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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
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Emergence of Cooperative Glucose-Binding Networks in Adaptive Peptide Systems

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Cite This: J. Am. Chem. Soc. 2023, 145, 9800−9807
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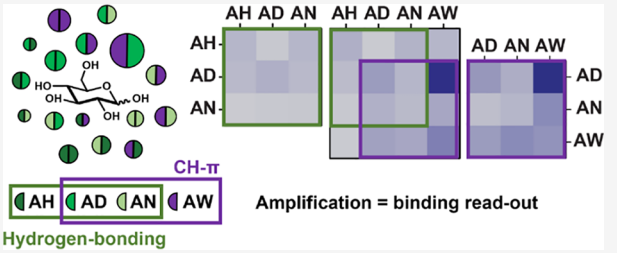
ABSTRACT: Minimalistic peptide-based systems that bind sugars in water are challenging to design due to the weakness of interactions and required cooperative contributions from specific amino-acid side chains. Here, we used a bottom-up approach to create peptide-based adaptive glucose-binding networks by mixing glucose with selected sets of input dipeptides (up to 4) in the presence of an amidase to enable in situ reversible peptide elongation, forming mixtures of up to 16 dynamically interacting tetrapeptides. The choice of input dipeptides was based on amino-acid abundance in glucose-binding sites found in the protein data bank, with side chains that can support hydrogen bonding and CH−π interactions. Tetrapeptide sequence amplification patterns, determined through LC−MS analysis, served as a readout for collective interactions and led to the identification of optimized binding networks. Systematic variation of dipeptide input revealed the emergence of two networks of non-covalent hydrogen bonding and CH−π interactions that can co-exist, are cooperative and context-dependent. A cooperative binding mode was determined by studying the binding of the most amplified tetrapeptide (AWAD) with glucose in isolation. Overall, these results demonstrate that the bottom-up design of complex systems can recreate emergent behaviors driven by covalent and non-covalent self-organization that are not observed in reductionist designs and lead to the identification of system-level cooperative binding motifs.



INTRODUCTION

Extensive studies and analyses of lectin X-ray crystal structures[1−3] have identified the key amino acids[4−6] and topological structures[7−10] in carbohydrate-binding modules which mainly rely on hydrogen bonding[11] and CH−π interactions (Figure 1A).[12,13] The ongoing study of protein−carbohydrate interactions[14,15] and underlying molecular recognition processes[16−18] have inspired the development of novel biomedical strategies[19−24] and supramolecular materials.[25−27] The multivalent mechanisms through which these interactions are strengthened highlight the need for a better understanding of the balance and combinatorial nature of carbohydrate-protein binding at a molecular (amino-acid specific) level, starting with minimalistic systems.

Several strategies have been employed in the development of water-based carbohydrate-binding peptides, including isolation of fragments[28] and reconstruction of spatially adjacent amino acids from lectin binding pockets[29] (Figure 1B), screening of peptide libraries through phage display,[30−32] and discovery of short peptide derivatives from dynamic combinatorial libraries.[33,34] Other non-peptide-based artificial designs are often developed in non-aqueous media to overcome the competition of water-like features of sugars,[35−38] with some water-based examples also reported.[39−44] The focus of these studies is usually on the identification of specific and high-affinity binders.

Systems chemistry[45−51] offers a unique approach to enhance the understanding of multi-component mixtures where combinatorial properties cannot be directly attributed to the sum of contributions from individual components in isolation. Inspired by chemical evolution through peptide-sequence exchange[52−54] and, in particular, open-ended enzyme-driven approaches to generate dynamic peptide libraries (DPLs),[55] we have developed a bottom-up approach to design and study complex adaptive systems that take advantage of self-organization and peptide-sequence exchange.[56] The composition of the system is determined through the selection of 'input' dipeptides that are expected to interact with a binding ligand or are prone to self-assembly, leading to dynamic self-organization. Further covalent and non-covalent self-organization is then enabled through enzymatic sequence (re-)combination and thermodynamic stabilization through self-assembly[57,58] or ligand binding.[59] This thermodynamic optimization process gives rise to the selection of self-assembling or binding sequences, with system-level peptide-

