ionization event.

Some molecular systems involve several coupled state coordinates. Multiscale free-energy calculations for ionizable photoswitchable eDHFR inhibitors consider the interaction between protonation and photoswitch-dependent molecular state [13]. Such systems demonstrate why the coordinates included in a thermodynamic model must follow the actual chemistry. Their photochemical features also restrict extrapolation to conventional ligands.

Protein-side protonation contributes additional complexity. Quantum calculations show that histidine charge state alters the character and energetics of noncovalent interactions involving aromatic and polar groups [14]. These pairwise effects are relevant mechanistic possibilities, but total binding affinity also includes conformational, solvation, and entropic contributions.

Recent predictive methods expand the ability to nominate protein protonation states. Protein language-model approaches use sequence information to estimate residue pKa values [15], while machine-learning classifiers provide alternative ways to characterize ionizable sites [16]. Their useful role in a binding study is to generate chemical-state hypotheses for further assessment, not to substitute for direct evidence of bound-state protonation.

Physical simulation methods are also changing. Constant-pH simulations using the polarizable AMOEBA force field allow ionization sampling within an electrostatic description that differs from conventional fixed-charge models [17]. This broadens the available physical representations, although polarizable calculations remain sensitive to their parameters, sampling, and validation endpoints.

Taken together, the evidence supports several distinct propositions: protonation can affect molecular interactions; binding environments can alter preferred states; and computational methods can explore these possibilities at increasing levels of sophistication. **Figure 1** represents their thermodynamic relationships while maintaining a distinction between established study-specific effects and states that remain unobserved in any particular drug–target system.

