When Are Two Hydrogen Bonds Better than One? Accurate First-Principles Models Explain the Balance of Hydrogen Bond Donors and Acceptors Found in Proteins

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Hydrogen bonds (HBs) play an essential role in the structure and catalytic action of enzymes, but a complete understanding of HBs in proteins challenges the resolution of modern structural (i.e., X-ray diffraction) techniques and mandates computationally demanding electronic structure methods from correlated wavefunction theory for predictive accuracy. Numerous amino acid sidechains contain functional groups (i.e., hydroxyls in Ser/Thr or Tyr and amides in Asn/Gln) that can act as either HB acceptors or donors (HBA/HBD) and even form simultaneous, ambifunctional HB interactions. To understand the relative energetic benefit of each interaction, we characterize the potential energy surfaces of representative model systems with accurate coupled cluster theory calculations. To reveal the relationship of these energetics to the balance of these interactions in proteins, we curate a set of 4,000 HBs, of which > 500 are ambifunctional HBs, in high-resolution protein structures. We show that our model systems accurately predict the favored HB structural properties. Differences are apparent in HBA/HBD preference for aromatic Tyr versus aliphatic Ser/Thr hydroxyls because Tyr forms significantly stronger O–H···O HBs than N–H···O HBs in contrast to comparable strengths of the two for Ser/Thr. Despite this residue-specific distinction, all models of residue pairs indicate an energetic benefit for simultaneous HBA and HBD interactions in an ambifunctional HB. Although the stabilization is less than the additive maximum due both to geometric constraints and many-body electronic effects, a wide range of ambifunctional HB geometries are more favorable than any single HB interaction.
When are two hydrogen bonds better than one?

Accurate first-principles models explain the balance of hydrogen bond donors and acceptors found in proteins

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ABSTRACT: Hydrogen bonds (HBs) play an essential role in the structure and catalytic action of enzymes, but a complete understanding of HBs in proteins challenges the resolution of modern structural (i.e., X-ray diffraction) techniques and mandates computationally demanding electronic structure methods from correlated wavefunction theory for predictive accuracy. Numerous amino acid sidechains contain functional groups (i.e., hydroxyls in Ser/Thr or Tyr and amides in Asn/Gln) that can act as either HB acceptors or donors (HBA/HBD) and even form simultaneous, ambifunctional HB interactions. To understand the relative energetic benefit of each interaction, we characterize the potential energy surfaces of representative model systems with accurate coupled cluster theory calculations. To reveal the relationship of these energetics to the balance of these interactions in proteins, we curate a set of 4,000 HBs, of which > 500 are ambifunctional HBs, in high-resolution protein structures. We show that our model systems accurately predict the favored HB structural properties. Differences are apparent in HBA/HBD preference for aromatic Tyr versus aliphatic Ser/Thr hydroxyls because Tyr forms significantly stronger O–H⋯O HBs than N–H⋯O HBs in contrast to comparable strengths of the two for Ser/Thr. Despite this residue-specific distinction, all models of residue pairs indicate an energetic benefit for simultaneous HBA and HBD interactions in an ambifunctional HB. Although the stabilization is less than the additive maximum due both to geometric constraints and many-body electronic effects, a wide range of ambifunctional HB geometries are more favorable than any single HB interaction.
1. Introduction
Noncovalent interactions are ubiquitous in biological systems, playing essential roles in both enzyme catalysis\(^1\) and the structural properties of both DNA\(^2\) and proteins\(^3-6\). Over the years, an increasing array of interactions including noncovalent carbon bonds\(^7-8\), \(n\) to \(\pi^*\) interactions\(^9-11\), protein–ligand cation–π, aromatic, salt bridges\(^12\), and other interactions\(^13-19\) have been studied to understand their potential roles in biomolecular structure and function. Among these, hydrogen bonds (HBs) are a particularly critical class of noncovalent interactions for biological function. The definition of the HB has become more encompassing over the years\(^20\), expanding to include a range of interactions such as \(N-H\cdots N\)^{21-24}, sulfur-containing\(^25-27\), \(X-H\pi\)^{28-29}, and \(C-H\cdots O\)^{30-34}, among others. Nevertheless, the HB is generally distinguished from other noncovalent interactions by its fairly strong electrostatic component\(^35\) with evidence of some covalent\(^36-38\) bond formation\(^20, 39\), as supported by the interaction being directional in nature\(^40\).

Given the inherently quantum mechanical (QM) nature of the HB, care must be taken in defining and observing it in structural or computational studies. Use of geometric considerations (i.e., van der Waals radii) alone to determine the presence or absence of HBs can lead to erroneous conclusions\(^39, 41-42\). Some classical electrostatic models or empirical correlations\(^43-45\) have been developed along with first-principles investigations\(^46-50\) to understand the strength and nature of hydrogen bonding. Nevertheless, HBs can challenge conventional modeling methods, including approximate density functional theory (DFT) treatments\(^51-53\) that fail to accurately model long-range electron correlation. Although protein structures can be used to validate DFT and more accurate correlated wavefunction theory (WFT) methods\(^15, 54-57\), challenges remain. For example, short hydrogen bonds are ubiquitous in proteins, but they are often penalized during structural refinement\(^58\). Many noncovalent distances in a range of 2.6–2.8 Å that would be
deemed to be unfavorable in crystal structures are instead observed to be favorable and weakly stabilizing when evaluated with correlated wavefunction theory. These interactions cannot be captured with the classical force fields predominantly used to simulate proteins. Similar observations have recently been made in noncovalent interactions in DNA. Although force fields have been noted in recent years to be broadly improving in agreement with experiment, they can fail to describe noncovalent interactions essential for modeling protein structure in globular or intrinsically disordered proteins due to inherent limitations in the physics that can be captured by their functional forms.

Cooperative, strong hydrogen bonds are an exemplary subset of HBs where modeling beyond the force field level is essential. In low-barrier hydrogen bonds (LBHBs), the barrier to hydrogen transfer is similar to the zero-point vibrational energy. These HBs are typically characterized by O···O separations around 2.6 Å or lower, and similarly short interactions are observed in charge-assisted hydrogen bonds and salt bridges. These functionally important interactions would be missed by standard force fields that do not treat charge transfer or polarization and disfavor short non-covalent distances.

The same assumptions that limit the modeling of LBHBs give rise to uncertainty about the interplay and balance of sidechain–sidechain, sidechain–backbone and intraresidue HBs in protein structure and function. For instance, it is poorly understood, given the number of potential hydrogen-bonding partners that a sidechain can form, the extent to which single or multiple hydrogen bonds are observed for a particular sidechain. Part of this challenge arises from the fact that the structure of most amino acids will require some compromise of the preferred linear and directional nature of the HB to form multiple interactions. This is in contrast to the lack of compromise required for base pairing interactions comprising simultaneous short,
linear N–H⋯O and N–H⋯N HBs in RNA\textsuperscript{77-78} and DNA\textsuperscript{79-80}. As an example of the potential functionality offered by multiple, compromised interactions, we recently observed that simultaneous donor and acceptor HB interactions between the aliphatic hydroxyl of a Thr sidechain and a neighboring Asn played an essential role in native substrate recognition of a non-heme iron halogenase\textsuperscript{81}. This interaction, validated by experimental mutagenesis\textsuperscript{82}, was revealed with QM modeling but was challenging to replicate with force fields. Similarly, a large-scale screen of protein interactions suggests that many simultaneous donor/acceptor interactions can form between the aromatic Tyr and Asn or Gln residues\textsuperscript{59}.

In this work, we thus investigate the balance of hydrogen bond acceptor (HBA) and donor (HBD) interactions in representative models of amino acid sidechains (i.e., Tyr/Ser/Thr with Asn/Gln) that can act simultaneously as HBAs and HBDs. Given the challenges with ensuring the accuracy of computational models of HBs, we first carry out careful coupled cluster theory modeling of individual HBA or HBD interactions. The WFT-level analysis carried out here is essential, as force-field modeling fails to reproduce essential differences between aromatic and aliphatic amino acids. We confirm the suitability of our models and level of theory for capturing the structures favored in a curated set of 4,000 HBs obtained from high-resolution (<1.5 Å) X-ray crystal structures of proteins. Using this analysis, we quantify the degree and nature of the benefit observed in the formation of ambifunctional (i.e., simultaneous HBA/HBD) HB interactions.

2. Curation Approach

We curated a data set of candidate hydrogen-bonding residues from the protein data bank (PDB)\textsuperscript{83} following a refinement of the procedure introduced in prior work\textsuperscript{59}. Candidate HBs between Tyr, Ser, or Thr and Asn or Gln were extracted from X-ray crystal structures with
resolution <1.5 Å, which was selected to ensure low positional uncertainty (ca. 0.03 Å) of heavy atoms\textsuperscript{84-85}. In accordance with prior work, all residues were both required to not be within close (i.e., hydrogen-bonding) distance of nonstandard residues or ligands and were taken from a subset of unique structures that had less than 90% sequence identity deposited in the PDB as of October 29, 2017. Residues were selected for the data set if the heavy-atom distances between O and N HB donors or acceptors from the relevant residue sidechains were within 120% of the sum of van der Waals’ (vdW) radii, which was longer by design than the cutoff for close contacts targeted in Ref. 59. To confirm that HB interactions were not an artifact of poorly solved structures, we retained residues following our prior approach\textsuperscript{59} using constraints on the difference of calculated and experimental structure factor amplitudes (i.e., $R$ factor $\leq$ 20%), good agreement on the held out set (i.e., $R_{\text{free}} - R \leq 0.07$), and a good Z-score of the real-space $R$-value (i.e., $\text{RSRZ} \leq 20\%$), which is evaluated against proteins of similar resolution. In addition to prior constraints, we confirmed density support\textsuperscript{86} (i.e., EDIA scores $> 0.8$) for the atoms of both residues in curated HB pairs. We subsequently refined the set based on quantum mechanical criteria (ESI Table S1).

From the resulting set of 6,114 residue pairs, we carried out further refinements to identify a subset for which HBs were most likely to be present. A wide range of HB distance (2.2–4.0 Å) and angle criteria (90–180°) have been proposed\textsuperscript{87-89} in the literature. Because consensus about HB distances and angles is not established, and the optimal HB distance or angle is strongly species dependent, we developed a quantum-mechanically derived approach to selecting distance and angle cutoffs. The presence of bond critical points (BCPs\textsuperscript{90}) from the quantum theory of atoms in molecules (QTAIM)\textsuperscript{91} and the potential energy density evaluated at that point provides a heuristic estimate of HB strength\textsuperscript{92}. After adding and optimizing hydrogen
atoms on representative (ca. 10%) residue pairs from PDB structures, we quantified the presence of BCPs and the potential energy density at the BCP using Multiwfn\textsuperscript{93} (ESI Text S1 and Table S2). Our final range of HBA/HBD distances (N···O: 2.5–3.2 Å, O···O Ser/Thr: 2.4–3.1 Å, and O···O Tyr: 2.4–3.2 Å) and angles (N–H···O: 105–180° and O–H···O: 110–180°) was selected based on structures where BCPs were detected for N–H···O and O–H···O HBs. Our definition of an ambifunctional HB requires at least one of the O···O and N···O HB distances to fall within their specified HB distance criteria, whereas the second must only be within the 120% vdW radii sum criteria. Using these distance criteria, the refined protein data set consists of 3,908 residue pairs. Hydrogen bond angle distributions were evaluated over all of these residues by an automated procedure that added hydrogen atoms, evaluated HB angles, and also classified N–H···O HBs as cis or trans (ESI Text S2).

3. Results and Discussion

3a. N–H···O Hydrogen Bonds.

Before we can evaluate the relative stabilization of the ambifunctional HB configuration, we first determine the strength of single HB (i.e., N–H···O or O–H···O) interactions with model system potential energy curves and compare these observations to geometries observed in X-ray crystal structures of analogous protein residues. Sidechain-based N–H···O HBs are formed when the sidechain amide hydrogen atoms of Asn/Gln act as HB donors to the HB acceptor sidechain hydroxyl oxygen of Ser, Thr, or Tyr (Figure 1). The aliphatic hydroxyl in Ser/Thr is expected\textsuperscript{94} to form stronger N–H···O HBs than the aromatic hydroxyl in Tyr because in the latter the resonance with the tolyl group induces less negative partial charge on the oxygen (Figure 1). We employ truncated models for these residues, i.e., acetamide for Asn/Gln and methanol for Ser/Thr or p-cresol for Tyr, which facilitates the use of high-accuracy methods\textsuperscript{95–96} with larger
basis sets (i.e., DLPNO-CCSD(T)/CBS, see Sec. 5) and simplifies the study of the HB interaction.

![Amino acid residues and HB interactions](image)

**Figure 1.** (Top) Amino acid residues in their zwitterionic form with three-letter codes along with the portion of sidechain highlighted in blue used for model system studies. (Bottom) The four HB conformations studied for both representative model systems, acetamide–methanol and acetamide–p-cresol. The \( \text{trans N–H} \cdots \text{O HB} \), \( \text{cis N–H} \cdots \text{O HB} \), ambifunctional HB, and \( \text{O–H} \cdots \text{O} \) HB interactions are shown from left to right with methanol and \( p \)-cresol distinguished by the R group as indicated at bottom left. Hydrogen-bonding interactions are shown as green dotted lines, and participating electronegative atoms are colored red for oxygen and blue for nitrogen.

In both the protein residues and their truncated models, the planar amide hydrogen atoms can be either \( \text{cis} \) or \( \text{trans} \) with respect to the C=O bond (Figure 1 and ESI Figure S1). The WFT N–H \( \cdots \) O HB interaction energies of \( \text{cis} \) and \( \text{trans} \) N–H \( \cdots \) O HBs agree within 1 kcal/mol for both models, so we select \( \text{cis} \) as the representative case for further comparison (Figure 2 and ESI Table S3). Between models, the interaction energies of N–H \( \cdots \) O HBs in acetamide–\( p \)-cresol are smaller for both conformations (-6.1 kcal/mol) than in the acetamide–methanol model (\( \text{cis: -7.0 kcal/mol} \)), consistent with our expectations (Figure 2 and ESI Table S3). Although the aromatic \( p \)-cresol hydroxyl exhibits weaker interaction energies than the aliphatic hydroxyl in methanol, the difference remains modest (< 1 kcal/mol, Figure 2 and ESI Table S3). The optimized geometries support energetic observations; longer N\( \cdots \) O HB distances (~0.1 Å) and larger HB
angles (~6.3°) are observed for p-cresol than methanol N–H···O HBs (ESI Tables S4–S5).

**Figure 2.** Comparison of interaction energies ($E_{int}$) in kcal/mol of HB conformations in acetamide–methanol (acet-MeOH, left lines) and acetamide–p-cresol (acet-cres, right lines) models. The four conformations compared are the trans N–H···O (trans NHO, in green), cis N–H···O (cis NHO, in blue), O–H···O (OHO, in red), and ambifunctional HBs (ambifunctional, in gray), and optimized model structures (carbon in gray, hydrogen in white, oxygen in red, and nitrogen in blue) are shown with hydrogen bonds drawn as black dotted lines.

To quantify how well our energetic models reproduce the N···O HB distances in high-resolution crystal structures, we calculated one-dimensional (1D) potential energy curves (PECs) (see Sec. 5) as a function of the N···O HB distance and compared distances in the protein data set to the representative model systems (Figure 1 and see Sec. 2). Features of the 1D PECs for the representative cis N–H···O HB conformation in both models are broadly consistent with the freely optimized model structures, both in terms of a deeper energy minimum for the methanol model (by ca. 0.9 kcal/mol) and a shorter (methanol: 2.94 Å vs p-cresol: 3.06 Å) N···O HB distance (Figure 3 and ESI Figures S2–S3).
Figure 3. Normalized histograms (blue, left axes) of heavy-atom HB distances (in Å, bin width of 0.1 Å) for Ser-Asn (top) and Tyr-Asn (bottom) X-ray crystal structures with the 1D PECs (red, right axes) for acetamide–methanol (top) and acetamide–p-cresol (bottom) overlaid. The left panes show the N···O HB distance histograms and PECs of cis N–H···O HBs, and the right panes show the O···O HB distance histograms and PECs of O–H···O HBs. The structure insets depict representative protein structure sidechains for the relevant HB, with the atom that corresponds to the Cα of the residues represented as a green sphere and the remaining atoms shown as sticks with carbon in gray, hydrogen in white, nitrogen in blue, and oxygen in red.