Received: February 13, 2023
Published: April 19, 2023

https://doi.org/10.1021/jacs.3c01620
J. Am. Chem. Soc. 2023, 145, 9800−9807

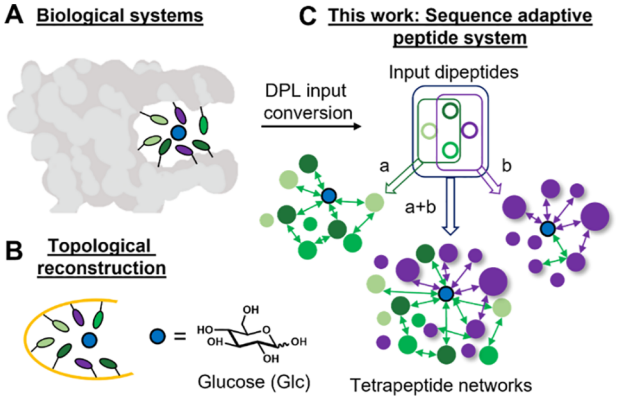


Figure 1. Comparison of Glc-binding approaches. (A) Glc-protein binding pocket depicting Glc and amino-acid side chains. (B) Topological reconstruction of binding-site amino acids. (C) Sequence-adaptive systems approach where circles represent unique peptide sequences, circle size represents their abundance, and arrows indicate interactions. Shades of green and purple represent tetrapeptides with hydrogen bonding (a) and aromatic (b) moieties, respectively, or both, giving rise to a cooperative binding network (a + b).

sequence adaptation observed when changing the conditions (e.g., changes in temperature or solvent composition).[58,59] In aqueous systems, enzymatic peptide hydrolysis is thermodynamically favored over the reverse, amide-bond formation through condensation, and the formation and amplification of longer peptide sequences are therefore only observed if they are stabilized through a binding or self-assembly advantage, therefore, peptide formation, even at low levels, serves as a proxy for stabilization through binding and aggregation.

Here, we apply the DPL approach to harness combinatorial peptide−carbohydrate interactions and study system-level adaptation through the analysis of formation of tetrapeptide sequence patterns, from mixtures of selected dipeptides, in the presence of glucose (Glc). Peptides that cooperatively interact with Glc through hydrogen bonding and/or CH−π interactions are identified, and we show that the interactions are strengthened by collective, system-level networks. Furthermore, we demonstrate that both types of interactions co-exist in cooperative networks with differential behaviors, drawing parallels to their co-existence in Glc-binding proteins (Figure 1C).

RESULTS AND DISCUSSION

We use a relatively non-specific endoprotease, *thermolysin*, to catalyze the reversible oligomerization of dipeptides (AX) (Figure 2A). The dipeptides were designed with Alanine (A) as the N-terminal amino acid to facilitate enzymatic sequence exchange with *thermolysin*, while minimizing N-terminus residue contribution to self-assembly (compared to previously

