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Influence of Chemical Denaturants on Solute Solvent Interactions of Aromatic Amino Acids During Protein Unfolding

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Abstract: ***Chemical denaturants such as urea and guanidinium hydrochloride (GdnHCl) are the most widely used tools for probing protein stability, yet the molecular basis of their action remains an active area of investigation. Because aromatic amino acids tryptophan, tyrosine, and phenylalanine carry large, polarizable side chains that are simultaneously hydrophobic and capable of specific electrostatic interactions, they are exceptionally sensitive reporters of how denaturants reorganize the solvent shell around a protein during unfolding. This paper reviews the mechanisms by which urea and GdnHCl alter solute solvent interactions of aromatic residues, integrating the direct-binding and indirect (water structure perturbation) models, preferential interaction thermodynamics, and transfer free energy data. We examine how denaturant molecules compete with water for access to aromatic ring surfaces, how this competition changes local hydration numbers and dielectric environment, and how cation–π and π–π contacts are modulated as the protein backbone becomes more solvent-exposed. The synthesis presented here supports a hybrid mechanism in which direct denaturant aromatic ring stacking dominates at the residue level, while bulk solvent reorganization contributes a smaller, cooperative component to net unfolding thermodynamics.***

Keywords: urea; guanidinium hydrochloride; aromatic amino acids; protein unfolding; preferential interaction; hydration shell

1. Introduction

Protein unfolding by chemical denaturants is one of the oldest and most reproducible experimental paradigms in biophysical chemistry. Urea and GdnHCl reliably convert compact, folded proteins into expanded, largely disordered chains at molar concentrations, and the free energy of this transition is routinely used to quantify protein stability. Despite decades of study, the question of how these small, water-miscible molecules accomplish unfolding at the level of individual side chains is still debated. Aromatic amino acids occupy a privileged position in this debate because their side chains are large enough to support direct denaturant contact, hydrophobic enough to be buried in the native core, and electronically rich enough to participate in cation–π, π–π, and CH–π interactions that are sensitive to the surrounding dielectric environment.

This review focuses specifically on the solute solvent interactions of tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe) as unfolding proceeds in urea and GdnHCl solutions. We first summarize the structural and physicochemical features that make aromatic residues distinctive solvation probes. We then examine the two dominant mechanistic frameworks for denaturant action direct binding and indirect solvent perturbation and show how data from aromatic side chains have been used to discriminate between them. Finally, we discuss preferential interaction coefficients and transfer free energies as the quantitative bridge between molecular-level solvation changes and macroscopic unfolding thermodynamics.

2. Structural and Physicochemical Properties of Aromatic Amino Acids

Tryptophan, tyrosine, and phenylalanine each present a conjugated ring system attached to the polypeptide backbone through a short methylene linker. Phenylalanine carries a simple, nonpolar phenyl ring; tyrosine adds a para-hydroxyl group that introduces both hydrogen-bonding capacity and a modest dipole; tryptophan's indole ring is the largest and most polarizable, with a permanent dipole moment and a nitrogen hydrogen donor that participates in hydrogen bonding even while the ring face remains hydrophobic. These differences produce measurably different hydration behavior: Trp and Phe are strongly excluded from bulk water and tend to bury in the native core, whereas Tyr, owing to its hydroxyl group, is more frequently found at the protein surface or in partially buried environments.

Because aromatic rings possess a quadrupole moment with a negative electrostatic potential above and below the ring plane and a positive potential at the edge, they engage in characteristic non-covalent interactions: face-to-face and edge-to-face π–π stacking with other aromatic rings, cation–π interactions with lysine, arginine, or the guanidinium group of GdnHCl itself, and CH–π interactions with aliphatic side chains. Any solvent species capable of mimicking or disrupting these geometries will perturb the local solvation shell in ways that are optically and thermodynamically detectable.

2.1 Aromatic Residues as Intrinsic Solvation Probes

The absorption and emission properties of aromatic side chains are exquisitely sensitive to the polarity, hydrogen bonding capacity, and mobility of their immediate environment. This sensitivity is what allows spectroscopic techniques to track denaturant-induced changes in solute solvent interactions without extrinsic labelling, and it is the physical basis for much of the mechanistic evidence discussed in this paper.

3. Mechanisms of Denaturant Action

Two historically competing models explain how urea and GdnHCl destabilize folded proteins. The direct interaction (or direct binding) model holds that denaturant molecules bind preferentially and directly to the protein surface, including backbone amide groups and aromatic side chains, lowering the free energy of the unfolded, more solvent exposed state relative to the compact native state. The indirect model proposes that denaturants act primarily by altering the structure and hydrogen bonding network of bulk water, weakening the hydrophobic effect that normally drives burial of nonpolar and aromatic residues. Contemporary evidence, drawn substantially from studies of aromatic amino acid solvation, favors a predominantly direct mechanism, with a smaller but non-negligible indirect contribution.