Despite differences in the hydroxyl placement on Ser or Thr, the most frequently observed N···O HB distances in Ser/Thr-Asn/Gln pairs agree well with the 1D PEC minimum for the acetamide–methanol model (Figure 3 and ESI Figures S2–S3). This good agreement further supports the choice of truncated models (i.e., methanol for both Ser and Thr) to represent the protein residues. Although a range of distances are observed over the full protein set, only a small fraction of structures have distances different from the value at the minimum of the
acetamide–methanol N···O HB 1D PEC (Figure 3). Consistent trends hold for HBs with Tyr, where the shallower 1D PEC model coincides with both the slightly wider range and longer distances of X-ray crystal structures (Figure 3). Overall, our truncated models capture key interactions from protein crystal structures whether in cis or trans N–H···O HB conformations, but sub-kcal/mol energetic differences (e.g., relative preference for cis or trans) in the models can be expected to be affected by competing backbone and environmental stabilization in the crystal structures (Figures 1–2, and ESI Text S3, Tables S3, S6–S7, and Figures S4–S5).

The directionality of HBs provides a key indicator of their strength and character. We thus evaluated N–H···O HB angle distributions over the X-ray crystal structures (see Sec. 2). Ser/Thr and Tyr N–H···O angle distributions exhibit similar trends, with the highest angle probability between 150 and 170° and a rapid decay outside of that range (Figure 4 and ESI Figures S6–S7). In our model systems, we had observed more obtuse N–H···O angles, with the acetamide–p-cresol angle (178°) larger than acetamide–methanol (171°), both of which exceed many of the angles observed in our X-ray structure data set (Figure 4). Because we approximate the placement of hydrogen atoms to compute angles in the X-ray structures (see Sec. 2), this additional uncertainty limits determination of the relative directionality of Tyr versus Ser/Thr N–H···O HBs with Asn/Gln amino acid pairs (Figure 4). For the N–H···O HB angles for X-ray structures of all amino acid pairs, average values (154°) are reduced by ca. 20° with respect to the model systems, suggesting a possible effect of the protein environment on favored HB angles (see Sec. 2 and ESI Table S6). In the extended X-ray crystal structures of amino acid pairs with reduced HB angles, interactions with solvent molecules, backbone (i.e., N–H hydrogen or carbonyl oxygen) atoms of nearby residues or other interactions in the greater environment absent from the models are present to varying degrees (ESI Figure S8).
Figure 4. Normalized histograms of N–H···O HB (left) and O–H···O HB (right) angles (in °) for Ser-Asn (top) and Tyr-Asn (bottom) residue pairs from X-ray crystal structures. All histograms have 10° bin widths. The structure insets depict the HB angle on representative protein structure sidechains with the corresponding Cα of the residues represented as a green sphere and the remaining atoms shown as sticks with carbon in gray, hydrogen in white, nitrogen in blue, and oxygen in red.

To examine the interplay of optimal HB distances and angles, we computed two-dimensional potential energy surfaces (PESs) of the N–H···O HB interaction. Qualitatively similar PES shapes are obtained for the acetamide–methanol and acetamide–p-cresol model systems (Figure 5). The main difference between the two arises from the less favorable global minimum in p-cresol that corresponds to a shallower overall PES, leading to more comparable interaction energies for the two models when the angles or distances are displaced from the
global minimum (Figure 5 and ESI Figures S9–S10). Although in both cases, the strongest interaction energies are at the expected HB distances (i.e., between 2.8 and 3.2 Å) and HB angles (i.e., between 160° and 180°), structures over a wide 140–180° angle range are within 1–2 kcal/mol of the global minimum (Figure 5). In both cases, structures at a fixed distance always favor larger HB angles, but displacement from the equilibrium angle incurs considerably less penalty than distance displacement (Figure 5).

Figure 5. The 2D PESs depicting interaction energies ($E_{\text{int}}$ in kcal/mol, colorbar at right) of N–H···O HBs (left) and O–H···O HBs (right) in acetamide–methanol (top) and acetamide–p-cresol (bottom). The heavy-atom (i.e., N···O and O···O) distances (in Å) and X–H···O angles (in °, X = N for the left panes and X = O for the right panes) are shown as labeled on the axes, and the same color scale is used for all PESs with 1 kcal/mol contour lines. The X-ray crystal structure distances and angles (translucent green circles) from the data set are overlaid onto the PESs for the corresponding Ser-Asn (labeled S-N) and Tyr-Asn (labeled Y-N) residue pairs.

Comparing protein structures to our model systems, the majority (i.e., over 85%) of Ser/Thr-Asn/Gln structures reside within 2 kcal/mol of the global minimum on the computed model 2D PES (Figure 5 and ESI Table S8). Results for Tyr-Asn/Gln are consistent, but the shallower nature of the 2D PES for p-cresol leads to a smaller fraction (70%) residing within 2 kcal/mol of the model global minimum (Figure 5 and ESI Table S8). Over all amino acid pairs, a minority (10%) of X-ray structures sample smaller than expected HB angles (i.e., between 110°
and 130°) that correspond to less favorable model interaction energies due to a short N···O distance but relatively long HBD to HBA (i.e., H···O) distance (ESI Table S8). Examining the full protein in representative cases reveals competing HB interactions in the surrounding protein environment with alternative HB partners (e.g., solvent or other amino acids) that likely compensate for the formation of these weaker N–H···O HBs (ESI Figure S11).

3b. O–H···O Hydrogen Bonds.

When the sidechain hydroxyls of Ser, Thr, or Tyr act as HBDs to the sidechain amide oxygen HBA of Asn or Gln, O–H···O HBs are formed instead of N–H···O HBs (Figure 1). In this case, an aromatic hydroxyl (e.g., Tyr or p-cresol) is expected to form stronger O–H···O HBs than the aliphatic hydroxyl (e.g., Ser/Thr or methanol) due to the resonance delocalization of the nonbonded electron pair of the hydroxyl oxygen into the aromatic ring that enhances O–H bond polarity. As expected, the interaction energy of O–H···O HB in acetamide–p-cresol is stronger (ca. 3 kcal/mol) than in acetamide–methanol (-11.0 kcal/mol vs -7.9 kcal/mol, Figure 2). Consistent with energetic trends, modest geometric differences (i.e., 0.05 Å shorter O···O HB distances for p-cresol than for methanol) are observed between the two models of O–H···O HBs (ESI Tables S4–S5). These geometric differences are similar to those we observed for N–H···O HBs despite the higher energetic differences for the two model systems’ O–H···O HBs (ESI Tables S4–S5).

While the 1D PEC O···O HB energetics are largely consistent with those of the freely optimized structures, some minor differences are apparent due to differences in the level of theory used (see Sec. 5, Figure 3 and ESI Figures S2–S3). The 1D PEC acetamide–p-cresol O···O heavy-atom HB distance is further reduced (2.73 Å vs 2.81 Å) with respect to methanol (Figure 3 and ESI Tables S4–S5). Overlaying X-ray crystal structure O···O distances on these
1D PECs confirms the suitability of the model systems for O–H···O HBs, with a significant fraction of Tyr-Asn/Gln O···O distances that are shorter than those for Ser/Thr-Asn/Gln (Figure 3 and ESI Figures S2–S3). The distribution of X-ray crystal structure O···O HB distances is especially narrow for Tyr HBs, consistent with the steeper 1D PEC for Tyr in comparison to Ser/Thr (Figure 3 and ESI Figures S2–S3). The most frequently observed O···O distances in X-ray crystal structures are slightly shorter (by ca. 0.1 Å) than the 1D PEC minima for both methanol and p-cresol (Figure 3 and ESI Figures S2–S3). This effect is relatively modest, as it corresponds to interaction energies approximately 0.3 kcal/mol above the model 1D PEC minimum. Both the omission of the protein environment and our neglect of quantum nuclear effects particularly relevant at short HB distances could explain this discrepancy.

Analyzing the O–H···O HB angle distribution over the X-ray crystal structure data set highlights a greater preference for near-linear (i.e., 170–180°) angles in comparison to N–H···O HB angles, especially for the Tyr residue pairs (Figure 4 and ESI Figures S6–S7). The greater strength of Tyr O–H···O HBs is consistent with a slightly higher fraction of the most linear angles in comparison to Ser or Thr (Figure 4 and ESI Figures S6–S7). Average O–H···O HB angles (ca. 165°) are comparable for all residue pairs and higher than those for N–H···O HB angles by around 11° (ESI Table S9). This increase in favored angles in the X-ray crystal structures suggests greater consistency with the O–H···O HB angles of fully optimized models in comparison to the N–H···O HB case (ESI Tables S4–S5).

Evaluation of the O–H···O HB 2D PESs highlights good agreement between the optimal model structures and observed X-ray crystal structures for all amino acid pairs (Figure 5 and ESI Figures S9–S10). In comparison to N–H···O HBs, the joint distribution of X-ray crystal structure distances and angles is both more compact and more aligned with the lowest-energy regions of
the model systems for both aromatic (i.e., Tyr or p-cresol) and aliphatic (i.e., Ser/Thr or methanol) hydroxyl HBDs (Figure 5 and ESI Figures S9–S10). Almost all (ca. 95%) of the Ser/Thr X-ray crystal structures sample distances and angles within 2 kcal/mol of the O–H⋯O model global minimum (Figure 5 and ESI Table S8). The percentage of Tyr-Asn/Gln O–H⋯O HB X-ray structures (ca. 85%) within 2 kcal/mol of the model 2D PES global minimum is also increased (Figure 5 and ESI Table S8). Thus, the steeper PES and greater directionality of O–H⋯O HBs is likely due to local interactions that are well described in gas-phase models.

Of the four HBs considered thus far, the acetamide–p-cresol O–H⋯O HB is significantly (~4.9 kcal/mol) stronger than the acetamide–p-cresol N–H⋯O HB as well as the HBs in the acetamide–methanol model (Figure 5 and ESI Table S3). Displacements of the acetamide–p-cresol O–H⋯O HB distance by ca. 0.7 Å or angle by ca. 60° lead to interaction energies still as strong as the alternative N–H⋯O HB (Figure 5 and ESI Figures S9–S10). The differentiation of HB strength for aromatic hydroxyls diverges from the aliphatic hydroxyl (i.e., the methanol model) where the O–H⋯O HB is only slightly stronger (0.9–1.4 kcal/mol) than the N–H⋯O HB (ESI Table S3).

Given the greater favorability of O–H⋯O over N–H⋯O HBs, we expected to observe a significantly larger number of O–H⋯O HBs especially for interactions with Tyr. Contrary to both our expectations and prior observations over a larger data set59, a greater number of N–H⋯O HBs is observed in our data set than O–H⋯O HBs for either Ser/Thr or Tyr with Asn/Gln (ESI Table S10). One potential source of this counterintuitive difference in HB abundance is the compensation of weaker N–H⋯O sidechain–sidechain HBs by additional sidechain–backbone or sidechain–solvent interactions. Indeed, inspection of the protein around representative N–H⋯O HBs reveals simultaneous formation of sidechain–backbone HBs and additional sidechain (i.e.,
Ser/Thr/Tyr hydroxyl or Asn/Gln carbonyl) to solvent HB interactions that could not form in O–H⋯O HB conformations (ESI Figures S8 and S12). Additionally, Asn/Gln can form two N–H⋯O HBs per sidechain, which we also observe in our data set (ESI Figure S13). Thus, raw counts in a limited data set likely capture the relative favorability of globally compensated HB interactions, whereas the relative strengths of the individual HBs appear better captured by comparison of the distributions of X-ray structures and model systems.

3c. Energetic Stabilization from Ambifunctional Hydrogen Bonds.

If oriented appropriately, the sidechain hydroxyl (i.e., of Ser, Thr, or Tyr) can simultaneously act as an HBD to the amide oxygen and an HBA to amide nitrogen of Asn or Gln, forming a conformation we refer to as an ambifunctional HB. Because this arrangement involves the combined formation of an O–H⋯O and cis N–H⋯O HB, its interaction strength could be as large as the sum of the two individual HBs (see Secs. 3a–3b). Unlike other noncovalent interactions in biological systems (e.g., RNA/DNA) where multiple HBA/HBDs can form in near-linear configurations, we can expect this sidechain–sidechain interaction to involve some compromise. Such a compromise in the ambifunctional HB can arise from differences of the distances/angles of the two HBs with respect to optimal values for individual HBs as well as from the electronic properties that dictate the participating atoms’ abilities to act as HBAs/HBDs (Figure 1). If these effects are modest, the ambifunctional HB interaction energy should by higher than either individual HB, and this conformation should be observed in protein structures.

At its upper limit, the ambifunctional HB would correspond to the sum of the two single HBs (methanol: -14.9 kcal/mol and p-cresol: -17.1 kcal/mol), with a more favorable acetamide–p-cresol ambifunctional HB expected due to its strong constituent O–H⋯O HB (ESI Table S11).
The acetamide-\(p\)-cresol ambifunctional HB (-12.2 kcal/mol) is indeed stronger by ca. 2 kcal/mol than that in acetamide–methanol (-10.6 kcal/mol), but both are weaker than the theoretical limit by a comparable 4–5 kcal/mol (Figure 2 and ESI Table S11). Despite the overall weaker interaction energy for methanol, its ambifunctional HB is significantly (ca. 3-4 kcal/mol) more favorable than its individual O–H···O or N–H···O HBs (Figure 2 and ESI Table S3). Conversely, the acetamide-\(p\)-cresol ambifunctional HB provides a limited (ca. 1 kcal/mol) benefit over the O–H···O HB conformation, suggesting the dominant role of the O–H···O HB even in the ambifunctional conformation (Figure 2 and ESI Table S3). Beyond this purely electronic interaction energy picture, the ambifunctional HB could be expected to incur a relative entropic penalty in comparison to the individual HBs. While relative free energies do disfavor the ambifunctional HB, this difference is modest (< 0.5 kcal/mol) with respect to the single O–H···O HB (ESI Table S12).

To understand if the reduced interaction energies in the ambifunctional HB arise due to a geometric compromise, we compared the geometry of the constituent HBs with the corresponding single O–H···O and \(\text{cis}\) N–H···O HBs. In both model system ambifunctional conformations, the N···O and O···O HB distances are mostly unchanged (shortened by ca. 0.05 Å and 0.1 Å, ESI Tables S4–S5 and S11). Comparing interaction energies at these shortened HB distances from the individual 1D PECs, we determine that no significant energy penalty is incurred (ESI Table S13). The ambifunctional HB angles are more distinct, being significantly reduced (O–H···O: ca. 10° and N–H···O: ca. 30-40°) with respect to their near-linear values in single HBs (ESI Tables S4–S5 and S11). In contrast to the negligible distance penalties, the penalty for displacing both HB angles from their optimal values comes at an energetic cost of 1.5 kcal/mol in both model systems (ESI Tables S11 and S13). The remaining difference between
the ambifunctional HB interaction energy and the additive sum of the single HBs (ca. 3 kcal/mol) is thus likely due to many-body, electronic effects such as those that limit the simultaneous HBA/HBD strength of the hydroxyl oxygen. Indeed, if we had used a typical force field for biomolecular simulations (i.e., the generalized amber force field, or GAFF\textsuperscript{98}) we would have failed to distinguish differences between the aromatic and aliphatic hydroxyls, resulting in underestimation of the higher O–H⋯O HB strength in p-cresol and failure to predict the relatively high benefit of the ambifunctional HB in the methanol model (ESI Table S14).