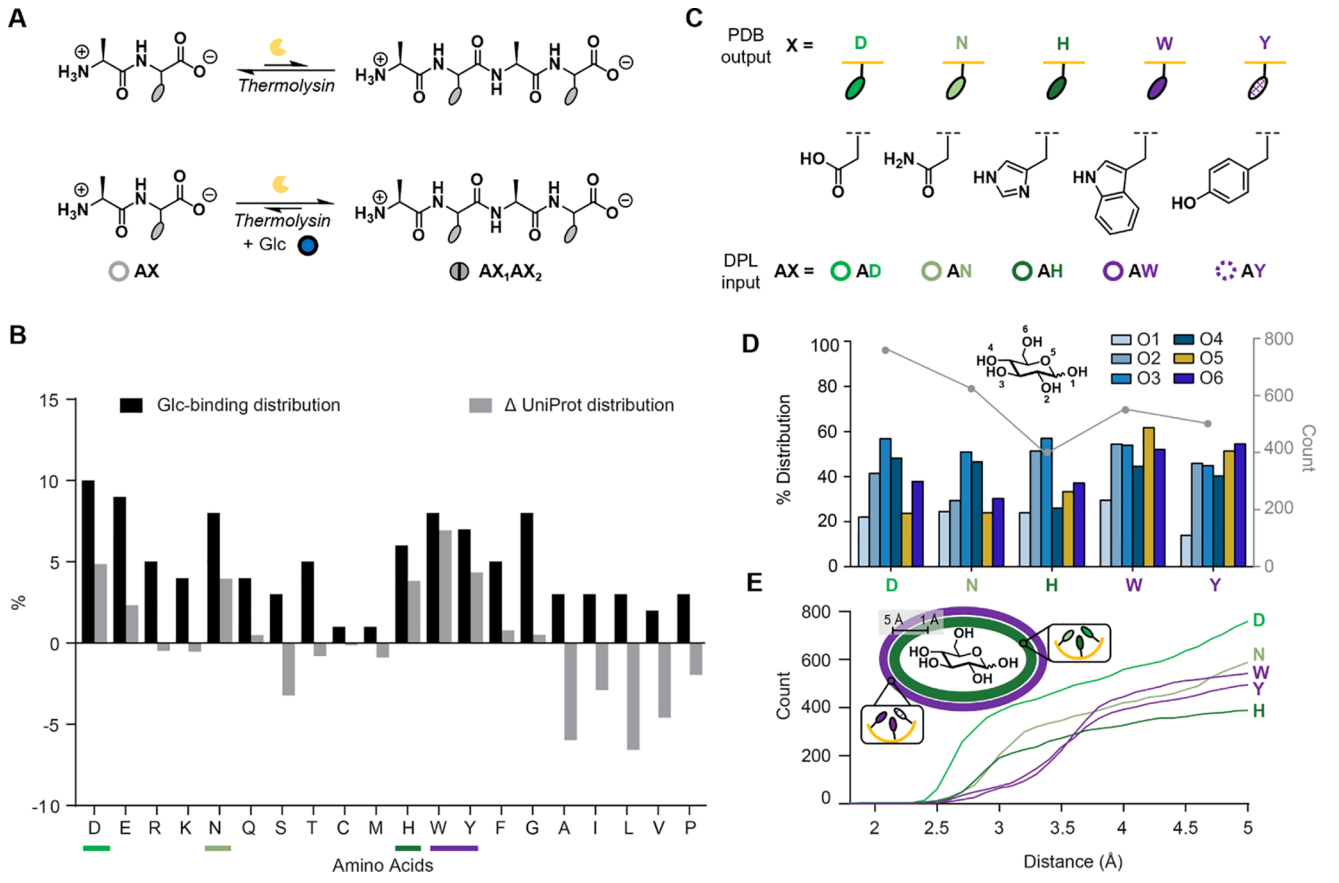


Figure 2. Dynamic peptide library input informed by PDB-mining of binding sites of Glc-binding proteins. (A) Enzyme-catalyzed peptide condensation showing equilibrium bias in the presence and absence of Glc. (B) Amino-acid distribution in Glc-binding sites and difference with general distribution (UniProt). (C). Selection of amino acids based on PDB analysis. (D) Atom-specific distribution of amino acids in Glc-binding sites (5 Å) normalized to overall amino-acid abundance at 5 Å. (E) Proximal cumulative occurrence of amino acids in Glc-binding shells and cartoon representation of spatial separation.