3.1 The Direct Binding Model

Molecular dynamics simulations and osmotic pressure measurements show that both urea and guanidinium ions accumulate at protein surfaces relative to bulk concentration, with guanidinium showing an especially strong preference for stacking against aromatic rings via a combination of cation–π and van der Waals interactions. Urea, lacking a permanent charge, interacts with aromatic surfaces primarily through van der Waals contact and weak hydrogen bonding involving its carbonyl oxygen and amine hydrogens, allowing it to occupy positions immediately above and below the ring plane in a geometry similar to a stacked aromatic partner.

This direct contact behavior explains why exposure of aromatic residues to denaturant during unfolding is thermodynamically favorable: the denaturant does not merely tolerate contact with the hydrophobic ring surface, it is attracted to it, in contrast to water, which pays an entropic and enthalpic penalty to solvate an extended nonpolar surface.

3.2 The Indirect, Water-Mediated Model

An alternative view holds that denaturants restructure hydrogen bonded water networks, reducing the free energy cost of exposing hydrophobic surface area to solvent. Spectroscopic studies of water dynamics near dissolved urea and GdnHCl show modest perturbation of the hydrogen-bond network, but the magnitude of this effect is generally too small to account for the full free energy of unfolding by itself. Nonetheless, this indirect contribution likely acts cooperatively with direct binding, particularly during the early stages of unfolding when only limited denaturant access to buried aromatic clusters is possible.

3.3 A Hybrid, Residue-Class-Dependent Mechanism

The balance between direct and indirect contributions appears to depend on the chemical nature of the exposed side chain. For aromatic residues specifically, direct contact interactions dominate because of the favourable stacking geometry available to both urea and guanidinium. For polar and charged residues, direct hydrogen bonding to backbone amides plays a larger relative role. This residue class dependence explains why aromatic residue based spectroscopic probes (fluorescence, near-UV circular dichroism) are so responsive to denaturant concentration even before global unfolding, as reported in Section 5.

4. Preferential Interaction and Hydration Shell Perturbation

Preferential interaction coefficients (Γ or ξ) quantify the excess (or deficit) of denaturant molecules in the local domain around a protein surface relative to bulk solution. Positive preferential interaction values for urea and GdnHCl at aromatic rich protein surfaces indicate local denaturant accumulation, consistent with the direct binding model. Because aromatic side chains have the largest solvent accessible surface area per residue among common amino acids and the strongest tendency to be buried in the native state, they contribute disproportionately to the total preferential interaction signal measured for whole proteins.

The hydration shell around a buried aromatic ring in the native state typically contains far fewer water molecules than the equivalent surface exposed after unfolding. As the polypeptide chain expands, aromatic rings gain access to bulk solvent, and in the presence of denaturant, a substantial fraction of the first solvation shell is occupied by urea or guanidinium rather than water. Estimates from simulation studies indicate that denaturant occupancy of the first solvation shell around exposed aromatic rings can approach or exceed 20–30% of the layer, a value markedly higher than the bulk mole fraction of denaturant in solution direct evidence of preferential, non-random accumulation.

4.1 Comparative Denaturant Strength: Urea versus GdnHCl

GdnHCl is roughly twice as effective as urea on a molar basis at destabilizing most proteins, and this potency difference is reflected in aromatic-residue solvation behaviour. The planar, resonance-stabilized guanidinium cation forms stronger and more persistent stacking interactions with aromatic rings than the smaller, more polar urea molecule, and it additionally screens electrostatic repulsion within the unfolded chain, promoting greater chain expansion.

Table 1: Summarizes representative comparative features

Property	Urea	Guanidinium Hydrochloride
Charge	Neutral	Cationic (Cl^- counterion)
Dominant aromatic interaction	van der Waals / weak H-bonding	Cation–π stacking
Relative denaturing strength	1× (reference)	~2–2.5× per mole
Typical unfolding [C] range	4–8 M	1.5–6 M
Effect on ionic side chains	Minimal direct effect	Screens electrostatics, disrupts salt bridges
m-value contribution per aromatic residue	Moderate	High

5. Cation–π and π–π Interactions Under Denaturing Conditions

Native protein cores frequently contain clusters of aromatic residues stabilized by π–π stacking, and surface aromatic residues may form cation–π interactions with nearby basic side chains. As denaturant concentration increases, guanidinium ions can substitute for these native cation–π partners, effectively competing with intramolecular arginine or lysine contacts for access to the aromatic ring face. This substitution has two consequences: it directly disrupts a native stabilizing interaction, and it provides a favourable alternative interaction in the unfolded state, lowering the relative free energy of the unfolded ensemble. Urea cannot replicate true cation–π stacking but can still intercalate between stacked aromatic pairs, weakening π–π contacts through steric and dielectric effects.