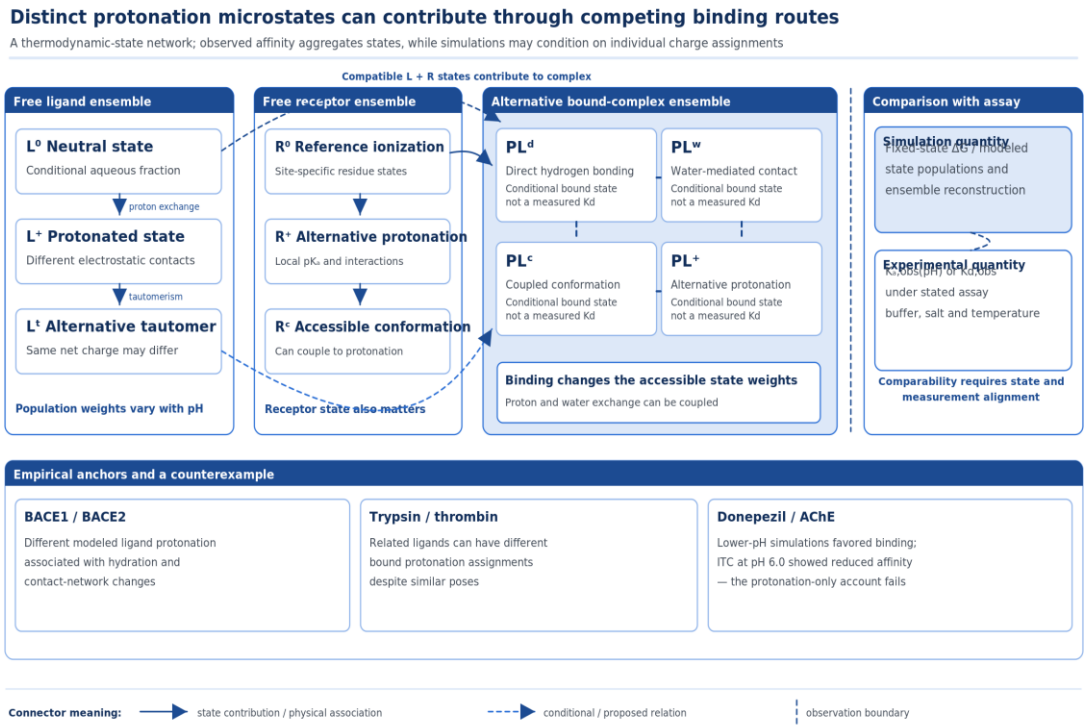


Figure 1. Chemical-state populations influence binding through several non-equivalent thermodynamic routes. Thermodynamic state network connecting free ligand microstates, receptor ionization states, and alternative bound-complex states. Horizontal relationships distinguish chemical association from protonation and tautomeric interconversion. Molecular consequences may include direct hydrogen bonding, water-mediated interactions, and changes in pocket organization, as supported by selected binding studies. The outer observation boundary separates calculated, state-conditioned free energies from pH-dependent experimental affinity. Solid connectors denote physically supported associations or equilibria; dashed connectors indicate conditional or proposed relationships. The donepezil–acetylcholinesterase discrepancy illustrates why a plausible protonation trend may fail to predict the measured response. L denotes ligand; R, receptor; PL, complex; AChE, acetylcholinesterase; BACE, β-secretase; ITC, isothermal titration calorimetry.

Unresolved relationships between simulation assumptions and experimental conditions

A constant-pH simulation is defined by its chemical-state space, energy function, and sampling algorithm. Recent implementation work in GROMACS extends the practical treatment of biomolecular titration [18]. Yet employing a constant-pH algorithm does not ensure that all binding-relevant states are accessible, or that the modeled solution conditions reproduce those of an experimental assay. Agreement requires separate consideration of chemistry, ensemble sampling, and measurement.

GPU-accelerated Hamiltonian interpolation can make protonation-state sampling more computationally accessible [19]. Adiabatic free-energy approaches provide another route to exploring dynamic ionization [20]. Neither computational efficiency nor convergence of a selected observable guarantees that an omitted tautomer, receptor conformation, or proton-coupled hydration state has become irrelevant. Convergence must be assessed for quantities that contribute to the proposed binding mechanism.

Force-field sensitivity further complicates comparisons. Reparameterized force fields can improve pKa predictions for particular classes of titratable residues [21]. Such improvements are encouraging but do not establish correct ligand microstate energetics or a valid free-energy difference between solution and complex. If the underlying protonation equilibrium is inaccurately represented, longer simulation time cannot necessarily correct the error.

Some processes require explicit chemical treatment. QM/MM investigations of biological protonation dynamics show how proton transfer and associated free-energy changes can be sampled when electronic rearrangement matters [22]. Their scope differs from that of equilibrium ligand binding: establishing a proton-transfer pathway does not determine its statistical importance to an assay-level binding constant.

Prediction models introduce an additional interpretive step. Atomic sensitivity analysis can make small-molecule pKa predictions more interpretable [23], but sensitivity of a predictor to a structural feature is not an observation of proton occupancy. A model may select a plausible state without establishing that the corresponding microstate dominates in the experimental buffer or bound complex.

Binding-site water represents a particularly consequential competing mechanism. Calculations of buried-water free energies demonstrate that water occupancy and sampling must be treated explicitly in some binding systems [24]. A computed affinity discrepancy that changes after improved hydration sampling should therefore not automatically be classified as evidence for proton linkage.

The unresolved problem is not simply whether protonation changes during binding. It is whether the hypothesized change has been isolated from other contributions that differ between the simulation and experiment. As summarized in **Table 1**, chemically comparable observations require matching state definitions, solution conditions, and measurement endpoints while maintaining independent assessments of sampling and model uncertainty.

Table 1. Distinct sources of discordance between protonation-dependent simulations and experimental binding measurements

Physical or analytical unit	Comparison required	Unresolved mechanism or uncertainty	Interpretation supported by the comparison	Proposed discriminating control
Free ligand ionization ensemble	Assigned microstates versus solution-state ionization evidence	Incorrect micro-pKa or omitted tautomer	State populations may be incorrectly weighted	Alternative microstate assignments constrained by independent titration evidence
Protein ionizable residues	Fixed residue charges versus pH-dependent protein states	Environmental pKa shift	Receptor charge assumptions may alter affinity estimates	Residue-state sensitivity and independently supported pKa constraints
Bound complex	Conditional free energy versus apparent experimental affinity	Proton linkage and state reweighting	Conditional and ensemble affinities may differ	Calculate consistently referenced free/bound state ensembles
Binding-site solvent	Simulated water occupancy versus alternative hydration configurations	Slow water exchange or water displacement	An affinity error may be solvent-mediated	Enhanced water sampling under matched chemical-state conditions
Electrostatic description	Fixed-charge versus polarizable or reparameterized models	Polarization and parameter error	Protonation attribution may be model-dependent	Cross-model electrostatic sensitivity comparison
Simulation and assay conditions	Nominal simulation pH versus measured assay environment	Ionic strength, buffer, temperature, or sampling mismatch	pH alone does not establish full condition equivalence	Reproduce documented assay conditions and assess convergence

Table note. Rows identify analytically different sources of uncertainty; they are not mutually exclusive causes of experimental discrepancies. The final column contains proposed controls, not validated performance standards. Evidence support is maintained in the citation audit and the surrounding manuscript discussion.