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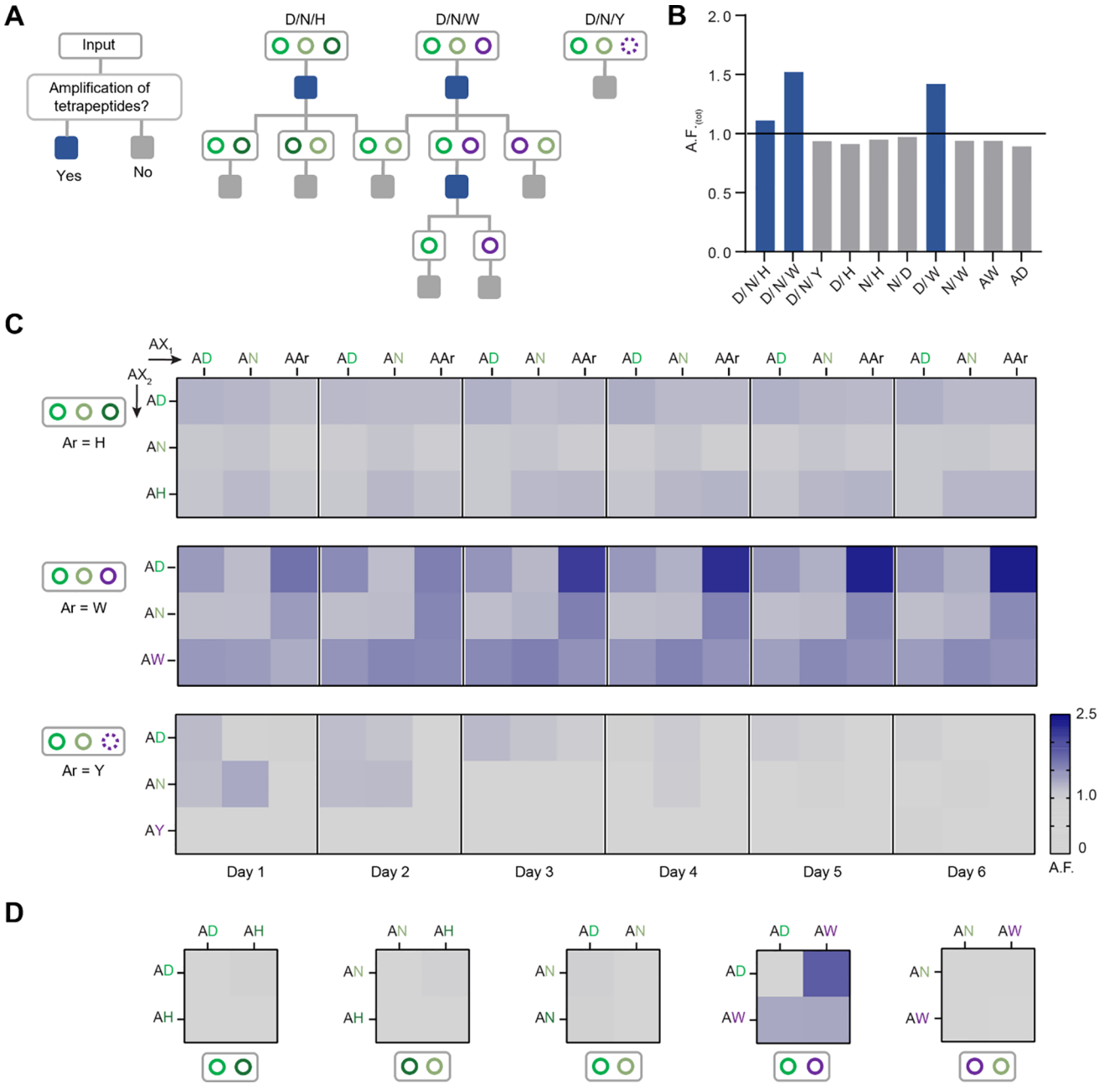

Figure 3. Amplification of tetrapeptides in Glc-containing peptide libraries. (A) Decision tree of sequential input variation depending on whether amplification is observed or not. (B) Histogram of A.F.$_{(tot)}$ in different systems. (C) Heatmaps of specific tetrapeptide A.F.s over 6 days of three component systems. (D) Heatmaps of specific tetrapeptide A.F.s of two component systems at day 6.