To quantify energetic relationships among the \textit{cis} N–H⋯O HB, O–H⋯O HB, and ambifunctional HB conformations, we identified a reaction coordinate for the minimal structural rearrangement that describes the transition between these conformations (see Sec. 5 and ESI Text S4). A suitable reaction coordinate to capture this transition is the rotation of the alcohol (i.e., methanol or p-cresol) with respect to the amide, a quantity well described by the (H)O⋯C=O intermolecular angle (ESI Figure S14). Although the intermolecular angle was constrained during optimization, the remaining degrees of freedom (e.g., HB distance and angle) were fully relaxed. This reaction coordinate can be transformed to the N–H⋯O angle between acetamide and the alcohol, a quantity more intuitively linked to the hydrogen bond (ESI Text S4 and Figure S15).

Along the reaction coordinate, increasing N–H⋯O angles correspond to the transformation from a single O–H⋯O HB to the formation of an additional N–H⋯O interaction in the ambifunctional HB until the O–H⋯O HB interaction is lost and only the N–H⋯O HB remains (Figure 6). The hydroxyl group of the alcohol in either model rotates freely along the reaction coordinate, making it difficult to map the reaction coordinate to the O–H⋯O angle. Still, the O–H⋯O angle generally behaves as expected with changing N–H⋯O angle: a near-
linear O–H···O angle must coincide with an acute N–H···O angle or vice versa (ESI Figure S16).

**Figure 6.** Interaction energies ($E_{\text{int}}$, in kcal/mol) of HB conformations shown (red dots) as a function of N–H···O HB angle (in °) and a corresponding 10-point running average (gray line) for (top) acetamide–methanol and (bottom) acetamide–p-cresol. The energies in the ambifunctional HB basin (i.e., below the energy of the O–H···O HB minimum) lie below the blue dashed line. Representative structures with measured O–H···O HB angles are shown for the O–H···O HB (top left inset), N–H···O HB (top right inset), and ambifunctional HB (bottom inset) with the relevant O–H···O HB angle annotated in black. The N–H···O HB interaction is shown as a gray line in the conformations where it is also present, and its value can be read from the x-axis. Select discontinuous (red symbol) points were pruned from the plots for clarity.

For both model systems, the N–H···O HB conformation appears only as a shoulder along the reaction coordinate and thus the transition to the lowest-energy ambifunctional HB conformation is barrierless (Figure 6). Conversely, while the O–H···O HB is a local minimum
along the reaction coordinate, the transition from the O–H⋯O HB conformation to the ambifunctional HB conformation has a small (methanol: 1.7 kcal/mol, p-cresol: 2.3 kcal/mol) barrier in both model systems (Figure 6 and ESI Table S15). For the acetamide–methanol model, it is apparent that this barrier can be partly attributed to the reduction of the O–H⋯O HB angle from its ideal value (180° to 160°) before the N–H⋯O angle approaches the larger values (ca. 120–140°) near the ambifunctional HB global minimum (Figure 6 and ESI Figure S16). However, no such geometric distortion is observed for the acetamide–p-cresol case (ESI Figure S16). The thermodynamic driving force for forming the ambifunctional HB (e.g., with respect to a single O–H⋯O HB) on the reaction coordinate is lower for p-cresol than methanol, despite both p-cresol conformations having stronger overall interaction energies (Figure 6). Exiting the ambifunctional HB global minimum requires 4–5 kcal/mol for both model systems to break the ambifunctional N–H⋯O interaction and reform a single O–H⋯O HB, with the greater stability of the ambifunctional HB in acetamide–methanol leading to a slightly higher energetic cost than for acetamide–p-cresol (Figure 6).

Although geometric arguments can partly explain the significant barrier on the acetamide–methanol reaction coordinate for rearranging from the single O–H⋯O HB to the ambifunctional HB, it does not explain the equally large barrier for the p-cresol model. To understand the electronic origins of the barrier for rearrangement, we used symmetry-adapted perturbation theory (i.e., at the SAPT2+3 level of theory99) to decompose relative electrostatic and dispersion contributions in both minima on the reaction coordinate as well as at the peak of the DLPNO-CCSD(T) barrier (ESI Table S16). This energy decomposition reveals that a loss of dispersion and induction stabilization occurs equivalently (by ca. 2 kcal/mol) for both model systems at the maximum energy point between the O–H⋯O and ambifunctional HB.
configurations (ESI Table S16). At this energetic peak on both reaction coordinates, dispersion stabilization is also weaker than at the N–H···O HB geometry (ESI Table S16). Although favorable electrostatic interactions also weaken at the barrier, this effect is counteracted by a reduction in the exchange repulsion (ESI Table S16). Thus, a delicate interplay of electronic effects can be expected to govern rearrangement between single and ambifunctional HB configurations. It is therefore unsurprising that using a standard biomolecular force field (e.g., GAFF⁹⁸) fails to capture this barrier for rearrangement in addition to underestimating the stabilization of the ambifunctional HB (ESI Figure S17 and Table S14).

In both model systems, we identify an ambifunctional HB basin as the range of reaction coordinate geometries around the global minimum that remain stabilized with respect to the most stable single HB (i.e., O–H···O) conformation (Figure 6). Due to the greater relative stability of the methanol ambifunctional HB with respect to the constituent HBs, its basin corresponds to a larger, nearly 3 kcal/mol energy window instead of approximately 1.5 kcal/mol for p-cresol (Figure 6). These differences are reflected in the geometric properties of structures within the basin: methanol N–H···O angles span a nearly 40° range (123 to 160°), whereas favorable p-cresol N–H···O angles span only 20° (126 to 146°, Figure 6 and ESI Figures S18–S19). An even wider range of O–H···O angles is observed for methanol (135-167°), whereas the favored O–H···O angles for p-cresol (151-168°) are more restrictive (Figure 6 and ESI Figures S18-S19). This observation confirms that for the aromatic hydroxyls in p-cresol or Tyr residues, the O–H···O interactions dominate and an ambifunctional HB provides limited additional stabilization.

Nevertheless, structural variations in the ambifunctional HB basin are comparable for both models, with the higher, productive O–H···O angles compensated by a monotonic linear reduction to less productive N–H···O angles or vice versa (ESI Figures S18–S19). Approaching
the ambifunctional HB global minimum from either side of the basin corresponds to a shortening of the heavy-atom distance for the forming HB (i.e., N···O or O···O) while the other HB distance changes minimally (ESI Figures S18–S19). As a result, the sum of the two HB distances is lowest in the ambifunctional HB global minimum and rises in either direction (ESI Figure S20). The basin O···O distances span a significantly narrower 0.18-Å range for p-cresol (2.72–2.90 Å) than the 0.55-Å range for methanol (2.75–3.30 Å, ESI Figures S18–S19).

Given the increased favorability of ambifunctional HBs over both O–H···O and N–H···O HBs for model systems, we expect to observe them in protein crystal structures. Indeed, we observe ambifunctional HBs between all pairs of amino acids, corresponding to around 15% (559 of 3,908) of all HBs in our data set (ESI Table S10). Although one may expect a more significant fraction of Ser/Thr HBs to be ambifunctional than Tyr HBs due to the enhanced relative benefit for the aliphatic hydroxyl, the difference in relative abundance is modest (ca. 15–18% vs 11–12%, ESI Table S10). This relative abundance in crystal structures is likely dictated by a combination of both geometric constraints for ambifunctional HB formation as well as competition with other interactions as previously observed for single HBs (see Sec. 3b).

To characterize the geometries of X-ray crystal structure ambifunctional HBs, we computed their joint N···O and O···O HB distance distributions (Figure 7 and ESI Figure S21). In this set, one of the HB distances is typically closer to its optimal value than the other, with a small (ca. 10%) fraction consisting of two HB distances close to their optimal values in single N–H···O or O–H···O HBs (Figure 7 and ESI Figures S21–S22 and Table S17). While competing interactions with solvent molecules or other residues can partially rationalize why symmetric ambifunctional HB interactions are infrequently observed, such competing interactions are also observed simultaneously with highly symmetric ambifunctional HBs (Figure 8). For the
asymmetric Ser/Thr pairs with Asn or Gln, a slight majority (57%) has shorter N···O than O···O HB distances, whereas for Tyr this subset represents a minority (43%), consistent with differences in relative O–H···O HB strength (Figure 7 and ESI Figure S21). Although the HB distances in many cases are asymmetric, the positioning of the residue pairs still orients both sets of HBAs and HBDs in sufficient proximity for two simultaneous interactions (Figure 8).

**Figure 7.** Normalized 2D histograms of O···O HB distance ($d(O···O)$ in Å) vs. N···O HB distance ($d(N···O)$ in Å) for residue pairs in the data set with normalized frequency colored according to the colorbar shown at right. The green rectangular box indicates the O···O and N···O HB distance ranges over which the strongest ambifunctional HBs are observed. The residue pairs shown are Ser-Asn/Gln (S-N/Q, 208 pairs shown, left), Thr-Asn/Gln (T-N/Q, 238 pairs shown, middle), and Tyr-Asn/Gln (Y-N/Q, 113 pairs shown, right).

|      | $d(O···O) > d(N···O)$ | $d(O···O) = d(N···O)$ | $d(O···O) < d(N···O)$ |
|------|----------------------|----------------------|----------------------|
| Ser-Asn | ![Ser-Asn](image1) | ![Ser-Asn](image2) | ![Ser-Asn](image3) |
| Tyr-Gln | ![Tyr-Gln](image4) | ![Tyr-Gln](image5) | ![Tyr-Gln](image6) |

**Figure 8.** Representative proteins showing the three different types of ambifunctional HBs in Ser-Asn (top) and Tyr-Gln (bottom) residue pairs in our data set: shorter $d(N···O)$ (left),
equivalent length \( d(N\cdots O) \) and \( d(O\cdots O) \) (middle), and shorter \( d(O\cdots O) \) (right). The Ser-Asn pairs correspond to PDB IDs (left to right): 2NWD, 2VOV, and 1MKK, and the Tyr-Gln pairs to PDB IDs (left to right): 4I71, 3BVU, and 4N1I. Specific HBs are indicated by black dashed lines between the heavy atoms in residue pairs with annotated distances (black, in Å) and N/O–H···O angles (light green, in °). Hydrogen atoms reported in the crystal structure (top left) are shown as solid spheres, whereas hydrogen atoms added for the remaining proteins and relaxed with constrained heavy atoms are shown as translucent spheres. The green dashed lines indicate additional stabilizing interactions observed with respect to the residue pair. All residues are labeled by one-letter amino acid codes and residue numbers.

In the model systems, we attributed lower than expected interaction energies to the difficulties associated with simultaneous formation of two productive HB angles. In the X-ray structure set, more ambifunctional HBs have near-linear (i.e., 170–180°) O–H···O angles and small (i.e., 110–130°) N–H···O angles than the reverse, but average angles differ only around 5° from the average values of the single HB angles in X-ray structures (Figure 8 and ESI Figure S23 and Tables S4–S5 and S18). Consistent with the HB distance analysis, a minority of all Tyr (9%) and Ser/Thr (20%) ambifunctional HBs simultaneously form relatively obtuse O–H···O and N–H···O angles as high as those observed in the model systems (Figure 8 and ESI Figure S23). This small fraction overlaps significantly with the minority of strong, symmetric ambifunctional HBs that nearly all have two obtuse angles (Figure 8 and ESI Figure S23). Consistent with observations on protein structures, model system geometries also exhibit a greater reduction of the N–H···O angle than the O–H···O angle, especially for \( p \)-cresol (20°) versus methanol (10°, ESI Tables S4–S5).

While ambifunctional HBs are apparent in protein structures, the benefit of a near-linear O–H···O angle especially in interactions with Tyr can be expected to dominate. Thus we can expect the Tyr hydroxyl to act as a simultaneous HBA and HBD only when limited deviation of HB angles is necessary. Conversely, we should anticipate this motif to be apparent in proteins with Ser or Thr in close proximity to Gln or Asn. We expect more such structures could be
uncovered with even larger-scale and more inclusive examination of proteins from crystal structures, molecular dynamics, or with other (e.g., NMR) spectroscopic techniques.

4. Conclusions

We combined accurate correlated wavefunction theory energetics of model systems and analysis of high-resolution X-ray crystal structures of proteins to understand the balance of individual or simultaneous hydrogen bond donor and acceptor interactions between sidechains in proteins. Using representative models of aliphatic hydroxyl (i.e., Ser/Thr) or aromatic hydroxyl (i.e., Tyr) groups with the amide sidechains of Asn/Gln, we obtained accurate potential energy curves that defined these hydrogen-bonding interactions. Analysis of the model systems confirmed expectations that aromatic hydroxyl groups form the strongest O−H···O HBs but considerably weaker N−H···O HBs, whereas these interactions were balanced for aliphatic hydroxyl groups. The model systems were deemed to be suitable representations of residue-residue interactions in proteins thanks to the good agreement of gas-phase and crystal structure geometries. Almost all HB distances obtained from protein crystal structures resided within 1–2 kcal/mol of the favored gas-phase minimum energy structure. Nevertheless, we observed limited correspondence between energetic favorability (i.e., O−H···O > N−H···O) and relative abundance in the data set, which we attributed partly to compensating intermolecular HBs that are more plentiful in N−H···O HB configurations.

We showed that simultaneous O−H···O and N−H···O interactions are stabilizing in an ambifunctional HB. While this energetic benefit was most significant for aliphatic hydroxyl groups, it was less than the theoretical limit (i.e., sum of two individual HBs) and only slightly more favorable than the O−H···O HB alone for aromatic hydroxyls. We determined this reduction in interaction strength was due both to geometric constraints on the formation of two
productive HB angles and distances along with the reduced ability of a single hydroxyl to act as a simultaneous HBA/HBD. These many-body effects could not be captured by conventional force fields widely used to study proteins. While evaluating the reaction coordinate that captured transformation between HB conformations revealed rearrangement from the N–H⋯O to ambifunctional HB to be barrierless, the basin of stable ambifunctional HB structures accommodated a wide range of distances and angles especially for Ser/Thr-Asn/Gln. Consistent with model system observations, we observed a range of ambifunctional HB structures in X-ray crystal structures, especially for Ser/Thr over Tyr. These studies set the stage for systematic and quantitative study of representative models to illuminate mechanistic roles for ambifunctional HBs that may have been missed when studied with conventional force fields. It is expected that the unique energetic and geometric properties of these hydrogen bonds could play an important role in substrate recognition and in controlling enzyme selectivity through substrate positioning.

5. Computational Details

Representative models of protein hydrogen-bonding interactions were studied using methanol and p-cresol as hydrogen bond donor or acceptor (HBD or HBA) models of Ser/Thr and Tyr, respectively, and acetamide as an HBD or HBA model of Asn/Gln (Figure 1). These choices were made to minimize the effect of sidechain truncation on interaction energies in comparison to the full residues while minimizing computational cost (ESI Table S19). Initial structures were built by hand and optimized with the MMFF94 force field using Avogadro v1.2.0. Geometries were prepared in four configurations containing up to two candidate hydrogen bonds for both unconstrained and constrained geometry optimizations on acetamide–methanol and acetamide–p-cresol model systems (Figure 1).
Unconstrained and constrained geometry optimizations were performed using both hybrid (i.e., B3LYP\textsuperscript{105-107}) density functional theory (DFT) and Møller–Plesset second-order perturbation theory (MP2). All optimizations were carried out using the 6-31G* basis set\textsuperscript{108}, followed by single-point energies evaluated using larger basis sets (ESI Tables S4–S5). Semi-empirical D3\textsuperscript{109} dispersion with Becke–Johnson\textsuperscript{110} damping was incorporated in the B3LYP optimizations, although its effect on geometries was limited (ESI Tables S4–S5). B3LYP-D3 DFT geometry optimizations were carried out in a developer version of TeraChem\textsuperscript{111} v1.9 in Cartesian coordinates using L-BFGS algorithm, as implemented in DL-FIND\textsuperscript{112}. Default thresholds of 4.5x10\textsuperscript{-4} hartree/bohr for the maximum gradient and 1x10\textsuperscript{-6} hartree for self-consistent field (SCF) convergence were employed. Geometry optimizations with MP2 in ORCA\textsuperscript{113} v.4.0.1.2 were carried out in redundant internal coordinates using the BFGS method with default thresholds of 3x10\textsuperscript{-4} hartree/bohr for the maximum gradient and 5x10\textsuperscript{-6} hartree for SCF convergence. The MP2 and B3LYP-D3 HB distances and angles as well as intramolecular bonds were comparable, with only equilibrium O⋯O distances exhibiting a slight dependence on basis set or method choice (ESI Tables S4–S5 and S20). Comparisons of single-point energies at higher levels of theory on MP2 and B3LYP-D3 geometries revealed very limited differences (\leq 0.1 kcal/mol) on evaluated interaction energies (ESI Table S21). All initial and optimized structures are provided in the ESI.