Development of the proposed protonation-aware conceptual framework

The preceding evidence supports a conditional interpretation built around three connected quantities: the distribution of unbound chemical states, the energetics and accessibility of bound configurations, and the observable reported by an assay. These quantities must refer to compatible thermodynamic conditions before a calculated and measured affinity can be compared meaningfully. Importantly, agreement at one pH does not alone demonstrate that their protonation mechanisms agree.

The first operation is chemical-state identification. Ligand and receptor microstates should be specified independently, including the ionizable groups and tautomeric alternatives that can materially change their interactions. Their populations must follow a consistent proton chemical potential and thermodynamic reference convention. Since polarizable force-field calculations can yield different predictions for residue ionization [25], uncertainty in the electrostatic description should accompany uncertainty in microstate assignment.

The second operation connects state populations to binding configurations. Proton uptake or release may modify direct electrostatic contacts, solvent organization, and conformational preferences. In one studied selectivity problem, proton-coupled conformational allostery provided a mechanistic explanation for inhibitor behavior across related proteases [26]. Such a pathway illustrates a broader possibility, but its applicability cannot be assumed for another receptor merely because that receptor contains titratable residues.

The third operation addresses experimental observability. The quantity compared with a calculation should be identified as an apparent equilibrium constant, a conditional state-specific affinity, a kinetic measurement, or another experimentally defined endpoint. The free and bound state ensembles must be weighted consistently for the specified assay pH and other material solution conditions. In the donepezil case, the computational and experimental trends diverged despite explicit attention to protonation [1]; this constitutes evidence that microstate weighting alone may be insufficient.

This interpretation admits distinct outcomes. In some systems, the principal correction may be adequate accounting for multiple ligand protonation states. Elsewhere, protein-side titration, binding-site water, or conformational changes may dominate. When two mechanisms produce similar apparent affinity trends, orthogonal ionization measurements, controlled chemical perturbations, and rival-sensitive simulations become necessary to identify their respective contributions.

Figure 2 connects these analytical levels and distinguishes the proposed reconciliation requirements from established computational and experimental observations. Its central implication is conditional: an apparent match in affinity is scientifically informative only to the extent that the compared state ensembles, measurement definitions, and dominant mechanisms have been specified or independently constrained.