studied systems based on F, L, W, and V),[58,59] thus allowing the navigation of shallow potential energy landscapes without the introduction of significant structural or kinetic bias. The selection of AX as input dipeptides and the use of an endoprotease ensures that system-level adaptation arises from Glc presence through interactions with C-terminal residues (X) and consequent amplification of $(AX)_N$ motifs, with all possible tetrapeptides motifs readily tractable by liquid-chromatography-mass-spectrometry (LC−MS). The choice of Glc-interacting residues (X) was informed by amino acids that are prominent in protein-Glc binding sites found in the protein data bank (PDB) (Supporting information Section II.2). Inspired by a previous study by Kiessling and Woolfson,[17] we extracted the distribution of each of the canonical amino acids in a 5 Å radius from Glc and compared it to their general abundance in proteins (Figures 2B and S1). Residues that simultaneously have a high occurrence in proximity to Glc within these proteins, and are additionally more abundant in Glc binding sites compared to their general distribution in proteins (high Glc-binding distribution and high Δ UniProt extracted from the Universal Protein Resource (UniProt)) were selected (Figure 2C) (D, N, H, W, and Y; E was omitted for its similarity to D). The spatial proximity of each amino-acid residue with specific Glc atoms (Figure 2D) reflects their preferred sites of interactions. H, D, and N are mainly found closer to equatorial oxygens (O2, O3, and O4), emphasizing their participation in hydrogen bonding interactions. CH−π interactions can be accounted for by

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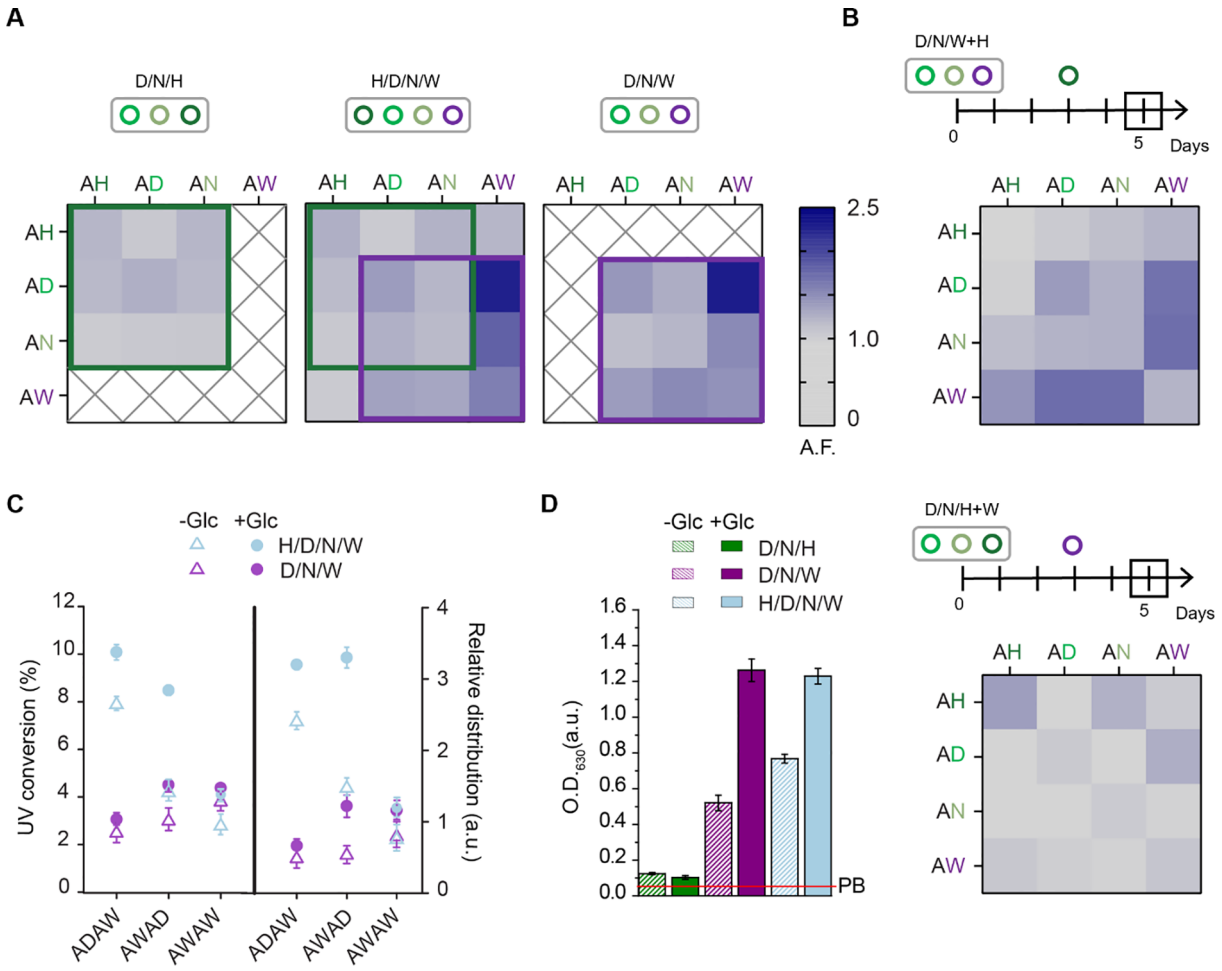