For the acetamide–methanol model system, single-point energy calculations were carried out on MP2/6-31G* geometries with both canonical coupled cluster singles doubles with perturbative triples (i.e., CCSD(T)) and domain-localized pair natural orbital CCSD(T) (i.e., DLPNO-CCSD(T)\textsuperscript{114-115}). Dunning-style correlation consistent double-ζ and triple-ζ (i.e., aug-cc-pVDZ and aug-cc-pVTZ) basis sets were employed to enable two-point\textsuperscript{116-118} extrapolation to
the complete basis set (CBS) limit. Given the larger size (i.e., 25 atoms) of the acetamide–p-
cresol system, only DLPNO-CCSD(T) single point energies were evaluated on MP2/6-31G*
structures with aug-cc-pVXZ (X=D, T) basis sets to enable extrapolation to the CBS limit (ESI
Table S3). All reported DLPNO-CCSD(T) energies correspond to those obtained from Tight
PNO thresholds, after testing the effect of threshold choice on interaction energies (ESI Table
S3). For the acetamide–methanol model system where canonical CCSD(T) could be carried out,
interaction energies obtained from DLPNO-CCSD(T)/CBS with Tight PNO thresholds and
CCSD(T)/CBS agreed to within 0.3 kcal/mol, with relative interaction trends in even closer
agreement (ESI Table S3).

One-dimensional (1D) potential energy curves (PECs) and two-dimensional (2D)
potential energy surfaces (PESs) were obtained by generating initial geometries for constrained
geometry optimizations. Constrained optimizations were all carried out in ORCA v4.0.1.2 at the
MP2/6-31G* level of theory, followed by single-point energies evaluated with DLPNO-
CCSD(T)/CBS or with the generalized amber force field (GAFF98). We select this protocol to
ensure we are predictive across full HB potential energy curves, but B3LYP-D3/aug-cc-pVTZ
interaction energies underestimate the DLPNO-CCSD(T)/aug-cc-pVTZ values by only ca. 1
kcal/mol, indicating limited method sensitivity of some properties (ESI Table S22). The 1D
PECs were obtained for cis N–H⋯O HBs and O–H⋯O HBs in acetamide–methanol and
acetamide–p-cresol systems by varying the constrained HB distance (i.e., N⋯O or O⋯O) in
steps of 0.01 Å from 2.4 Å to 4.0 Å. The 2D PESs were obtained for the cis N–H⋯O HB and O–
H⋯O HB of acetamide–methanol and acetamide–p-cresol by varying the constraining HB
distance from 2.40 up to 5.00 Å in steps of 0.05 Å and simultaneously varying the angle from
110° to 180° in steps of 5°. Reaction coordinates for the transformation between N–H⋯O,
ambifunctional, and \( \text{O–H} \cdots \text{O} \) HBs for the acetamide–methanol and acetamide–\( \text{p} \)-cresol model systems were sampled from initial geometries in which HB partners were translated and rotated with respect to each other using an in-house Python script. The reaction coordinate corresponded to an intermolecular angle, which was selected by trial and error, and initial geometries for constrained optimizations were generated by rotation of this angle in 0.1° increments (ESI Text S4).

ASSOCIATED CONTENT

Electronic Supplementary Information.
Refinement of protein crystal structures from the data set; topology analysis of PDB files from protein data set; QTAIM analysis for some PDB files forming single HBs; addition of hydrogen atoms to PDB files; optimized geometries of cis and trans \( \text{N–H} \cdots \text{O} \) HBs of acetamide-methanol; acetamide-methanol/\( \text{p} \)-cresol interaction energies at higher levels of theory; HB energies and geometries for all acetamide-methanol and acetamide-\( \text{p} \)-cresol HB conformations; distance histograms overlaid onto 1D PECs for Ser/Tyr-Gln and Thr-Asn/Gln residue pairs; presence of more number of trans than cis \( \text{N–H} \cdots \text{O} \) HBs in data set; cis-trans \( \text{N–H} \cdots \text{O} \) HBs, mean and standard deviation of HB angles in data set; comparison of cis and trans \( \text{N–H} \cdots \text{O} \) HB energies of Ser-Asn; optimized geometries of cis and trans \( \text{N–H} \cdots \text{O} \) HBs of Ser-Asn; representative proteins showing cis and trans \( \text{N–H} \cdots \text{O} \) HBs; angle histograms for Ser/Tyr-Gln and Thr-Asn/Gln residue pairs in data set; representative protein showing stabilizing interactions; 2D contours for Ser/Tyr-Gln and Thr-Asn/Gln residue pairs in data set; distribution of PDB files in two innermost contours of 2D energy surfaces; representative stabilizing interactions with small HB angles; mean and standard deviation of \( \text{O–H} \cdots \text{O} \) HBs in data set; number of \( \text{N–H} \cdots \text{O} \), \( \text{O–H} \cdots \text{O} \), and ambifunctional HBs in data set; representative proteins showing \( \text{N–H} \cdots \text{O} \) and \( \text{O–H} \cdots \text{O} \) HBs; representative protein showing two simultaneous \( \text{N–H} \cdots \text{O} \) HBs; geometries and energies of single HBs with ambifunctional HB distances; thermodynamic corrections for acetamide-methanol/\( \text{p} \)-cresol HB energies; geometries and energetic penalties in ambifunctional HBs; comparison of GAFF and DLPNO-CCSD(T)/CBS energies for select HBs; details of reaction coordinate construction and transformation; reaction coordinate intermolecular angle in acetamide-methanol; translated reaction coordinate as \( \text{N–H} \cdots \text{O} \) angle in both model systems; \( \text{O–H} \cdots \text{O} \) angle as a function of \( \text{N–H} \cdots \text{O} \) angle in reaction coordinate plots; energies of \( \text{O–H} \cdots \text{O} \) HB and transition state in reaction coordinate figures; comparison of SAPT energies of select HB configurations; reaction coordinate plots with GAFF interaction energies; plots of \( \text{O–H} \cdots \text{O} \) vs \( \text{N–H} \cdots \text{O} \) angle and \( \text{O–O} \) vs \( \text{N–N} \) distance in ambifunctional HB basin of acetamide-methanol and acetamide-\( \text{p} \)-cresol; plots of sum of HB distances vs \( \text{N–H} \cdots \text{O} \) HB angle in ambifunctional HB basin of both model systems; 2D distance histograms of ambifunctional HBs in the data set; 2D distance histograms of single HBs in the data set; number of moderately and extremely strong ambifunctional HBs in data set; 2D angle histograms of ambifunctional HBs in the data set; mean \( \text{N–H} \cdots \text{O} \) and \( \text{O–H} \cdots \text{O} \) HB angles in ambifunctional HBs in data set; HB energies of formamide/acetamide/propanamide and methanol/ethanol; basis-set dependence of optimized
geometries of acetamide-methanol; comparison of HB energies of MP2 and B3LYP-D3 optimized geometries; comparison of B3LYP-D3 and DLPNO-CCSD(T) energies. (PDF)

PDB information of structures in the refined data set, i.e., PDBID, residue names and numbers, HB distances and angles, type of HB for N–H···O HB in text files; CSV files containing information about the resolution and where available, electron density score for individual atoms (EDIA) for structures in the refined data set; O···O and N···O HB distance information in single HBs; initial and optimized geometries of model systems obtained at different levels of theory and with 6-31G* basis set; an Excel file with information about HB energies of a representative set of PDB structures obtained from topology analysis for single and ambifunctional HBs. (ZIP)

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Notes

The authors declare no competing financial interest.

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Electronic Supplementary Information for

_When are two hydrogen bonds better than one? Accurate first-principles models explain the balance of hydrogen bond donors and acceptors found in proteins_

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| Refinement criteria       | S-N  | S-Q  | T-N  | T-Q  | Y-N  | Y-Q  | Total |
|---------------------------|------|------|------|------|------|------|-------|
| resolution < 1.5 Å        | 1,284| 854  | 1,504| 1,011| 843  | 618  | 6,114 |
| HB distance cutoff applied| 889  | 545  | 1,016| 591  | 578  | 416  | 4,035 |
| HB angle cutoff applied   | 856  | 526  | 988  | 582  | 562  | 394  | 3,908 |

Text S1. Topology analysis of PDB files from protein data set.

To identify the HB distance criteria, residue pairs were selected at random from the initial protein data set. Hydrogen atoms were added to the PDB files using Avogadro v1.2.0\(^1\) and the added hydrogen atoms were force-field optimized with MMFF94\(^2\) while the heavy atoms were held fixed. Hydrogen atom positions were further refined with constrained geometry optimizations at the B3LYP-D3/6-31G* level of theory. The hybrid DFT optimization was selected for consistency with the level of theory with which the topology analysis could be readily carried out. Topology analysis was carried out to identify bond critical points (BCPs) and evaluate HB energies where BCPs were observed\(^3\)-\(^5\). Negative energies were not used alone to determine the presence of HBs from the potential energy densities using Espinosa’s equation (i.e., \(E_{HB} = V_{BCP}/2\)) because they can be significant overestimates\(^3\). They were used only for qualitative assessments.

Based on these considerations, we determined HBs to exist only for those structures for which HB energies computed from potential energy densities were equal to or stronger than -4 kcal/mol. N–H···O HBs satisfying this criterion were found between 2.50 and 3.20 Å in Ser/Thr/Tyr-Asn/Gln, and O–H···O HBs were found between 2.40 and 3.10 Å in Ser/Thr-Asn/Gln systems or between 2.40 and 3.20 Å in Tyr-Asn/Gln systems. To confirm that strong HBs were indeed absent in structures with HB distances outside of the above set HB distance criteria, we selected at random 10% of all the PDB structures that fell outside of the HB distance criteria as a representative sample. This corresponds to PDB structures with N···O distance < 2.50 Å or > 3.20 Å for Ser/Thr/Tyr-Asn/Gln systems and PDB structures with O···O distance < 2.40 Å or > 3.10 Å (3.20 Å) for Ser/Thr-Asn/Gln (Tyr-Asn/Gln) systems. We then carried out topology analysis for all the selected systems and computed HB energies from potential energy densities of the BCPs (when present). We found the resulting HB energies to be consistent with the set HB distance criteria, i.e., HBs of significant strength were absent from this set.

Following a similar protocol, HB angle criteria for N–H···O HBs and O–H···O HBs were determined. We performed topology analysis for all the structures with HB angles ≤ 110°. We found that four N–H···O HB systems showed strong HBs with HB angles < 110° (105.0°, 107.3°, 109.0°, and 109.9°) and very short HB distances (2.70 Å, 3.20 Å, 2.69 Å, and 2.68 Å, respectively). We did not observe strong O–H···O HBs with HB angles ≤ 110°. The complete set of results of these calculations is tabulated in an Excel file in Supporting Information.
Table S2. QTAIM analysis for some of the PDB files for the N–H···O and O–H···O HBs present between Ser/Thr/Tyr and Asn/Gln on B3LYP-D3/6-31G* geometry optimized PDB structures with heavy atoms constrained. The N–H···O and O–H···O HB distances for these PDB structures fall outside the HB distance criteria, and hence, HBs are either not observed or the observed HBs are very weak, as corroborated by the HB energy estimates. The HB distance and angle information are given in Å and °, respectively. The energy of HB (E_{HB}), which is estimated as half of the potential energy at the BCP obtained from QTAIM topology analysis, is given in kcal/mol.

| PDB ID | Chain Name | Residue 1 | Residue 2 | HB distance (Å) | HB angle (°) | E_{HB} (kcal/mol) |
|--------|------------|-----------|-----------|-----------------|-------------|------------------|
| N–H···O HBs | | | | | | |
| 3ir4   | A          | S169      | N165      | 3.34            | 138.7       | -2.0             |
| 5vn4   | A          | N213      | S210      | 3.26            | 104.6       | ---              |
| 4esp   | A          | S37       | Q4        | 3.67            | 73.7        | -0.5             |
| 1llf   | A          | Q62       | S59       | 3.64            | 91.0        | -1.2             |
| 5akr   | A          | N305      | T252      | 3.33            | 101.0       | -1.2             |
| 5ta0   | A          | T505      | N348      | 3.38            | 111.7       | -1.0             |
| 3e2d   | B          | Q364      | T283      | 3.62            | 104.7       | -0.5             |
| 2fgq   | X          | T226      | Q224      | 3.64            | 108.2       | -0.5             |
| 1lg9   | A          | N62       | Y51       | 3.60            | 94.2        | -0.6             |
| 3i45   | A          | Y24       | N12       | 3.47            | 134.1       | -1.2             |
| 5js4   | B          | Q267      | Y265      | 3.30            | 108.9       | ---              |
| 4brc   | B          | Q142      | Y138      | 3.62            | 66.0        | -0.6             |
| O–H···O HBs | | | | | | |
| 5fls   | B          | N115      | S113      | 3.48            | 79.7        | ---              |
| 3gzb   | E          | S48       | N45       | 3.56            | 157.8       | -1.1             |
| 3b7e   | B          | Q226      | S181      | 3.15            | 159.3       | -3.4             |
| 2x9g   | D          | Q236      | S233      | 3.51            | 155.4       | -1.5             |
| 2imi   | B          | T54       | N50       | 3.10            | 150.1       | -3.4             |
| 2r01   | A          | T37       | N34       | 3.53            | 161.9       | -1.3             |
| 5hv    | A          | Q98       | T95       | 3.47            | 142.7       | ---              |
| 5a71   | A          | Q46       | T43       | 3.40            | 162.5       | -2.0             |
| 3a72   | A          | Y241      | N180      | 3.32            | 95.6        | -0.8             |
| 1u3w   | A          | N114      | Y110      | 3.36            | 155.2       | -2.0             |
| 5epu   | E          | Y52       | Q31       | 3.56            | 98.0        | ---              |
| 4gwb   | A          | Q101      | Y63       | 3.60            | 113.6       | ---              |

Text S2. Addition of hydrogen atoms to PDB files.

PDB structures in the protein data set do not contain hydrogen atoms. So, an in-house Python script was used to add hydrogen atoms to select atoms in the PDB files.

\[ N\text{–}H\cdots\text{O HBs} \]

Since the atoms involved in an N–H···O HB in Ser/Thr/Tyr-Asn/Gln systems are the side-chain hydroxyl oxygen of Ser/Thr/Tyr and side-chain amide nitrogen and hydrogen atoms of Asn/Gln, hydrogen atoms were added only to the side-chain amide nitrogen of Asn/Gln using an in-house Python script. The script reads each PDB file and writes the coordinates of side-chain hydroxyl oxygen of Ser/Thr/Tyr, side-chain amide carbon, oxygen, and nitrogen atoms of Asn/Gln to an XYZ file. Based on the following assumptions, two hydrogen atoms were added to the nitrogen atom in the XYZ file.

(i) Carbon, oxygen, nitrogen, and hydrogen atoms of the amide side-chain of Asn/Gln are in the same plane owing to the partial double-bond character of the C–N bond.
(ii) The $\angle$C–N–H angle is 120.6° in Ser/Thr-Asn/Gln systems and 120.4° in Tyr-Asn/Gln systems. These angles correspond to the mean value of $\angle$C–N–H angle of cis and trans N–H···O HBs observed in MP2/6-31G* optimized geometries of acetamide–methanol and acetamide–p-cresol N–H···O HBs, respectively.

(iii) The N–H distance is 1.02 Å in all the systems as observed in the MP2/6-31G* optimized geometries of acetamide–methanol and acetamide–p-cresol N–H···O HBs.

Each of the above assumptions leads to one mathematical equation, as elaborated below in the order of assumptions.