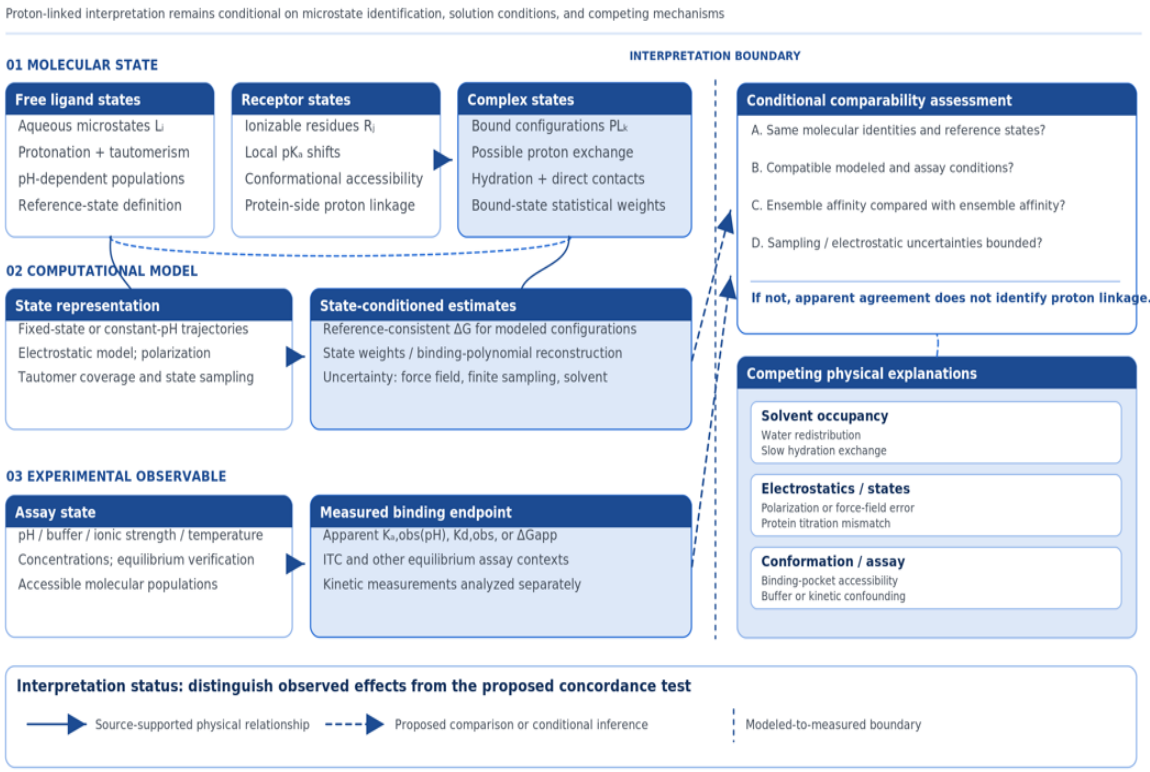


Figure 2. State-resolved binding claims require agreement across molecular, computational, and assay levels. The multilevel reconciliation map distinguishes the free ligand, receptor and complex-state ensembles (top), computational state representation and conditional free-energy estimation (middle), and experimental assay state and measured binding endpoint (bottom). The interpretation boundary separates those levels from the proposed comparability assessment, which considers molecular identity, reference states, assay conditions, ensemble affinity, and model uncertainty. Competing explanations involving solvent occupancy, electrostatics, conformational sampling, and assay conditions remain explicit. Solid arrows denote source-supported physical relationships; dashed arrows denote conditional or proposed comparisons; the vertical dashed line marks the model-to-measurement interpretation boundary. The figure reports no new experimental validation.

Implications for molecular simulation and ligand optimization

Protonation-aware ligand optimization requires decisions about both chemical-state representation and the experimental quantity against which a prediction will be judged. A substituent that changes ligand basicity may alter the solution population of a binding-competent microstate, its intrinsic association free energy, or both. These contributions can produce different apparent affinity trends even when the simulated bound geometry remains similar. Statistically replicated relative binding free-energy calculations provide a basis for assessing whether calculated ligand rankings are sufficiently precise to inform optimization [27]. Chemical-state uncertainty must be evaluated alongside numerical uncertainty.

A practical comparison begins by identifying the ionizable groups affected by a proposed chemical modification. Where alternative states are appreciably populated, the relative free energies should be evaluated using thermodynamically consistent state definitions. Independent calculations across justified charge assignments can establish the sensitivity of predicted ligand ranking to this choice. Comparisons across free-energy estimators or molecular dynamics engines then address numerical reproducibility [28]. Agreement among implementations strengthens confidence in numerical consistency but cannot independently establish that the correct protonation ensemble was sampled.

Receptor flexibility and hydration require parallel attention. Accelerated sampling of receptor conformational and solvation responses can change the energetic treatment of ligand association [29]. If changing a ligand substituent also modifies water displacement or a protein conformational equilibrium, a single protonation correction may misattribute the origin of its observed affinity. Ligand optimization should consequently distinguish predicted changes in chemical-state abundance from changes in interactions within a particular state.

Equilibrium and kinetic optimization also require separate endpoints. Computational approaches developed to estimate ligand binding and dissociation rates address information that an equilibrium binding free energy does not determine uniquely [30]. A ligand may exhibit a favorable equilibrium affinity without possessing the desired residence time, and changes in kinetic behavior cannot automatically be interpreted as proton exchange during association or dissociation.

These considerations yield a set of comparison principles for chemical-state agreement between simulation and experiment **(Table 2)**. Their purpose is to identify which additional evidence would make a particular optimization decision defensible and when an apparently favorable computed ranking remains conditional.