Figure 4. Context-dependent co-existence of hydrogen bonding and CH−π interactions and comparison of analytical approaches. (A) Side-by-side comparison of A.F. heatmaps in the D/N/H (re-ordered), H/D/N/W, and D/N/W systems. (B) Delayed addition of dipeptides AH and AW to an equilibrating mixture results in different compositional distribution and amplification of tetrapeptides. (C) Comparison of relative distributions and UV-determined conversions of a selection of tetrapeptides. (D) Optical density values of the D/N/H, D/N/W, and H/D/N/W system mixtures at day 6.

considering residue proximity to the ring oxygen (O5), which shows a clear preference for W and Y out of all amino acids, being the residues with the highest propensities for CH−π interactions (Figure S2).[17] This is also reflected in the Glc atom-specific distribution of W and Y residues which have a noticeably higher proximity occurrence to O5 compared to H, D, and N (Figure 2D). The accumulative abundance of these 5 amino acids as a function of distance from Glc (Figure 2E) further shows a clear distinction between H, D, and N that occur between 2.5 and 3.5 Å (hydrogen bonding distance) and aromatic residues W and Y, appearing at longer distances (3−5 Å). We hypothesized that, in a DPL containing both types of residues, the two modes of interactions would generate cooperative hydrogen bonding and/or CH−π interaction networks.

We started with mixtures of three AX dipeptides with X = D and N and either H, W, or Y to compare the roles of the aromatic residues. Concentrations of aromatic amino acids (50 mM) were kept lower than polar amino acids (100 mM) to avoid self-assembly-driven condensation from dominating the system and outweighing Glc−peptide interactions (that are expected to be much weaker). Dipeptides and *thermolysin* were mixed in phosphate buffer (PB, pH 6.5) and heated with gentle shaking at 37 °C with 0 or 200 mM Glc (Supporting Information Section II.3). Analysis by LC−MS coupled with targeted database screening (using TraceFinder) allowed the detection, separation, and identification of all produced AX_1AX_2 tetrapeptides. Amplification factors (A.F.)[60−63] based on electrospray ionization (ESI) peak areas (Supporting Information Section III), were used to evaluate the strength of Glc-binding interactions. Considering the total amplification of tetrapeptides ($A.F._{(tot)}$ from the sum of tetrapeptide peak areas), we observed moderate amplifications in the D/N/H and D/N/W systems ($A.F._{(tot)}$ = 1.13 and 1.42 respectively) while in the D/N/Y system, no increase in tetrapeptide areas occurred ($A.F._{(tot)}$ = 0.93, tetrapeptide formation disfavored). These results suggest that Glc-binding, while weak, is sufficient to enable amide equilibrium shifts toward condensation in the D/N/H and D/N/W systems. The contrasts between these three systems can be rationalized by the differences in nature and strength of the interactions involved, where we speculate that the D/N/H system is mainly driven by weak hydrogen bonds, while in the D/N/W system, CH−π interactions strengthen Glc−peptide interactions. These are expected to be weaker in the D/N/Y system,[12,13] and, in this context, are not sufficient to enable peptide elongation. To identify the minimum and essential components required for binding-induced amplification in the D/N/H and D/N/W systems, we