(i) We obtained the equation of plane formed by the side-chain amide carbon, oxygen, and nitrogen atoms using their coordinates. Because hydrogen atoms lie in the same plane, the x, y, and z coordinates of each hydrogen atom must satisfy the equation of plane formed by side-chain amide carbon, oxygen, and nitrogen atoms.

$$ah_x + bh_y + ch_z + d = 0$$

where a, b, c, and d are known and $h_x$, $h_y$, and $h_z$ are the x, y, and z coordinates of a hydrogen atom.

(ii) x, y, and z coordinates of the hydrogen atom must satisfy the dot-product equation of C–N and N–H bonds.

$$\cos (\angle C–N–H) = \frac{(C - N) \cdot (N - H)}{||C - N|| \cdot ||N - H||}$$

where $N - H = (n_x - h_x, n_y - h_y, n_z - h_z)$.

(iii) x, y, and z coordinates of hydrogen atom must satisfy the equation of norm of $N - H$.

$$||N - H|| = \sqrt{(n_x - h_x)^2 + (n_y - h_y)^2 + (n_z - h_z)^2} = 1.02 \text{ Å}$$

The Python script solves the above three equations for the three unknowns. The presence of square terms in the equation of norm leads to two sets of coordinates, which correspond to the two hydrogen atoms. The script then writes the coordinates of hydrogen atoms to the XYZ file.

The distances between side-chain hydroxyl oxygen of Ser/Thr/Tyr and side-chain amide hydrogen atoms of Asn/Gln were measured. The hydrogen atom closest to the side-chain hydroxyl oxygen would result in favorable hydrogen bonding interaction. The position of this hydrogen atom with respect to the amide oxygen determines whether the N–H···O HB is cis or trans. The script classifies the HBs as cis or trans, and also computes the N–H···O HB angle.

O–H···O HBs. Addition of a hydrogen atom to side-chain hydroxyl oxygen of Ser/Thr/Tyr follows a protocol very similar to the one followed while adding hydrogen atoms to N–H···O HB systems. The hydrogen atom is added using an in-house Python script only to the side-chain hydroxyl oxygen since the atoms involved in O–H···O HB are side-chain amide oxygen of Asn/Gln and side-chain hydroxyl group of Ser/Thr/Tyr.

The script reads each PDB file and writes the coordinates of side-chain hydroxyl oxygen, $C\alpha$, and $C\beta$ of Ser/Thr, and side-chain amide oxygen of Asn/Gln to an XYZ file. For Tyr systems, the coordinates of side-chain hydroxyl oxygen, $C\varepsilon$, and $C\zeta$ of Tyr, and side-chain amide oxygen of Asn/Gln are written to an XYZ file. Based on the following assumptions, a hydrogen atom was added to the hydroxyl oxygen atom in the XYZ file.

(i) O–H distance is 0.98 Å in Ser/Thr-Asn/Gln systems and 0.99 Å in Tyr-Asn/Gln systems as observed in the MP2/6-31G* optimized geometry of acetamide-methanol O–H···O HB.
The $\angle C\beta-O-H$ angle is 106.4° in Ser/Thr-Asn/Gln systems and $\angle C\zeta-O-H$ angle is 108.7° in Tyr-Asn/Gln systems as observed in MP2/6-31G* optimized geometry of acetamide-methanol $O-H\cdots O$ HB.

(iii) $C\alpha$, $C\beta$, hydroxyl oxygen and hydrogen atoms of Ser/Thr lie in the same plane. $C\epsilon$, $C\zeta$, hydroxyl oxygen and hydrogen atoms of Tyr lie in the same plane.

Each of the above assumptions leads to one mathematical equation. Although, the third assumption is not true, assuming this results in three equations in three unknowns as elaborated below.

(i) $x$, $y$, and $z$ coordinates of hydrogen atom must satisfy the equation of norm of $O-H$.

$$||O-H|| = (o_x - h_x)^2 + (o_y - h_y)^2 + (o_z - h_z)^2 = 0.98 \text{ Å (Ser/Thr systems)}$$

$$||O-H|| = (o_x - h_x)^2 + (o_y - h_y)^2 + (o_z - h_z)^2 = 0.99 \text{ Å (Tyr systems)}$$

(ii) $x$, $y$, and $z$ coordinates of hydrogen atom must satisfy the dot-product equation of $C\beta-O$ and $O-H$ bonds for Ser/Thr systems. Similarly, $x$, $y$, and $z$ coordinates of hydrogen atom must satisfy the dot-product equation of $C\zeta-O$ and $O-H$ bonds for Tyr systems.

$$\cos (\angle C\beta-O-H) = (C\beta - \vec{O} \cdot O-H)/(||C\beta-O|| ||O-H||)$$

$$\cos (\angle C\zeta-O-H) = (C\zeta - \vec{O} \cdot O-H)/(||C\zeta-O|| ||O-H||)$$

where $\vec{O} = (o_x - h_x, o_y - h_y, o_z - h_z)$.

(iii) We obtain the equation of plane formed by $C\alpha$, $C\beta$, and hydroxyl oxygen of Ser/Thr, and that formed by $C\epsilon$, $C\zeta$, and hydroxyl oxygen of Tyr. From the third assumption, the $x$, $y$, and $z$ coordinates of hydrogen atom must satisfy this equation of plane.

$$a_h x + b_h y + c_h z + d = 0$$

where $a$, $b$, $c$, and $d$ are known and $h_x$, $h_y$, and $h_z$ are the $x$, $y$, and $z$ coordinates of the hydrogen atom.

The Python script solves the above three equations in three unknowns. The presence of square terms in the equation of norm leads to two sets of coordinates, of which the set of coordinates closest to the side-chain amide oxygen of Asn/Gln is selected using the script. With these coordinates of hydrogen atom as the starting point, the $O-H$ bond is rotated around $C\beta-O$ bond (Ser/Thr) or $C\zeta-O$ bond (Tyr) from 0° to 360° in steps of 0.1°. At each step of rotation, the distance between side-chain amide oxygen of Asn/Gln and hydrogen atom is measured. The coordinates of hydrogen atom in the configuration with shortest distance between hydrogen and amide oxygen are written to the XYZ file. The script then computes $O-H\cdots O$ HB angle using these hydrogen atom coordinates.

Ambifunctional HBs. For ambifunctional HBs, two hydrogen atoms were added to the sidechain amide nitrogen of Asn/Gln and one hydrogen atom was added to the sidechain hydroxyl oxygen of Ser/Thr/Tyr. The addition of amide hydrogen atoms was done as per the protocol mentioned above for adding hydrogen atoms to residue-pairs forming N–H⋯O HBs, and the addition of sidechain hydroxyl hydrogen was done following the protocol for adding hydrogen atoms to residue-pairs forming O–H⋯O HBs.
Table S3. Interaction energies (in kcal/mol) with larger basis sets and higher levels of theory using the MP2/6-31G*-optimized geometries of all four acetamide–methanol and acetamide–p-cresol HB conformations: N–H···O cis HB, N–H···O trans HB, O–H···O HB, and ambifunctional (ambi.) HB. The interaction energy is obtained as the difference in energy of the dimer from that of the isolated molecules, all evaluated on MP2/6-31G*-optimized geometries. The two-point extrapolation formula based on the aug-cc-pVDZ and aug-cc-pVTZ energies is used to extrapolate to the limit following Refs. 6-8 for DLPNO-CCSD(T) and CCSD(T). Here, Normal refers to default thresholds of TCutPairs = 10⁴, TCutPNO = 3.33 x 10⁻⁷, TCutMKN = 10⁻³, and Tight refers to default thresholds of TCutPairs = 10⁻⁵, TCutPNO = 1.00 x 10⁻⁷, TCutMKN = 10⁻³. The interaction energy of cis N–H···O HB obtained from CCSD(T)/CBS calculations was 0.5 kcal/mol stronger than that of trans N–H···O HB of acetamide–methanol, while the interaction energy of trans N–H···O HB obtained from DLPNO-CCSD(T)/CBS calculations is 0.1 kcal/mol stronger than that of cis N–H···O HB of acetamide–p-cresol. DLPNO-CCSD(T)/CBS calculations also reveal that cis and trans N–H···O HBs of acetamide–methanol are 0.9 kcal/mol and 0.3 kcal/mol stronger than those of acetamide–p-cresol, respectively.

| Method/Basis | Interaction Energy (kcal/mol) | \( \text{N–H} \cdots \text{O} \) cis HB | \( \text{N–H} \cdots \text{O} \) trans HB | \( \text{O–H} \cdots \text{O} \) HB | Ambi. HB |
|--------------|-------------------------------|---------------------------------|---------------------------------|---------------------------------|--------|
| acetamide–methanol |                               |                                 |                                 |                                 |        |
| DLPNO-CCSD(T) / aug-cc-pVDZ (Normal) | -6.9                          | -7.3                            | -12.1                           | -12.7                           |        |
| DLPNO-CCSD(T) / aug-cc-pVDZ (Tight)      | -7.2                          | -7.4                            | -12.4                           | -13.1                           |        |
| CCSD(T) / aug-cc-pVDZ                     | -7.4                          | -7.6                            | -12.7                           | -13.5                           |        |
| DLPNO-CCSD(T) / aug-cc-pVTZ (Normal)      | -6.2                          | -6.4                            | -11.2                           | -12.1                           |        |
| DLPNO-CCSD(T) / aug-cc-pVTZ (Tight)      | -6.4                          | -6.5                            | -11.4                           | -12.5                           |        |
| DLPNO-CCSD(T) / aug-cc-pVTZ (Tight)      | -6.4                          | -6.5                            | -11.4                           | -12.5                           |        |
| CCSD(T)/CBS                              | -6.1                          | -6.2                            | -11.0                           | -12.2                           |        |
| acetamide–p-cresol |                               |                                 |                                 |                                 |        |
| DLPNO-CCSD(T) / aug-cc-pVDZ (Normal) |                                 |                                 |                                 |                                 |        |
Table S4. Comparison of interaction energies (in kcal/mol) and optimized geometries (relevant distances in Å and angles in °) of all four acetamide–methanol HB configurations obtained using the listed methods with and without incorporating semi-empirical dispersion (D3) for the hybrid DFT (B3LYP\textsuperscript{10-12}) results. The counterpoise corrected results were obtained on the B3LYP-D3/6-31G* geometries. HB distances and angles from B3LYP and B3LYP-D3 calculations were found to differ by at most 0.05 Å and 6.4°, respectively, and the interaction energies from B3LYP-D3 calculations were stronger than those from B3LYP calculations by at most 2.3 kcal/mol. Comparison of optimized geometries obtained from B3LYP-D3 and MP2 calculations revealed that the HB distances and angles differed by at most 0.06 Å and 1.6°, respectively, and the B3LYP-D3 interaction energies were found to be stronger than those obtained from MP2 calculations by at most 1.9 kcal/mol. As a result, MP2-optimized geometries are used throughout in this work.

| Method/Basis                   | Int. E (kcal/mol) | O–H⋯O=C (Å) | H⋯O⋯N (Å) | O⋯O (Å) | N⋯O (Å) | ∠O–H⋯O (°) | ∠N–H⋯O (°) |
|-------------------------------|-------------------|-------------|-----------|---------|---------|------------|------------|
| N–H⋯O cis HB                  |                   |             |           |         |         |            |            |
| B3LYP-D3/6-31G*               | -10.1             | --          | 1.91      | --      | 2.92    | --         | 169.8      |
| Counterpoise-corrected B3LYP-D3/6-31G* | -7.8           | --          | --        | --      | --      | --         | --         |
| B3LYP/6-31G*                  | -8.1              | --          | 1.95      | --      | 2.96    | --         | 170.6      |
| MP2/6-31G*                    | -9.1              | --          | 1.96      | --      | 2.97    | --         | 171.4      |
| N–H⋯O trans HB                |                   |             |           |         |         |            |            |
| B3LYP-D3/6-31G*               | -8.9              | --          | 1.94      | --      | 2.92    | --         | 161.1      |
| Counterpoise-corrected B3LYP-D3/6-31G* | -6.8           | --          | --        | --      | --      | --         | --         |
| B3LYP/6-31G*                  | -7.1              | --          | 1.97      | --      | 2.97    | --         | 167.5      |
| MP2/6-31G*                    | -8.0              | --          | 1.99      | --      | 2.97    | --         | 162.3      |
| O–H⋯O HB                      |                   |             |           |         |         |            |            |
| B3LYP-D3/6-31G*               | -12.2             | 1.85        | --        | 2.80    | --      | 161.8      | --         |
| Counterpoise-corrected B3LYP-D3/6-31G* | -8.7           | --          | --        | --      | --      | --         | --         |
| B3LYP/6-31G*                  | -9.9              | 1.86        | --        | 2.82    | --      | 164.9      | --         |
| MP2/6-31G*                    | -10.5             | 1.90        | --        | 2.85    | --      | 162.7      | --         |
| Ambifunctional HB             |                   |             |           |         |         |            |            |
| B3LYP-D3/6-31G*               | -15.9             | 1.83        | 1.96      | 2.75    | 2.83    | 153.3      | 140.8      |
| Counterpoise-corrected B3LYP-D3/6-31G* | -12.3           | --          | --        | --      | --      | --         | --         |
| B3LYP/6-31G*                  | -13.6             | 1.86        | 1.98      | 2.78    | 2.85    | 152.8      | 141.4      |
| MP2/6-31G*                    | -14.0             | 1.90        | 2.01      | 2.81    | 2.88    | 152.6      | 141.8      |
Table S5. Comparison of interaction energies (in kcal/mol) and optimized geometries (relevant distances in Å and angles in °) of all four acetamide–p-cresol HB configurations obtained using the listed methods with and without incorporating semi-empirical dispersion (D3⁹) for the hybrid DFT (B3LYP¹⁰–¹²) results. The counterpoise corrected results were obtained on the B3LYP-D3/6-31G* geometries. B3LYP and B3LYP-D3 calculations were found to differ by at most 0.07 Å and 7.4°, respectively, and the interaction energies from B3LYP-D3 calculations were stronger than those from B3LYP calculations by at most 3.7 kcal/mol. Comparison of optimized geometries obtained from B3LYP-D3 and MP2 calculations revealed that the HB distances and angles differed by at most 0.05 Å and 1.0°, respectively, and the B3LYP-D3 interaction energies were found to be stronger than those obtained from MP2 calculations by at most 2.0 kcal/mol. For the ambifunctional HB in both model systems, the N···O HB distance and N–H···O HB angle are more distorted than the O···O HB distance and O–H···O HB angle with respect to their equilibrium values in a single HB configuration. This effect is most pronounced with p-cresol, which has both a shorter O···O HB distance and longer N···O HB distance (both by ca. 0.05 Å) and a more linear O–H···O HB angle at the cost of a less favorable N–H···O HB angle (both by 4°). These observations are in line with a shallower PEC of the N–H···O HB than that of O–H···O HB in p-cresol.