Table 2. Chemical-state agreement criteria for interpreting ligand optimization predictions against experimental binding assays

Optimization decision unit	Computational quantity to examine	Experimental correspondence required	Principal ambiguity	Proposed evaluation principle
Ionizable substituent change	Relative microstate populations and conditional binding energies	Affinity measured under documented pH and solution conditions	Population shift versus intrinsic interaction improvement	Evaluate both contributions before attributing structure–affinity trends
Ligand tautomer modification	State-specific association free energies and accessible tautomer populations	Chemical-state evidence where experimentally obtainable	Incorrect bound-state tautomer assignment	Test alternative state definitions and report sensitivity
Protein–ligand electrostatics	Residue charge assignments and electrostatic model dependence	Protein ionization constraints and binding measurements	Force-field error versus proton linkage	Compare defensible parameterizations independently of sampling precision
Binding-pocket hydration	Water occupancy and solvent-dependent free-energy contributions	Affinity or structural observations informative about hydration	Water rearrangement mistaken for a protonation effect	Reassess chemical-state trends under improved solvent sampling
Candidate affinity ranking	Ensemble-conditioned free energies with statistical uncertainty	Matched-assay equilibrium affinity ranking	Numerical convergence versus chemical correctness	Require both reproducibility and chemically consistent state accounting
Residence-time optimization	Association/dissociation kinetics	Independently measured kinetic endpoints	Equilibrium affinity confused with kinetic persistence	Interpret kinetic predictions separately from proton-linked equilibrium affinity

Table note. Evaluation principles are original theoretical proposals informed by the cited computational and molecular evidence. They are not established regulatory criteria or experimentally validated optimization thresholds. The underlying chemical-state and assay conditions determine their applicability.

Testable predictions, competing explanations, and evidence requirements

The central account is falsifiable when protonation-dependent predictions are compared with measurements that independently constrain chemical-state populations. Its first prediction concerns the relationship between assay pH and apparent binding affinity. If binding is accompanied by a change in mean proton occupancy, the pH dependence of the association constant should be thermodynamically consistent with that linkage under matched equilibrium conditions. The direction and magnitude cannot be assigned without knowing the relevant states and their populations.

A discriminating experiment would measure equilibrium affinity across a controlled pH range while independently characterizing accessible ligand ionization states. Buffer composition, ionic strength, temperature, and protein stability should be assessed so that their effects do not masquerade as proton linkage. Where experimentally feasible, proton uptake measurements, spectroscopic state assignments, and perturbation of specific ionizable groups could provide complementary constraints. A measured affinity trend inconsistent with independently supported protonation changes would weaken the central interpretation.

The computational counterpart should compare chemically justified fixed-state and multistate treatments under comparable sampling conditions. Predictions should be tested against independently measured affinities rather than evaluated solely by internal free-energy convergence. Systematic improvement restricted to ligands with accessible alternative protonation states would strengthen the proposed account. Similar improvement for chemically nonionizable controls could instead implicate unrelated methodological changes.

Competing explanations require explicit controls. Enhanced water sampling, alternative electrostatic descriptions, and independent receptor conformational sampling can determine whether a protonation-sensitive result remains stable when other physical uncertainties are addressed. Multiple reactive-state docking offers a related methodological example of why the chemical identity of the modeled enzyme–ligand system matters [31]. Reactive-state predictions nevertheless require distinct evidence before they can establish equilibrium proton linkage.

Absolute binding free-energy calculations have also been used to support virtual-screening and hit-discovery decisions [32]. Within protonation-sensitive series, such calculations could be evaluated against matched pH-dependent measurements to test whether ensemble-state treatment improves experimentally relevant predictions. A positive result would support the proposed comparison strategy within the tested domain; it would not prove a universal correction method.

The most challenging counterexample remains a persistent simulation–assay disagreement despite well-constrained chemical-state populations. If solvent or conformational corrections resolve such a discrepancy while protonation-state reweighting does not, the evidence would favor the competing mechanism. Theoretical validity therefore depends on the ability to reject protonation as the dominant explanation when independent observations warrant doing so.