Because cation–π and π–π interactions contribute several kilojoules per mole of stabilization at native aromatic clusters, their disruption by denaturant is a significant, residue specific contributor to the overall free energy of unfolding one that is captured poorly by generic hydrophobicity scales but well captured by explicit solvent simulations and near-UV CD measurements of aromatic asymmetry loss.

6. Transfer Free Energies and the Tanford Framework

The transfer free energy (Δg_{tr}) of an amino acid side chain from water to a concentrated denaturant solution provides a quantitative, additive measure of denaturant affinity. Classic Tanford style transfer experiments, later refined using the solubility and vapor pressure methods of Bolen and coworkers, show that aromatic side chains have among the most favorable (negative) transfer free energies to urea and GdnHCl solutions of any residue class, second only to some large aliphatic side chains. Approximate literature-derived values place the transfer free energy of tryptophan side chain analogs at roughly −0.4 to −0.8 kcal/mol per molar urea and correspondingly larger favorable values in GdnHCl, with tyrosine and phenylalanine showing somewhat smaller but still substantial favorable transfer energies.

Because m-values (the denaturant concentration dependence of unfolding free energy) correlate strongly with the change in solvent accessible surface area upon unfolding, and because aromatic residues contribute disproportionately large surface area changes, they are major determinants of the experimentally measured m-value for a given protein. This provides a direct, quantitative link between the microscopic solute solvent interactions discussed above and the macroscopic denaturation curves measured in standard chemical denaturation assays.

7. Implications for the Mechanism of Unfolding

Taken together, the evidence reviewed here supports a model in which denaturant molecules do not simply weaken water's hold on the protein; they actively compete for the same stacking, van der Waals, and cation–π contacts that stabilize the native aromatic core. This has practical implications for interpreting denaturation experiments: apparent two-state behavior at the level of global structure can mask a more graded, residue-specific sequence of solvation events in which surface-exposed aromatic clusters exchange native contacts for denaturant contacts before the hydrophobic core is fully disrupted. Understanding this sequence is essential for correctly interpreting m-values, for engineering protein stability, and for predicting how mutations at aromatic positions will alter denaturant sensitivity.

8. Computational and Simulation Evidence

Molecular dynamics simulations have been central to resolving the direct versus indirect debate because they provide atomistic, time-resolved detail that is inaccessible to bulk experimental measurements. All atom explicit solvent simulations of model peptides and small proteins in urea and GdnHCl consistently show denaturant molecules occupying the first solvation shell of exposed aromatic rings at densities exceeding bulk concentration, with residence times at the ring face substantially longer than residence times of water molecules at equivalent nonpolar surfaces. These simulations also resolve the specific orientation of bound denaturant: urea tends to lie with its planar amide face parallel to the aromatic ring, maximizing van der Waals contact, while guanidinium adopts a nearly coplanar stacking geometry that maximizes cation–π overlap.

8.1 Free Energy Perturbation and Potential of Mean Force Calculations

Potential of mean force (PMF) calculations, in which the free energy of separating a denaturant molecule from an aromatic ring is computed as a function of distance, show a deep, well defined minimum at contact distances consistent with direct stacking for both urea and guanidinium, with the guanidinium minimum being 1.5 to 2 fold deeper than the urea minimum. These PMF-derived binding free energies correlate well with experimentally measured transfer free energies (Section 6), providing an important cross validation between computational and experimental approaches and lending confidence to the direct binding interpretation.

8.2 Dispersion Interactions and the 2-Stage Unfolding Hypothesis

Simulation studies decomposing the interaction energy between denaturant and protein into electrostatic and dispersion (van der Waals) components have shown that dispersion interactions, rather than hydrogen bonding, account for the majority of the favorable urea protein interaction energy, particularly at aromatic and other nonpolar surfaces. This observation underlies the two-stage unfolding hypothesis, in which an initial, dispersion-driven adsorption of denaturant onto exposed hydrophobic and aromatic patches at the protein surface is followed by a second stage of cooperative backbone solvation and hydrogen-bond disruption that completes global unfolding. Aromatic residues, by virtue of their large polarizable surface area, are predicted by this hypothesis to be among the earliest and most extensively denaturant-coated residues during the first stage.

9. Residue-Specific Differences Among Trp, Tyr, and Phe

Although tryptophan, tyrosine, and phenylalanine are often discussed collectively as the aromatic residue class, their solute solvent interactions with denaturants differ in experimentally and computationally resolvable ways that reflect their distinct ring chemistries.