| Method/Basis       | Int. E (kcal/mol) | O–H···O=O=C (Å) | H–O···H–N (Å) | O···O (Å) | N···O (Å) | <O–H···O (°) | <N–H···O (°) |
|--------------------|------------------|-----------------|----------------|-----------|-----------|------------|------------|
|                    |                  |                 | N–H···O cis HB |           |           |            |            |
| B3LYP-D3/6-31G*    | -8.8             | --              | 2.01           | --        | 3.03      | --         | 177.0      |
| Counterpoise       | -6.5             | --              | --             | --        | --        | --         | --         |
| Corrected          |                   |                 |                |           |           |            |            |
| B3LYP-D3/6-31G*    | -6.5             | --              | 2.06           | --        | 3.07      | --         | 177.9      |
| MP2/6-31G*         | -8.3             | --              | 2.06           | --        | 3.07      | --         | 177.7      |
|                    |                  |                 | N–H···O trans HB |          |           |            |            |
| B3LYP-D3/6-31G*    | -8.5             | --              | 2.03           | --        | 3.01      | --         | 163.9      |
| Counterpoise       | -6.3             | --              | --             | --        | --        | --         | --         |
| Corrected          |                   |                 |                |           |           |            |            |
| B3LYP-D3/6-31G*    | -5.1             | --              | 2.07           | --        | 3.08      | --         | 171.3      |
| MP2/6-31G*         | -7.7             | --              | 2.07           | --        | 3.06      | --         | 164.8      |
|                    |                  |                 | O–H···O HB     |           |           |            |            |
| B3LYP-D3/6-31G*    | -15.5            | 1.78            | --             | 2.75      | --        | 165.9      | --         |
| Counterpoise       | -12.0            | --              | --             | --        | --        | --         | --         |
| Corrected          |                   |                 |                |           |           |            |            |
| B3LYP-D3/6-31G*    | -11.8            | 1.80            | --             | 2.77      | --        | 167.7      | --         |
| MP2/6-31G*         | -13.5            | 1.83            | --             | 2.80      | --        | 166.0      | --         |
|                    |                  |                 | Ambifunctional HB |          |           |            |            |
| B3LYP-D3/6-31G*    | -17.2            | 1.77            | 2.07           | 2.71      | 2.90      | 157.1      | 136.7      |
| Counterpoise       | -13.6            | --              | --             | --        | --        | --         | --         |
| Corrected          |                   |                 |                |           |           |            |            |
| B3LYP-D3/6-31G*    | -14.0            | 1.79            | 2.12           | 2.73      | 2.95      | 157.3      | 136.9      |
| MP2/6-31G*         | -15.5            | 1.82            | 2.11           | 2.76      | 2.94      | 156.6      | 137.7      |
**Figure S2.** Normalized histograms (blue, left axes) of heavy-atom HB distances (in Å, bin width of 0.1 Å) for Ser-Gln (top) and Tyr-Gln (bottom) X-ray crystal structures with the 1D PECs (red, right axes) for acetamide–methanol (top) and acetamide–p-cresol (bottom) overlaid. The left panes show the N···O HB distance histograms and PECs of cis N–H···O HBs, and the right panes show the O···O HB distance histograms and PECs of O–H···O HBs. The interaction energies ($E_{\text{int}}$) shown are obtained from DLPNO-CCSD(T)/CBS calculations. The structure insets are representative PDB structures for the relevant HB where the HB distance is indicated by black dotted lines. C$\alpha$ of the residues in the insets is represented as a green sphere indicating that the residues are truncated to show only the side-chains, and the remaining atoms in the side-chains are shown as sticks with carbon in gray, hydrogen in white, nitrogen in blue, and oxygen in red. C$\alpha$ of the residues in the insets is represented as a green sphere indicating that the residues are truncated to show only the side-chains, and the remaining atoms in the side-chains are shown as sticks with carbon in gray, hydrogen in white, nitrogen in blue, and oxygen in red.
Figure S3. Normalized histograms (blue, left axes) of heavy-atom HB distances (in Å, bin width of 0.1 Å) for Thr-Asn (top) and Thr-Gln (bottom) X-ray crystal structures with the 1D PECs (red, right axes) for acetamide–methanol (top and bottom) overlaid. The left panes show the N–O HB distance histograms and PECs of cis N–H···O HBs, and the right panes show the O–O HB distance histograms and PECs of O–H···O HBs. The interaction energies \( E_{\text{int}} \) shown are obtained from DLPNO-CCSD(T)/CBS calculations. The structure insets are representative PDB structures for the relevant HB where the HB distance is indicated by black dotted lines. Cα of the residues in the insets is represented as a green sphere indicating that the residues are truncated to show only the side-chains, and the remaining atoms in the side-chains are shown as sticks with carbon in gray, hydrogen in white, nitrogen in blue, and oxygen in red.

Text S3. Presence of a higher number of trans N–H···O HBs than cis N–H···O HBs in the protein data set.

Given the ability of our models to recapitulate key differences between Ser/Thr and Tyr residues, we return to the question of the extent to which the models could capture other kcal/mol-scale trends. Specifically, the DLPNO-CCSD(T)/CBS model energetics for methanol (i.e., representing Ser/Thr) had exhibited a weak preference for cis over trans N–H···O HBs, whereas they were degenerate for the Tyr-model p-cresol (main text Figure 2 and ESI Table S3). Analysis of the number of cis and trans N–H···O HB structures from the protein data set indicates a weak preference towards the formation of trans N–H···O HBs over cis N–H···O HBs for all amino acid pairs (ESI Table S6). These differences could arise due to our neglect of the
We thus compared our model acetamide–methanol cis and trans N–H···O HB interaction energies to those of full Ser and Asn residues (see Sec. 5). The full Ser-Asn model trans N–H···O HB is stronger than that of the cis N–H···O HB due to the presence of two additional backbone HBs in the trans conformation (by ca. 4.6 kcal/mol, ESI Table S7 and Figure S4). Over the full protein data set, we observed similar additional HB interactions in many of the trans N–H···O HB conformations (ESI Figure S5). Hence, while our models accurately capture key structural features, the higher abundance of trans N–H···O HBs in X-ray crystal structures is likely due to their higher compatibility with simultaneous backbone–backbone stabilization.

From the DLPNO-CCSD(T)/CBS results and the MP2/6-31G* optimized geometries, we inferred that additional HB interactions are present in trans N–H···O HBs (ESI Table S7 and Figure S4). Given that these interactions are observed between the backbones of the hydrogen bonding residues, it is very likely that trans N–H···O HBs are observed between residues on adjacent beta sheets or loops (ESI Figure S5). Since the backbones of residues were not observed to hydrogen bond in cis N–H···O HB, it is likely that the cis N–H···O HBs are observed between residues with one residue on an alpha helix while the other is on a beta sheet or a loop (ESI Figure S5). These features may not be observed in all the cis and trans N–H···O HBs, but they might be part of why we see more trans N–H···O HBs than cis N–H···O HBs despite similar energetics in model systems.

Table S6. Number of cis and trans N–H···O HBs in Ser-Asn, Ser-Gln, Thr-Asn, Thr-Gln, Tyr-Asn, and Tyr-Gln residue pairs in the protein data set, mean N–H···O angle, and standard deviation of N–H···O angle of N–H···O HBs.

| Residue pair | Number of cis N–H···O HBs | Number of trans N–H···O HBs | Mean N–H···O HB angle | Standard deviation |
|--------------|--------------------------|----------------------------|----------------------|-------------------|
| Ser-Asn      | 153                      | 260                       | 153.3°               | 16.7°             |
| Ser-Gln      | 73                       | 209                       | 153.4°               | 14.6°             |
| Thr-Asn      | 157                      | 280                       | 156.6°               | 16.2°             |
| Thr-Gln      | 85                       | 240                       | 155.0°               | 16.4°             |
| Tyr-Asn      | 87                       | 187                       | 152.4°               | 15.7°             |
| Tyr-Gln      | 54                       | 133                       | 154.5°               | 15.7°             |

Table S7. Comparison of interaction energies (Int. E, in kcal/mol) of cis and trans N–H···O HBs of Ser and Asn with N-terminal acetyl (ACE) and C-terminal N-methyl (NME) capping for both the residues. The interaction energy is obtained as the difference in energy of the dimer from that of the isolated molecules, all evaluated on MP2/6-31G*-optimized geometries. The two-point extrapolation formula based on the aug-cc-pVDZ and aug-cc-pVTZ energies is used to extrapolate to the complete basis set limit following Refs. 6–8 for DLPNO-CCSD(T) and canonical CCSD(T). Here, Tight refers to default thresholds of TCutPairs = 10^5, TCutPNO = 1.00 × 10^7, and TCutMKN = 10^3.

| Method/Basis                  | cis N–H···O HB Int. E (kcal/mol) | trans N–H···O HB Int. E (kcal/mol) | cis-trans (kcal/mol) |
|------------------------------|----------------------------------|----------------------------------|----------------------|
| DLPNO-CCSD(T) / aug-cc-pVDZ (Tight) | -15.7                            | -21.7                            | -6.0                 |
| DLPNO-CCSD(T) / aug-cc-pVTZ (Tight) | -15.0                            | -20.1                            | -5.1                 |
| DLPNO-CCSD(T)/CBS (Tight)     | -14.8                            | -19.4                            | -4.6                 |
**Figure S4.** MP2/6-31G* geometry-optimized structures of Ser-Asn N–H···O HBs with N-terminal acetyl (ACE) and C-terminal N-methyl (NME) capping for backbones of both residues: (a) *cis* N–H···O HB (b) *trans* N–H···O HB. Oxygen, nitrogen, carbon, and hydrogen atoms are shown in red, blue, gray, and white colors, respectively. The black dashed lines indicate the N–H···O HB of Ser and Asn sidechains. The green dashed lines indicate the additional HB interactions between the residues. In the case of *cis* N–H···O HB, an additional HB is present between sidechain amide carbonyl oxygen of Asn and backbone N–H hydrogen of Ser. For *trans* N–H···O HB, additional HBs are observed between the backbone carbonyl oxygen and N–H hydrogen of both the residues.

**Figure S5.** Representative proteins showing the HB interactions observed in the surrounding protein environment of N–H···O HBs between Thr and Gln. The protein cartoon is shown in translucent gray. The hydrogen bonding residues Thr and Gln are shown in green sticks while the nearby residues involved in HB interactions are shown in orange sticks. Oxygen and nitrogen atoms are shown in red and blue, respectively. Sidechain carbon atoms are shown in gray. (a) *cis* N–H···O HB between Thr located on a loop and Gln located on an alpha helix in the protein (PDB ID: 4URF) is shown in black dashed lines. HB between sidechain amide carbonyl oxygen of Gln and a nearby backbone N–H hydrogen is shown in orange dashed lines. (b) *trans* N–H···O HB between Thr and Gln in the protein (PDB ID: 4MHP) is shown in black dashed lines. Thr and Gln are located on adjacent beta sheets. The HBs between the backbone carbonyl oxygen and N–H hydrogen of the adjacent beta sheets are shown in orange dashed lines. The HB of Gln sidechain amide carbonyl oxygen with a nearby solvent molecule (red sphere) is shown in green dashed lines. Thr and Gln residues are labeled with the one-letter amino acid code followed by the residue number.
Figure S6. Normalized histograms of N–H···O HB (left) and O–H···O HB (right) angles (in °) for Ser-Gln (top) and Tyr-Gln (bottom) residue pairs from X-ray crystal structures. All histograms have 10° bin widths. The insets depict the HB angle on representative PDB structures with the corresponding Cα of the residues represented as a green sphere, and the remaining atoms are shown as sticks with carbon in gray, hydrogen in white, nitrogen in blue, and oxygen in red.
Figure S7. Normalized histograms of N–H···O HB (left) and O–H···O HB (right) angles (in °) for Thr-Asn (top) and Thr-Gln (bottom) residue pairs from X-ray crystal structures. All histograms have 10° bin widths. The insets depict the HB angle on representative PDB structures with the corresponding Cα of the residues represented as a green sphere, and the remaining atoms are shown as sticks with carbon in gray, hydrogen in white, nitrogen in blue, and oxygen in red.
**Figure S8.** A representative protein (PDB ID: 2XJP) showing the additional stabilizing HB interactions observed in the surrounding protein environment of an N–H···O HB between Tyr and Asn (HB angle shown in black dashed lines). The protein cartoon is shown in translucent gray. The hydrogen bonding residues Tyr and Asn are shown in green sticks while the nearby residues involved in additional HB interactions with Asn and Tyr are shown in orange sticks. Oxygen, nitrogen, and hydrogen atoms are shown in red, blue, and white, respectively. Sidechain carbon atoms are shown in gray. Tyr, Asn, Pro, Cys, Val, and Ser residues are labeled with the one-letter amino acid code followed by the residue number. HBs between the Asn sidechain amide carbonyl oxygen and a nearby Val backbone N–H hydrogen, the Asn sidechain amide hydrogen and a nearby Pro backbone carbonyl oxygen, the Tyr sidechain hydroxyl hydrogen and a nearby Ser backbone carbonyl oxygen are shown in orange dashed lines.

**Figure S9.** 2D PESs depicting interaction energies ($E_{\text{int}}$ in kcal/mol, colorbar at right) of N–H···O HBs (left) and O–H···O HBs (right) in acetamide–methanol (top) and acetamide–$p$-cresol (bottom). The heavy-atom (i.e., N···O and O···O) distances (in Å) and X–H···O angles (in °) are shown as labeled on the axes, where X–H···O corresponds to N–H···O (left) or O–H···O (right). The same color scale is used for all inset PESs with 1 kcal/mol contour lines. The X-ray crystal structure distances and angles (translucent green circles) from the data set are overlaid onto the PESs for the corresponding Ser–Gln (labeled as S-Q) and Tyr–Gln (labeled as Y-Q) residue pairs.
Figure S10. 2D PESs depicting interaction energies ($E_{\text{int}}$ in kcal/mol, colorbar at right) of N–H···O HBs (left) and O–H···O HBs (right) in acetamide-methanol (top and bottom). The heavy-atom (i.e., N···O and O···O) distances (in Å) and X–H···O angles (in °) are shown as labeled on the axes, where X–H···O corresponds to N–H···O (left) or O–H···O (right). The same color scale is used for all inset PESs with 1 kcal/mol contour lines. The X-ray crystal structure distances and angles (translucent green circles) from the data set are overlaid onto the PESs for the corresponding Thr-Asn (labeled as T-N) and Thr-Gln (labeled as T-Q) residue pairs.

Table S8. Distribution of PDB structures inside and outside the first two contours of the 2D potential energy surfaces of N–H···O and O–H···O HBs for Ser-Asn, Ser-Gln, Thr-Asn, Thr-Gln, Tyr-Asn and Tyr-Gln residue pairs in the protein data set, i.e., the number of PDB structures within 2 kcal/mol of the strongest interaction energy (columns 2 and 4) and the number of PDB structures outside this energy range (columns 3 and 5). Columns 6 and 7 indicate the number of PDB structures with less favorable HB angles (i.e., between 110° and 130°) that correspond to less favorable model interaction energies due to a short N···O distance but relatively long HBD to HBA (i.e., H···O) distance. The H···O distance in these N–H···O HBs ranges from 1.81 Å to 2.75 Å, and in O–H···O HBs, it ranges from 2.03 Å to 2.61 Å.

| Residue pair | No. of N–H···O HBs inside | No. of N–H···O HBs outside | No. of O–H···O HBs inside | No. of O–H···O HBs outside | No. of N–H···O HBs with 110-130° angles | No. of O–H···O HBs with 110-130° angles |
|--------------|----------------------------|----------------------------|---------------------------|---------------------------|---------------------------------|----------------------------------|
| Ser-Asn      | 345                        | 68                         | 289                       | 24                        | 50                              | 19                               |
| Ser-Gln      | 246                        | 36                         | 159                       | 7                         | 21                              | 7                                |
| Thr-Asn      | 386                        | 51                         | 367                       | 8                         | 43                              | 7                                |
| Thr-Gln      | 278                        | 47                         | 187                       | 8                         | 35                              | 9                                |
| Tyr-Asn      | 190                        | 84                         | 186                       | 34                        | 32                              | 6                                |
| Tyr-Gln      | 129                        | 58                         | 138                       | 24                        | 14                              | 4                                |
Figure S11. A representative protein (PDB ID: 4F1V) showing the additional stabilizing HB interactions observed in the surrounding protein environment of the N–H⋯O HB between Ser and Asn. The protein cartoon is shown in translucent gray. The hydrogen bonding residues Ser and Asn are shown in green sticks while the nearby Asp residue involved in HB interaction with Ser is shown in orange sticks. Oxygen, nitrogen, and hydrogen atoms are shown in red, blue, and white, respectively. Sidechain carbon atoms are shown in gray. The N⋯O HB distance and N–H⋯O HB angle are shown in black dashed lines. The N⋯O and H⋯O HB distances are 2.99 Å and 2.46 Å, respectively, and the N–H⋯O HB angle is 113.1°. Ser, Asn, and Asp residues are labeled with the one-letter amino acid code followed by their residue numbers. Orange dashed lines are shown for the HB between Asn sidechain amide hydrogen and Ser backbone carbonyl oxygen, HB between Ser sidechain hydroxyl hydrogen, and a nearby Asp sidechain carboxylate oxygen. Green dashed lines are shown for the HB of an Asn sidechain amide hydrogen atom with a solvent molecule, a HB between a Ser sidechain hydroxyl oxygen and the solvent molecule, and a HB between an Asp sidechain carboxylate oxygen with the solvent molecule.