https://doi.org/10.1021/jacs.3c01620
J. Am. Chem. Soc. 2023, 145, 9800−9807

reduced the systems' compositions by systematically removing dipeptides from the starting mixture (Figure 3A). Reducing the D/N/H system to two components did not result in any amplification indicating a cooperative network of interacting tetrapeptides where all three complementary residues are essential. In contrast, the deconstruction of the D/N/W system showed that D/W-containing tetrapeptides (D/W system) are capable of stabilizing Glc in the absence of N residues albeit to a lesser extent ($A.F._{(tot)}$ = 1.32). Further deconstruction to individual dipeptide systems (AD and AW) did not yield amplification of the corresponding tetrapeptides (ADAD and AWAW) (Figure 1A), conclusively demonstrating that cooperativity is essential. Heatmaps of sequence-specific tetrapeptide A.F.s over time are shown in Figure 3C. Amplified tetrapeptides in the D/N/H system show a distribution around comparable A.F. values indicating that there is a collective stabilization of Glc by all tetrapeptides, with similar strengths. In the D/N/W system, a clear bias toward the amplification of W-containing sequences is observed, suggesting that the system collectively self-organizes in the presence of Glc, favoring sequences that provide stronger stabilizing interactions (CH−π). This adaptation is also sequence-specific, with AWAD being 1.6× more amplified than ADAW (A.F. = 2.3 and 1.4, respectively, Figure S4), highlighting the selection of specific residue positioning, rather than side-chain preference alone. In the D/N/Y system, initial amplification of tetrapeptides with polar residues (ADAD, ADAN, ANAD, and ANAN) can be observed but does not persist as these systems proceed toward thermodynamic equilibrium where no amplification is observed. In both the D/N/H and D/N/W systems, the amplification of these same tetrapeptides persists and amplifies over time, suggesting network cooperativity.

To determine whether CH−π interactions out-compete hydrogen bonding, we combined the input dipeptides of the D/N/H and D/N/W systems to create a H/D/N/W system, at the same concentrations of starting dipeptides, leading to 16 unique tetrapeptides. All tetrapeptide sequences that this system had in common with the three-input systems had closely comparable A.F.s (Figure S6). Consequently, the sequence-specific A.F. heatmap of the combined system appeared to be a superimposition of the individual ones (Figure 4A). The results confirm that the stabilization from hydrogen bonding and CH−π interactions are orthogonal to each other, and can occur and emerge simultaneously in two distinct networks that do not interfere or out-compete each other while maintaining their respective strengths and sequence selectivity. We note that the same effect is observed under different conditions, such as a change in pH (Figure S7) showing the robustness of their co-existence independent of conditions. To assess whether the observed amplification patterns are substrate-specific or general to carbohydrates, we studied the D/N/H, D/N/W, and H/D/N/W systems in the presence of sucrose and found that the extent of amplification decreased as compared to Glc, with changes in amplification patterns also observed (Figure S8).

The effect of compositional history on system-level adaptation was studied by the introduction of one key dipeptide (AH or AW) to the D/N/W and D/N/H mixtures, respectively, after three days of equilibration. The results show that the systems, while having the same overall chemical composition, reached different distributions reflected by their A.F. patterns (Figure 4B). Owing to the differences in interaction strength, the D/N/W network is less affected by the perturbation (addition of AH), whereas the weaker network (D/N/H), and mainly D and N containing tetrapeptides, is destabilized by the addition of AW, resulting in a very different pattern of tetrapeptide amplification. The responses clearly depend on the system's history and may therefore suggest the possibility of developing chemical systems with structural memory.

Sequence-specific, and collective amplification of tetrapeptides based on A.F., while well-suited to illustrate adaptability in response to a stabilizing event, does not provide quantitative information on the actual distribution of species in a system. To gain quantitative insights, conversions of a selection of tetrapeptides (ADAW, AWAD, and AWAW) were calculated from UV-absorbance calibrated response (Supporting Information Section III.4). The conversions to tetrapeptides are modest (2−10%, Table S1), reflecting the thermodynamic cost of amide-bond formation in water and the weak nature of the interactions. Relative distributions, obtained from ESI peak correction to account for differential ionization efficiency for each species (Supporting Information Section III.3), show similar distribution trends between sequences and reaction mixtures, with some subtle differences that can be attributed to error margins (Figure 4C). This analysis, therefore, provides an alternative approach to obtaining compositional information (Figure S9) for a comprehensive analysis of compositionally diverse complex systems.