Discussion and future research directions

The theoretical value of treating protonation as a thermodynamic variable lies in specifying the chemical and experimental conditions under which a molecular binding prediction has meaning. A simulation may accurately characterize a particular bound microstate while still leaving unresolved its contribution to the observed affinity. Conversely, a favorable match between calculated and measured affinity can conceal compensating errors in protonation, hydration, and conformational sampling.

Solvent behavior illustrates this challenge. Grand canonical Monte Carlo approaches permit exchange of water molecules with a defined chemical reservoir and can assist binding-site hydration sampling [33]. Such methods address a physical uncertainty that may coexist with protonation linkage. Combining appropriate solvent treatment with chemically consistent titration methods could help distinguish whether ligand modification affects microstate populations, water displacement, or both.

Future research should prioritize coordinated experimental–computational comparisons across structurally related compounds whose ionizable groups can be characterized independently. Studies should document assay conditions and distinguish conditional state-specific affinities from apparent ensemble affinities. Ideally, several complementary measurements would constrain the same mechanism, including solution ionization behavior, equilibrium binding, and structural or spectroscopic evidence where obtainable.

Method development should also examine the interaction between chemical-state enumeration and molecular sampling. More elaborate protonation algorithms may have limited interpretive benefit if important conformational states remain inaccessible. Similarly, advances in pKa prediction should be evaluated for their influence on downstream binding predictions rather than through pKa benchmark accuracy alone. Independent tests across charge classes, receptor environments, and experimental conditions would be especially informative.
A further priority is distinguishing thermodynamic and kinetic consequences. Proton exchange may alter accessible conformations or intermediate states and thereby affect association or dissociation behavior. Such effects would require kinetic measurements and mechanistic simulations beyond equilibrium state weighting. Evidence of pH-dependent residence time should not automatically be interpreted as proof that proton uptake directly controls the rate-limiting step.
For pharmaceutical development, the most useful outcome would be a transparent attribution of uncertainty. A ligand-ranking prediction could then identify which portion depends on experimentally constrained chemistry, which reflects model-dependent assumptions, and which remains sensitive to alternative physical mechanisms. This would make protonation-aware analysis valuable even where it cannot deliver a uniquely determined affinity prediction.

Limitations and boundary conditions
This account derives its central thermodynamic relationships from established equilibrium principles and its mechanistic examples from heterogeneous computational and experimental studies. It introduces no new simulation dataset, binding measurements, or prospective validation. Its proposed diagnostic comparisons therefore remain theoretical recommendations requiring independent testing.
Chemical-state enumeration can be incomplete, particularly for coupled protein–ligand ionization, metal-associated binding, unusual tautomers, and reactions involving proton transfer. Macroscopic ionization constants may not uniquely identify the relevant microscopic states. Force-field, solvent, and finite-sampling errors may also remain correlated, making their individual contributions difficult to estimate.
The equilibrium treatment assumes that relevant states can be represented consistently under a defined proton chemical potential and standard-state convention. It may be inadequate for systems with irreversible binding, nonequilibrium chemistry, strong time-dependent hysteresis, or measurements dominated by kinetic processes. Experimental assay artifacts and protein instability introduce additional limitations beyond molecular equilibrium theory.
Finally, protonation sensitivity may be negligible within a given experimentally accessible pH interval. In that circumstance, elaborate multistate treatment need not yield a meaningful improvement over an adequately validated fixed-state representation. The strength of the argument resides in its conditional predictions and explicit alternatives, not in an expectation that protonation dominates every drug-binding calculation.

CONCLUSION

Protonation becomes a consequential variable in molecular dynamics of drug binding when accessible ligand, receptor, or complex states change their statistical contributions to association under the conditions relevant to experimental measurement. A fixed-state free-energy estimate and an apparent assay affinity can therefore describe different thermodynamic quantities even when both are calculated or measured accurately within their respective definitions.
Meaningful agreement requires chemically consistent state definitions, appropriate ensemble treatment, documented assay conditions, and explicit consideration of solvent, polarization, conformational, and kinetic alternatives. The proposed mechanism-based account provides falsifiable criteria for distinguishing proton linkage from these competing explanations. Its pharmaceutical value lies in improving the interpretation of ligand optimization predictions without treating protonation-aware simulation as universally sufficient or already empirically validated.

ACKNOWLEDGMENTS: None

CONFLICT OF INTEREST: None

FINANCIAL SUPPORT: None

ETHICS STATEMENT: None

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