Table S9. Mean O–H⋯O angle (in °) and standard deviation (in °) of O–H⋯O angle of O–H⋯O HBs in Ser-Asn, Ser-Gln, Thr-Asn, Thr-Gln, Tyr-Asn, and Tyr-Gln residue pairs in the protein data set.

| Residue pair | Mean O–H⋯O HB angle | Standard deviation |
|--------------|----------------------|--------------------|
| Ser-Asn      | 163.7°               | 15.4°              |
| Ser-Gln      | 161.5°               | 14.8°              |
| Thr-Asn      | 165.6°               | 13.0°              |
| Thr-Gln      | 163.5°               | 14.8°              |
| Tyr-Asn      | 168.3°               | 11.2°              |
| Tyr-Gln      | 168.0°               | 12.4°              |
Table S10. Number of N–H···O HBs, O–H···O HBs, and ambifunctional HBs in Ser-Asn, Ser-Gln, Thr-Asn, Thr-Gln, Tyr-Asn, and Tyr-Gln residue pairs in the protein data set.

| Residue pair | No. of N–H···O HBs | No. of O–H···O HBs | No. of ambifunctional HBs |
|--------------|---------------------|---------------------|---------------------------|
| Ser-Asn      | 413                 | 313                 | 130                       |
| Ser-Gln      | 282                 | 166                 | 78                        |
| Thr-Asn      | 437                 | 375                 | 176                       |
| Thr-Gln      | 325                 | 195                 | 62                        |
| Tyr-Asn      | 274                 | 220                 | 68                        |
| Tyr-Gln      | 187                 | 162                 | 45                        |

Figure S12. Representative proteins showing the HB interactions observed in the surrounding protein environment of N–H···O (left, PDB ID: 3VLA) and O–H···O (right, PDB ID: 3ZOJ) HBs between Ser and Asn. The protein cartoon is shown in translucent gray. The hydrogen bonding residues Ser and Asn are shown in green sticks while the nearby residues involved in HB interactions are shown in orange sticks. Oxygen atoms are shown in red, nitrogen in blue, sidechain carbon atoms in gray, and hydrogen atoms are shown in white. Ser, Asn, and Ile residues are labeled with the one-letter amino acid code followed by their residue numbers. In addition to the N–H···O HB between Ser and Asn (left, shown with black dashed lines), the sidechain hydroxyl of Ser interacts with two nearby backbone N–H (shown in orange dashed lines) and three nearby solvent molecules (shown in green dashed lines), while the sidechain amide oxygen of Asn interacts with two nearby solvent molecules (shown in green dashed lines). In addition to the O–H···O HB between Ser and Asn (right, shown with green dashed lines), there is only one other interaction of sidechain hydroxyl oxygen of Ser with a nearby solvent molecule (shown in green dashed lines).
Figure S13. A representative protein (PDB ID: 1XMK) showing the presence of two simultaneous N–H···O HBs of Asn with Ser (trans N–H···O HB) and Tyr (cis N–H···O HB) residues (shown in black dashed lines). The protein cartoon is shown in translucent gray. The hydrogen bonding residues Ser, Tyr and Asn are shown in green sticks while the nearby Ser (S314) residue involved in a HB interaction with Ser (S313) is shown in orange sticks. Oxygen atoms are shown in red, nitrogen atoms in blue, and sidechain carbon atoms are shown in gray. Ser, Asn, and Tyr residues are labeled with the one-letter amino acid code followed by their residue numbers. Green dashed lines represent the HB interactions of the Asn sidechain amide oxygen with three nearby solvent molecules. Orange dashed lines represent the HB interactions of sidechain hydroxyl group of Ser with nearby backbone nitrogen and sidechain hydroxyl oxygen atoms.

Table S11. O···O and N···O HB distances observed in ambifunctional HBs of acetamide–methanol and acetamide–p-cresol and the corresponding O–H···O and N–H···O HB angles, compared alongside the O–H···O and N–H···O HB interaction energies in the respective single O–H···O and N–H···O HBs, obtained from the optimized geometries on their 1D PECs.

|                | O···O dist. (Å) | N···O dist. (Å) | O–H···O angle (°) | N–H···O angle (°) | $E_{\text{int}}$ O–H···O (kcal/mol) | $E_{\text{int}}$ N–H···O (kcal/mol) |
|----------------|----------------|----------------|------------------|------------------|---------------------------------|---------------------------------|
| **Acetamide–methanol** | 2.81           | 2.88           | 162.2            | 171.1            | -7.9                            | -7.0                            |
| **Acetamide–p-cresol** | 2.76           | 2.94           | 166.3            | 176.5            | -11.1                           | -6.0                            |
Table S12. Comparison of zero point vibrational energies (ZPE in units of kcal/mol), ZPE + total thermal correction (E(ZPE) + E(trans) + E(rot) + E(vib)) to the electronic energy (E_{el}), entropic contribution (TS in units of kcal/mol, where T = 298.15 K), and the energy required to transform E_{el} into Gibbs free energy (G - E_{el}), i.e., E(ZPE) + E(trans) + E(rot) + E(vib) + k_{B}T, where k_{B}T is the thermal enthalpy correction with a value of 0.6 kcal/mol, for all four acetamide–methanol and acetamide–p-cresol HB configurations obtained using MP2/6-31G* optimized geometries evaluated at the MP2/6-31G* level of theory. We also report the net effect of G - E_{el} relative to cis N–H···O energies that Gibbs free energy corrections are expected to have on electronic energies.

| HB configuration | ZPE (kcal/mol) | ZPE + total thermal correction (kcal/mol) | Entropic contribution (TS) (kcal/mol) | ZPE+total thermal + enthalpy – entropic corrections (kcal/mol) |
|------------------|----------------|------------------------------------------|--------------------------------------|---------------------------------------------------------------|
| acetamide–methanol |               |                                          |                                      |                                                               |
| cis N–H···O      | 81.4          | 87.0                                    | 27.0                                 | 60.6(0.0)                                                      |
| trans N–H···O    | 81.4          | 87.1                                    | 27.4                                 | 60.3(-0.3)                                                     |
| O–H···O          | 81.6          | 87.1                                    | 26.9                                 | 60.8(0.2)                                                      |
| ambifunctional   | 82.2          | 88.0                                    | 27.3                                 | 61.3(0.7)                                                      |
| acetamide–p-cresol |            |                                          |                                      |                                                               |
| cis N–H···O      | 131.2         | 139.9                                   | 34.8                                 | 105.7(0.0)                                                     |
| trans N–H···O    | 131.5         | 140.6                                   | 35.1                                 | 106.1(0.4)                                                     |
| O–H···O          | 132.0         | 140.9                                   | 34.5                                 | 107.0(1.3)                                                     |
| ambifunctional   | 132.3         | 141.0                                   | 34.4                                 | 107.2(1.5)                                                     |

Table S13. Total energetic penalty (ΔE_{total}) in ambifunctional HBs due to shorter HB distances and smaller HB angles. The energetic cost due to shorter HB distances in ambifunctional HBs was evaluated using energies of single HBs with HB distances observed in ambifunctional HBs but near-linear HB angles observed in freely optimized geometries of single HBs, obtained from the 2D energy surfaces (E_{dist}, column 3). We then evaluated the difference in energies between E_{dist} and the energies of freely optimized single HBs (E_{single}, column 2) in both the model systems. We used a similar approach to evaluate the penalty related to smaller HB angles, i.e., we obtained energies of single HBs with HB angles observed in ambifunctional HBs but HB distances observed in freely optimized single HBs from the 2D contours (E_{angle}, column 5). We then evaluated the difference in energies between E_{angle} and the energies of single HBs in both the model systems.

| HB    | E_{single} (kcal/mol) | E_{dist} (kcal/mol) | Dist. (Å), angle (°) of E_{dist} | E_{angle} (kcal/mol) | Dist. (Å), angle (°) of E_{angle} | ΔE_{dist} (kcal/mol) | ΔE_{angle} (kcal/mol) | ΔE_{total} (kcal/mol) |
|-------|-----------------------|--------------------|---------------------------------|---------------------|---------------------------------|---------------------|----------------------|-----------------------|
| Acetamide–methanol |            |                    |                                 |                     |                                 |                     |                      |                       |
| N–H···O | -6.9                  | -6.9               | 2.90, 170                       | -5.6                | 3.00, 140                       | 0.0                 | 1.3                  | 1.5                   |
| O–H···O | -7.9                  | -7.9               | 2.80, 165                       | -7.7                | 2.85, 155                       | 0.0                 | 0.2                  |                       |
| Acetamide–p-cresol |            |                    |                                 |                     |                                 |                     |                      |                       |
| N–H···O | -6.1                  | -6.0               | 2.95, 180                       | -4.9                | 3.05, 140                       | 0.1                 | 1.2                  | 1.5                   |
| O–H···O | -11.0                 | -11.1              | 2.75, 165                       | -10.7               | 2.80, 155                       | -0.1                | 0.3                  |                       |
Table S14. Comparison of generalized amber force field (GAFF)\(^{13}\) and DLPNO-CCSD(T)/CBS (using the default Tight PNO thresholds of TCutPairs = 10\(^{-5}\), TCutPNO = 1.00 x 10\(^{-7}\), TCutMKN = 10\(^{-3}\)) energetics for select intermediates along the reaction coordinate: the cis N–H⋯O, O–H⋯·O, ambifunctional (ambi) intermediates, the barrier between the ambifunctional and O–H⋯·O configuration, and the difference between the ambifunctional and O–H⋯·O configurations as indicated as well as the relative stabilization of \(p\)-cresol (p) versus methanol (m) as indicated in the legends in the table. All energies are listed in kcal/mol.

| HB configuration | GAFF (kcal/mol) | DLPNO-CCSD(T)/CBS (kcal/mol) |
|-----------------|----------------|-------------------------------|
| p-cresol | methanol | diff | p-cresol | methanol | diff |
| cis N–H⋯O (1) | -4.2 | -7.1 | -2.9 | -6.1 | -7.1 | -1.0 |
| O–H⋯·O (2) | -9.1 | -8.5 | 0.6 | -11.0 | -8.0 | 3.0 |
| ambi (3) | -10.3 | -10.2 | 0.1 | -12.2 | -10.9 | 1.3 |
| barrier | -8.7 | -7.8 | 0.9 | -8.6 | -6.2 | 2.4 |
| (3) – (2) | -1.2 | -1.7 | -0.5 | -1.2 | -2.9 | -1.7 |

Text S4. Details of reaction coordinate construction and transformation.

The (H)O⋯·C=N angle was selected by trial and error to construct a reaction coordinate (ESI Figure S14). This angle was then varied in increments of 0.1° such that methanol or \(p\)-cresol rotates around acetamide resulting in singly- (single HBs) and doubly-hydrogen-bonded (ambifunctional HB) conformations (Figure S14). Constrained optimizations were carried out on the resulting structures at the MP2/6-31G* level of theory by constraining the (H)O⋯·C-N angle that accounts for simultaneous rotation and translation of methanol or \(p\)-cresol along with the O⋯·O=C-N dihedral angle to prevent unphysical orientation of the molecules. The geometry optimizations were repeated on most of the structures until converged results were obtained. Single points were then computed on the converged geometries at the DLPNO-CCSD(T)/CBS level of theory using Tight PNO thresholds. Here, Tight refers to default thresholds of TCutPairs = 10\(^{-5}\), TCutPNO = 1.00 x 10\(^{-7}\), TCutMKN = 10\(^{-3}\).

We then measured the N–H⋯·O angles in all the optimized conformations sampled along the reaction coordinate for both the model systems. The N–H⋯·O angles increase from one conformation to another up to 180°, after which the smaller of [N–H⋯·O angle, 360° - N–H⋯·O angle] is reported. We then obtained potential energy surfaces of the interaction energies of our model systems as a function of the N–H⋯·O angle (ESI Figure S15).
Figure S14. (a) Trajectory of the acetamide–methanol system with respect to reaction coordinate, (H)O···C=N intermolecular angle colored by progress along the reaction coordinate (from red to blue). (b) (H)O···C-N reaction coordinate angle on a single representative structure with atoms colored as C in gray, O in red, N in blue, and H in white.

Figure S15. The translated reaction coordinate, N–H···O angle, in (a) the acetamide–methanol model system and (b) the acetamide–p-cresol model system. Oxygen, nitrogen, carbon, and hydrogen atoms are shown in red, blue, gray, and white, respectively.
Figure S16. O–H···O angle vs N–H···O angle in (a) acetamide–methanol and (b) acetamide–p-cresol model systems. The black line in each plot indicates the linear fit through the plot while the red circles are the data points. The slope of the linear fit for acetamide–methanol is -0.99 and for acetamide–p-cresol, it is -0.70. Discontinuous data points were pruned in the plots.

Table S15. DLPNO-CCSD(T)/CBS energies of O–H···O HB and the transition state corresponding to its transition to ambifunctional HB for acetamide–methanol and acetamide–p-cresol model systems. This transition state is qualitative in nature and represents the maximum energy point along this minimum energy pathway obtained through constrained optimizations.

| Model system        | O–H···O HB energy (kcal/mol) | Transition state energy (kcal/mol) |
|---------------------|------------------------------|------------------------------------|
| Acetamide–methanol  | -7.9                         | -6.2                               |
| Acetamide–p-cresol  | -11.0                        | -8.7                               |

Table S16. Comparison of different components of symmetry-adapted perturbation theory (SAPT) energies (in kcal/mol) obtained using MP2/6-31G*-optimized geometries for all four acetamide–methanol and acetamide–p-cresol HB configurations and also the HB configurations at the top of the energy barriers in the reaction coordinate (RC) plots evaluated at SAPT2+3/aug-cc-pVTZ level of theory, as implemented in Psi4.