9.1 Tryptophan

The indole ring of tryptophan combines the largest surface area of the three aromatic residues with a permanent electric dipole and an N–H hydrogen-bond donor. This dual character allows tryptophan to engage denaturants through both dispersion-dominated ring face stacking and, at the indole nitrogen, weak hydrogen bonding to urea's carbonyl oxygen. Because of its large surface area, tryptophan residues typically show the greatest per residue transfer free energy magnitude among the aromatic set, and are correspondingly the residues most responsible for large protein-level m-values in tryptophan-rich proteins.

9.2 Tyrosine

The para-hydroxyl substituent on tyrosine's ring introduces a competing hydrogen bonding site that can be satisfied by water, by an intramolecular partner, or by a denaturant molecule positioned at the ring edge rather than the ring face. This partially redirects denaturant interaction away from pure ring stacking toward a mixed edge/face binding mode, and helps explain why tyrosine residues, despite their substantial hydrophobic ring area, show somewhat smaller and more variable transfer free energies than tryptophan across different denaturant systems.

9.3 Phenylalanine

Lacking any heteroatom substituent, phenylalanine presents the most chemically simple aromatic surface, interacting with denaturants almost exclusively through dispersion and quadrupole mediated electrostatics. Because it cannot participate in hydrogen bonding, phenylalanine's transfer free energy behavior is the most cleanly attributable to nonpolar surface burial among the three residues, and it is frequently used in model compound studies as a reference nonpolar aromatic probe distinct from the more chemically complex tryptophan and tyrosine side chains.

10. Temperature and Concentration Dependence of Aromatic Solvation Changes

The magnitude of denaturant-aromatic interactions is not constant across experimental conditions. Increasing temperature generally weakens direct denaturant binding to aromatic surfaces due to increased entropic cost of ordering denaturant molecules at the ring face, partially offsetting the temperature-dependent weakening of the hydrophobic effect itself. At low denaturant concentrations, aromatic ring binding sites are not saturated, and preferential interaction coefficients increase approximately linearly with bulk denaturant concentration; at high concentrations approaching neat denaturant, binding site saturation and altered bulk solvent structure produce measurable deviations from strict linearity, a factor that must be considered when applying the linear extrapolation method discussed in the companion spectroscopy paper to data spanning wide denaturant concentration ranges.

11. Broader Implications for Related Cosolvent Systems

The direct binding framework developed for urea and GdnHCl extends, with appropriate modification, to other cosolvents that interact with aromatic side chains, including alcohols, sugars, and ionic liquids used in protein refolding and formulation. Aliphatic alcohols such as ethanol and 2,2,2-trifluoroethanol, for instance, interact with aromatic rings through similar dispersion dominated contacts but simultaneously lower the dielectric constant of the bulk solvent, producing a distinct balance of direct and bulk-solvent effects compared to urea and GdnHCl. Understanding aromatic solute solvent interactions in the classical denaturant systems discussed here therefore provides a transferable conceptual foundation for interpreting protein behavior across the broader landscape of cosolvent modulated folding and stability studies.

11.1 Ionic Liquids and Deep Eutectic Solvents

More recently, ionic liquids and deep eutectic solvents have been explored both as denaturing media and, in some formulations, as stabilizing cosolvents for proteins. Cation components of many ionic liquids, particularly imidazolium based cations, present an aromatic or pseudo-aromatic ring capable of π–π stacking with protein aromatic residues in a manner directly analogous to guanidinium, and the denaturing potency of a given ionic liquid correlates well with the strength of this predicted stacking interaction. This convergence of mechanism across chemically distinct cosolvent classes reinforces the conclusion that aromatic ring stacking is a general, transferable determinant of denaturant efficacy rather than an idiosyncratic feature specific to urea and GdnHCl.

12. Conclusion

Aromatic amino acids serve as unusually informative reporters of denaturant mechanism because their large, polarizable ring systems support specific, energetically favorable interactions with both urea and guanidinium ions. The balance of evidence favors a direct interaction mechanism, in which denaturants accumulate preferentially at aromatic surfaces and substitute for native π–π and cation–π contacts, supplemented by a smaller indirect contribution from bulk water restructuring. Continued integration of simulation, preferential interaction thermodynamics, and spectroscopic measurement the subject of the companion paper in this series will further refine our quantitative understanding of how chemical denaturants unfold proteins one solute solvent contact at a time.

Looking forward, emerging techniques such as two-dimensional infrared spectroscopy, neutron scattering with

selective deuteration, and machine-learning-accelerated free energy calculations promise to further sharpen the residue-resolved picture of aromatic solute solvent interaction developed in this review. In particular, the ability to resolve individual aromatic side-chain solvation shells in real time during a denaturation jump, rather than only at thermodynamic equilibrium, will help determine whether the sequential exposure of aromatic clusters inferred from equilibrium spectroscopy (Section 5 of the companion paper) also holds kinetically, a question with direct relevance to understanding the earliest events in both protein unfolding and denaturant-associated misfolding pathways discussed in the third paper of this series.

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