We note that the highest yields are obtained in the system with the highest number of components (H/D/N/W). For instance, the yield of AWAD increases from 2.1 to 4.4 to 8.4% in the D/W, D/N/W and H/D/N/W systems respectively. This system-level property, whereby tetrapeptide conversion increases with initial component complexity (Table S1) can be understood in terms of an increase in overall concentration of peptide species, resulting in a more crowded and hydrophobic environment of interacting peptides that collectively stabilize the system and complexes formed. We measured the optical density of the D/N/H, D/N/W, and H/D/N/W systems at day 6 and found that the two W-containing systems have higher turbidity than the D/N/H systems, which increases further in the presence of Glc (Figure 4D). Dynamic light scattering (DLS) measurements also indicated the presence of micron-size aggregates in samples exhibiting high turbidity (Figure S10). These results underline the role of self-assembly propensity in enhancing binding interactions, presumably through more pronounced hydrophobic effect. Fluorescence spectroscopy was used to assess the extent of π-stacking driven aggregation in W-containing systems. In the presence of Glc, we observe an enhancement in fluorescence compared to systems without Glc (Figure S11), which can be interpreted as CH−π interactions competing with π−π interactions, which typically cause quenching of fluorescence, providing evidence of interactions between W side-chains and Glc.

To further confirm Glc-binding interactions in the systems, we selected the most amplified tetrapeptide (AWAD) and performed a fluorescence titration with this peptide and increasing amounts of Glc (Figure S12). A dose-dependent enhancement in fluorescence was observed, and the titration response was found to fit a 2:1 binding model.[64] Similarly, the addition of increasing amounts of Glc resulted in doubling of peptide peaks by ^{1}H-NMR (Figure S13). The diffusion coefficients of the two populations were found to be lower compared to peptide alone (1.15×10^{-10} and 1.39×10^{-10} m^2 s^{-1}, respectively, Figures S14 and S15). While these

https://doi.org/10.1021/jacs.3c01620
J. Am. Chem. Soc. 2023, 145, 9800−9807

studies were performed on an isolated system that only contained one tetrapeptide and Glc, contrasting the previously discussed dynamic heterogeneous mixtures, they demonstrate a clear cooperative mode of binding which may be anticipated to be present, and potentially further enhanced in a dynamic mixture of peptides with a more prominent hydrophobic effect. As our results demonstrate, changes in initial composition and/or removal of any peptide component (Figure 3A) have a direct effect on the systems' compositional distribution and behavior; therefore binding parameters using isolated components do not provide an accurate representation of the system.

Designed sequence-adaptive peptide systems have allowed us to identify co-existing networks of interacting tetrapeptides that simultaneously stabilize Glc through complementary and cooperative hydrogen bonding and CH−π interactions. Our approach highlights collective behaviors and identifies subtle nuances between functional amino-acid features of similar nature relevant to understanding cooperativity and residue-specific balance of carbohydrate−amino acid interactions. These insights are relevant from a fundamental biochemistry point of view, but also for future designs relevant to biomedical[19−24] and supramolecular materials[25−27] applications that require mimicry of protein−carbohydrate interactions. More generally, these systems' adaptive self-organization and history-dependent nature suggest opportunities to study molecular learning and, ultimately, the design of structural memory.[65]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.3c01620.

Supplementary figures, experimental section and analytical methodology (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding

R.V.U. and S.K.: AFoSR grant FA9550-21-1-0091 R.V.U.: Office of Naval Research Vannevar Bush Faculty Fellowship (grant N00014-21-1-2967).

Notes

The authors declare no competing financial interest.
Figure [1],A was created with BioRender.com

■ ACKNOWLEDGMENTS

R.V.U. acknowledges funding from Office of Naval Research for the Vannevar Bush Faculty Fellowship (grant N00014-21-1-2967). We thank the Air Force Office of Scientific Research (AFoSR) for funding of R.V.U., and S.K. (grant FA9550-21-1-0091). We thank Dr. Denize C. Favaro for assisting with NMR data acquisition.

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