| HB configuration       | Electrostatics (kcal/mol) | Exchange (kcal/mol) | Induction (kcal/mol) | Dispersion (kcal/mol) | Total SAPT2+3 (Col. 1+2+3+4, in kcal/mol) |
|------------------------|---------------------------|---------------------|----------------------|-----------------------|---------------------------------------------|
|                        | acetamide–methanol        |                     |                      |                       |                                             |
| cis N–H···O            | -10.2                     | 11.3                | -3.7                 | -4.9                  | -7.5                                        |
| trans N–H···O          | -8.3                      | 8.5                 | -2.5                 | -4.2                  | -6.5                                        |
| O–H···O                | -12.6                     | 14.3                | -4.5                 | -5.6                  | -8.4                                        |
| ambifunctional         | -19.2                     | 22.1                | -7.0                 | -7.3                  | -11.4                                       |
| HB at RC energy barrier| -7.6                      | 7.2                 | -2.6                 | -3.4                  | -6.4                                        |
|                        | acetamide–p-cresol        |                     |                      |                       |                                             |
| cis N–H···O            | -8.4                      | 9.8                 | -3.1                 | -5.0                  | -6.7                                        |
| trans N–H···O          | -7.1                      | 8.6                 | -2.0                 | -5.7                  | -6.2                                        |
| O–H···O                | -16.0                     | 18.7                | -6.4                 | -8.4                  | -12.1                                       |
| ambifunctional         | -20.2                     | 23.5                | -8.2                 | -8.7                  | -13.6                                       |
| HB at RC energy barrier| -9.9                      | 9.8                 | -4.0                 | -4.9                  | -9.0                                        |
Figure S17. Interaction energies ($E_{\text{int}}$, in kcal/mol) obtained from the Generalized Amber Force Field (GAFF) of HB conformations shown (red dots) as a function of N–H⋯O HB angle (in °) and a corresponding 10-point running average (gray line) for (top) acetamide–methanol and (bottom) acetamide–$p$-cresol. Representative structures with measured O–H⋯O HB angles are shown for the O–H⋯O HB (top left inset), cis N–H⋯O HB (top right inset), and ambifunctional HB (bottom inset) with the relevant O–H⋯O HB angle annotated in black. The value of the N–H⋯O HB angle can be read from the x-axis. Discontinuous data points were pruned in the plots. The $p$-cresol points have a greater number of discontinuities due to changes in the unconstrained degrees of freedom.
Figure S18. Plots of (a) O–H···O angle (in °) vs N–H···O angle (in °), (b) O···O distance (in Å) vs N···O distance (in Å), (c) O···O distance (in Å) vs N–H···O angle (in °) and (d) N···O distance (in Å) vs N–H···O angle (in °) observed in structures in the ambifunctional HB basin of acetamide–methanol model system. (a) The vertical and horizontal gray lines indicate the N–H···O and O–H···O angles observed in the most stable ambifunctional HB, respectively. The data points to the left of the vertical gray line indicate structures from the transition state (connecting O–H···O HB and ambifunctional HB) moving towards the ambifunctional HB configuration while those to the right indicate the structural rearrangements from N–H···O HB to the ambifunctional HB. Two insets are shown, where one exhibits a strong O–H···O HB but a weak N–H···O HB (left) while the other shows the reverse trend (right). (b) The vertical and horizontal gray lines indicate the N···O and O···O distances observed in the most stable ambifunctional HB, respectively. The data points to the left of the vertical gray line indicate the structural rearrangements from N–H···O HB to the ambifunctional HB while those to the right are structures from the transition state (connecting O–H···O HB and ambifunctional HB) moving towards the ambifunctional HB configuration (i.e., the reverse of the orientation on the left plot). Two insets are shown, where one exhibits a strong N–H···O HB but a weak O–H···O HB (left) while the other shows the reverse trend (right). (c) The vertical and horizontal gray lines indicate the N···O angle and O···O distance observed in the most stable ambifunctional HB, respectively. (d) The vertical and horizontal gray lines indicate the N–H···O angle and N···O distance observed in the most stable ambifunctional HB, respectively. For both (c) and (d), the data points to the left of the vertical gray line indicate structures from the transition state (connecting O–H···O HB and ambifunctional HB) moving towards the ambifunctional HB configuration while those to the right indicate the structural rearrangements from N–H···O HB to the ambifunctional HB. Two insets are shown, where one exhibits a strong O–H···O HB but a weak N–H···O HB (left) while the other shows the reverse trend (right). Discontinuous data points were pruned in the plots.
Figure S19. Plots of (a) O–H···O angle (in °) vs N–H···O angle (in °), (b) O···O distance (in Å) vs N···O distance (in Å), (c) O···O distance (in Å) vs N–H···O angle (in °) and (d) N···O distance (in Å) vs N–H···O angle (in °) observed in structures in the ambifunctional HB basin of acetamide–p-cresol model system. (a) The vertical and horizontal gray lines indicate the N–H···O and O–H···O angles observed in the most stable ambifunctional HB, respectively. The data points to the left of the vertical gray line indicate structures from the transition state (connecting O–H···O HB and ambifunctional HB) moving towards the ambifunctional HB configuration while those to the right indicate the structural rearrangements from N–H···O HB to the ambifunctional HB. Two insets are shown, where one exhibits a strong O–H···O HB but a weak N–H···O HB (left) while the other shows the reverse trend (right). (b) The vertical and horizontal gray lines indicate the N···O and O···O distances observed in the most stable ambifunctional HB, respectively. The data points to the left of the vertical gray line indicate the structural rearrangements from N–H···O HB to the ambifunctional HB while those to the right are structures from the transition state (connecting O–H···O HB and ambifunctional HB) moving towards the ambifunctional HB configuration (i.e., the reverse of the left plot). Two insets are shown, where one exhibits a strong N–H···O HB but a weak O–H···O HB (left) while the other shows the reverse trend (right). (c) The vertical and horizontal gray lines indicate the N–H···O angle and O···O distance observed in the most stable ambifunctional HB, respectively. (d) The vertical and horizontal gray lines indicate the N–H···O angle and N···O distance observed in the most stable ambifunctional HB, respectively. For both (c) and (d), the data points to the left of the vertical gray line indicate structures from the transition state (connecting O–H···O HB and ambifunctional HB) moving towards the ambifunctional HB configuration while those to the right indicate the structural rearrangements from N–H···O HB to the ambifunctional HB. Two insets are shown, where one exhibits a strong O–H···O HB but a weak N–H···O HB (left) while the other shows the reverse trend (right). Discontinuous data points were pruned in the plots.
Figure S20. Plots showing the sum of HB distances as a function of N–H⋯O HB angle for the structures in the ambifunctional HB basin for (a) acetamide–methanol and (b) acetamide–p-cresol model systems. The data point corresponding to the most stable ambifunctional HB is circled in black. The gray vertical line indicates the N–H⋯O HB angle in the most stable ambifunctional HB. Discontinuous data points were pruned in the plots.
**Figure S21.** Normalized 2D histograms of O···O HB distance \((d(O\cdots O)\text{ in Å})\) vs. N···O HB distance \((d(N\cdots O)\text{ in Å})\) of residue pairs in high-resolution crystal structures from our data set that we classify as forming ambifunctional HBs, and the normalized frequency is colored according to the colorbar. The green rectangular box indicates the O···O and N···O HB distance ranges over which the strongest ambifunctional HBs are observed. The residue pairs shown are Ser-Asn (top left), Ser-Gln (top right), Thr-Asn (middle left), Thr-Gln (middle right), Tyr-Asn (bottom left), and Tyr-Gln (bottom right). The bin width along each axis is 0.1 Å.
Figure S22. Normalized 2D histograms (frequency in colorbar at right) of $O\cdots O$ HB distance ($d(O\cdots O)$ in Å) vs $N\cdots O$ HB distance ($d(N\cdots O)$ in Å) of all residue pairs in with single HBs in the high-resolution crystal structure data set. The green square indicates the singly hydrogen bonded $N-H\cdots O$ HBs while the orange square indicates the singly hydrogen bonded $O-H\cdots O$ HBs. The residue pairs shown are Ser-Asn/Gln (top), Thr-Asn/Gln (middle), and Tyr-Asn/Gln (bottom). The residue counts of both singly hydrogen bonded $N-H\cdots O$ and $O-H\cdots O$ HBs (N) are shown in the top right corners of the plots. The bin width along each axis is 0.1 Å.
**Table S17.** Number of extremely strong ambifunctional HBs (column 1), moderately strong ambifunctional HBs (column 2), and total number of ambifunctional HBs (column 3 = column 1 + column 2) in Ser-Asn, Ser-Gln, Thr-Asn, Thr-Gln, Tyr-Asn, and Tyr-Gln residue pairs in the protein data set. The distance criteria for extremely strong ambifunctional HBs is: N···O HB distance ranges between 2.5 Å and 3.2 Å for Ser/Thr/Tyr-Asn/Gln systems while O···O HB distance ranges from 2.4–3.1 Å (3.2 Å) for Ser/Thr-Asn/Gln (Tyr-Asn/Gln) systems, respectively. Moderately strong HBs comprise of one of the HB distances within the above-mentioned range while the other HB distance is outside this range.

| Residue pair | Extremely strong | Moderately strong | Total |
|--------------|------------------|-------------------|-------|
| Ser-Asn      | 5                | 125               | 130   |
| Ser-Gln      | 9                | 69                | 78    |
| Thr-Asn      | 14               | 162               | 176   |
| Thr-Gln      | 11               | 51                | 62    |
| Tyr-Asn      | 4                | 64                | 68    |
| Tyr-Gln      | 6                | 39                | 45    |
Figure S23. (Left) Normalized 2D histograms of O–H···O HB angle (in °) vs N–H···O HB angle (in °) of residue pairs that show ambifunctional HBs in the high-resolution crystal structure data set. The green square indicates the most favorable O–H···O and N–H···O HB angles. The orange rectangle indicates the regions with most favorable O–H···O HB angles but poor N–H···O HB angles while the red rectangle indicates the regions with most favorable N–H···O HB angles but poor O–H···O HB angles. (Right) Normalized 2D histograms of O–H···O HB angle (in °) vs. N–H···O HB angle (in °) of residue pairs that show strong ambifunctional HBs with both moderate O···O and N···O HB distances in the high-resolution crystal structure data set. On both the left and the right, the residue pairs shown are Ser-Asn/Gln (top), Thr-Asn/Gln (middle), and Tyr-Asn/Gln (bottom). The residue counts (N) are shown in the bottom left corners of the plots. The bin width along each axis is 10°, and the frequency is indicated by the colorbar to the right of each graph.
Table S18. Mean N–H⋯O angle and O–H⋯O angle of all ambifunctional HBs (columns 2 and 3), strong ambifunctional HBs with both moderate HB distances (columns 4 and 5), and moderately strong ambifunctional HBs with one moderate and the other longer HB distance (columns 6 and 7) in Ser-Asn, Ser-Gln, Thr-Asn, Thr-Gln, Tyr-Asn, and Tyr-Gln residue pairs in the protein data set.

| Residue pair | Mean N–H⋯O HB angle | Mean O–H⋯O HB angle | Mean N–H⋯O HB angle (strong ambi.) | Mean O–H⋯O HB angle (strong ambi.) | Mean N–H⋯O HB angle (moderately strong ambi.) | Mean O–H⋯O HB angle (moderately strong ambi.) |
|--------------|---------------------|---------------------|-----------------------------------|-----------------------------------|-----------------------------------------------|-----------------------------------------------|
| Ser-Asn      | 135.8°              | 157.3°              | 126.2°                            | 146.0°                            | 136.2°                                        | 157.7°                                        |
| Ser-Gln      | 136.4°              | 154.4°              | 118.2°                            | 145.4°                            | 138.8°                                        | 155.5°                                        |
| Thr-Asn      | 131.3°              | 160.5°              | 128.5°                            | 163.7°                            | 131.5°                                        | 160.2°                                        |
| Thr-Gln      | 135.3°              | 159.0°              | 130.7°                            | 152.3°                            | 136.3°                                        | 160.4°                                        |
| Tyr-Asn      | 131.7°              | 156.7°              | 129.0°                            | 155.6°                            | 131.8°                                        | 156.8°                                        |
| Tyr-Gln      | 127.1°              | 158.8°              | 130.0°                            | 159.6°                            | 126.6°                                        | 158.7°                                        |

Table S19. Interaction energies (Int. E, in kcal/mol) obtained from DLPNO-CCSD(T)/CBS calculations using the MP2/6-31G*-optimized geometries of ambifunctional HB configurations of formamide–methanol, acetamide–methanol, propanamide–methanol, and acetamide–ethanol. The interaction energy is obtained as the difference in energy of the dimer from the isolated molecules, all evaluated on MP2/6-31G*-optimized geometries. The two-point extrapolation formula based on the aug-cc-pVDZ and aug-cc-pVTZ energies is used to extrapolate to the complete basis set (CBS) limit following Refs. 6-8 for DLPNO-CCSD(T). Here, Tight refers to default thresholds of TCutPairs = 10\(^{-5}\), TCutPNO = 1.00 \times 10^{-7}, TCutMKN = 10\(^{-3}\). The interaction energies of models where Asn/Gln is modeled by formamide, acetamide, and propanamide differ by no more than 0.34 kcal/mol. The interaction energies of models where Ser/Thr is modeled by methanol and ethanol differ by no more than 0.18 kcal/mol. While formamide is computationally cheaper to model than acetamide and propanamide, the latter would be more accurate for modeling Asn or Gln. Propanamide is computationally expensive and gives fairly comparable results to acetamide (within 0.06 kcal/mol), and hence, acetamide has been used to model both Asn and Gln.

| Representative Models | Int. E (kcal/mol) |
|-----------------------|-------------------|
| Formamide–methanol ambifunctional HB | -10.30 |
| Acetamide–methanol ambifunctional HB | -10.64 |
| Propanamide–methanol ambifunctional HB | -10.58 |
| Acetamide–ethanol ambifunctional HB | -10.82 |

Table S20. Key distances (in Å) and angles (in °) in MP2-optimized geometries of an acetamide–methanol ambifunctional HB configuration using different basis sets.

| Basis       | O–H⋯O=O (Å) | H⋯O⋯H–N (Å) | O⋯O (Å) | N⋯O (Å) | ∠OH⋯O (°) | ∠NH⋯O (°) |
|-------------|-------------|-------------|---------|---------|-----------|-----------|
| 6-31G*      | 1.90        | 2.01        | 2.81    | 2.88    | 152.6     | 141.8     |
| 6-31++G**   | 1.90        | 2.02        | 2.80    | 2.88    | 150.5     | 140.5     |
| cc-pVDZ     | 1.85        | 1.98        | 2.77    | 2.85    | 153.9     | 140.7     |
| cc-pVTZ     | 1.83        | 1.99        | 2.75    | 2.85    | 155.7     | 140.1     |
| aug-cc-pVDZ | 1.87        | 2.01        | 2.78    | 2.88    | 154.1     | 140.5     |
| aug-cc-pVTZ | 1.84        | 2.01        | 2.76    | 2.86    | 154.7     | 140.1     |
Table S21. Comparison of interaction energies (Int. E, in kcal/mol) obtained using MP2/6-31G*-optimized geometry or B3LYP-D3/6-31G*-optimized geometries of the acetamide-methanol ambifunctional HB configuration evaluated at several levels of theory. The CBS limit was extrapolated based on a two-point formula. Differences in structures lead to differences in single point energy-evaluated interaction energies of no more than 0.1 kcal/mol for each level of theory.

| Method/Basis                          | B3LYP-D3 struct. Int. E (kcal/mol) | MP2 struct. Int. E (kcal/mol) | Col. 1-2 (kcal/mol) |
|---------------------------------------|-----------------------------------|-------------------------------|---------------------|
| DLPNO-CCSD(T) / aug-cc-pVDZ (Normal)  | -10.6                             | -10.7                         | 0.1                 |
| DLPNO-CCSD(T) / aug-cc-pVDZ (Tight)   | -10.9                             | -10.9                         | 0.0                 |
| CCSD(T) / aug-cc-pVDZ                 | -11.1                             | -11.2                         | 0.1                 |
| DLPNO-CCSD(T) / aug-cc-pVTZ (Normal)  | -10.5                             | -10.5                         | 0.0                 |
| DLPNO-CCSD(T) / aug-cc-pVTZ (Tight)   | -10.7                             | -10.7                         | 0.0                 |
| CCSD(T) / aug-cc-pVTZ                 | -10.9                             | -10.9                         | 0.0                 |
| DLPNO-CCSD(T)/CBS (Tight)             | -10.9                             | -10.9                         | 0.0                 |

Table S22. Comparison of interaction energies (Int. E, in kcal/mol) obtained using MP2/6-31G*-optimized geometries for all four acetamide–methanol and acetamide–p-cresol HB configurations evaluated at B3LYP-D3/aug-cc-pVTZ and DLPNO-CCSD(T)/aug-cc-pVTZ (Tight) levels of theory. Differences in single point energy-evaluated interaction energies of both the levels of theory are no more than 0.8 kcal/mol for each configuration. Here, Tight refers to default thresholds of TCutPairs = 10^-5, TCutPNO = 1.00 x 10^-7, TCutMKN = 10^-3.

| HB configuration | B3LYP-D3/aug-cc-pVTZ Int. E (kcal/mol) | DLPNO-CCSD(T)/aug-cc-pVTZ (Tight) Int. E (kcal/mol) | Col. 1-2 (kcal/mol) |
|------------------|---------------------------------------|-----------------------------------------------------|---------------------|
| acetamide–methanol |                                       |                                                     |                     |
| cis N–H···O      | -6.6                                  | -7.1                                                | 0.5                 |
| trans N–H···O    | -6.0                                  | -6.6                                                | 0.6                 |
| O–H···O          | -7.8                                  | -8.0                                                | 0.2                 |
| ambifunctional   | -10.5                                 | -10.7                                               | 0.2                 |
| acetamide–p-cresol |                                     |                                                     |                     |
| cis N–H···O      | -5.8                                  | -6.4                                                | 0.6                 |
| trans N–H···O    | -5.7                                  | -6.5                                                | 0.8                 |
| O–H···O          | -11.0                                 | -11.4                                               | 0.4                 |
| ambifunctional   | -12.0                                 | -12.5                                               | 0.5                 |
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