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Deciphering the alphabet of disorder — Glu and Asp act differently on local but not global properties

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Abstract: Compared to folded proteins, the sequences of intrinsically disordered proteins (IDPs) are enriched in polar and charged amino acids. Glutamate is one of the most enriched amino acids in IDPs, while the chemically similar amino acid aspartate is less enriched. So far, the underlying functional differences of glutamates and aspartates in IDPs remain poorly understood. In this study, we examine the differential effects of aspartate and glutamates in IDPs by comparing the function and conformational ensemble of glutamate and aspartate variants of the disordered protein Dss1, using a range of assays, including interaction studies, nuclear magnetic resonance spectroscopy, small angle X-ray scattering and molecular dynamics simulation. First, we analyze the sequences of the rapidly growing data base of experimentally verified IDPs (DisProt) and show that the glutamate enrichment is not caused by a taxonomy bias in IDPs. From analyses of local and global structural properties as well as cell growth and protein-protein interactions using model acidic IDP from yeast and three Glu/Asp variants, we find that while Glu/Asp support similar function and global dimensions, the variants differ in their binding affinities and population of local transient structural elements. We speculate that these local structural differences may play roles in functional diversity where glutamates can support increased helicity important for folding and binding, while aspartates support extended structures and form helical caps, as well as playing more relevant roles in e.g., transactivation domains and ion-binding.

Keywords: Dss1; Intrinsically disordered protein; IDPs; molecular dynamics; NMR; sequence composition; SAXS.

1. Introduction

Intrinsically disordered proteins (IDPs) are involved in various cellular processes, including cell cycle regulation, cellular signalling, and protein degradation. Malfunctioning or aggregation of IDPs can cause severe diseases such as cancer, diabetes, Parkinson's disease, and Alzheimer's disease [1, 2]. IDPs are characterized by not adapting one certain spatial structure, instead fluctuating between multiple dynamic conformations, which distinguishes them from folded proteins. While the sequence-structure-function paradigm is by now well established for folded proteins, the link between sequence and function for IDPs is still poorly understood [3]. Understanding the relationship between sequence, conformational ensemble and function is important for understanding the structural basis of fundamental processes in life and for reaching treatment options for complex diseases.

The pioneering bioinformatics work in the early 2000s recognised IDPs as a separate class of proteins and showed that the amino acid sequence composition of IDPs was distinct from folded proteins. The most significant characteristics were a low content of hydrophobic residues and a high content of charged and polar residues [4, 5]. Some amino acids were found to be significantly more enriched or depleted in IDPs [6] and subsequent studies by Uversky et al from 2007 and 2013 [7, 8] found a similar enrichment. Surprisingly, glutamate is one of the most enriched amino acids in IDPs, while aspartate, which differs from glutamate only by having one less methylene group in the sidechain (Figure 1A) is not enriched. The same can be observed for the two similar amino acids glutamine and asparagine, where glutamine is more enriched in IDPs than asparagine, with no explanation for these biases having been provided. One reason for the enrichment of glutamate could be that glutamate has disorder promoting properties distinct from those of aspartate. The extra methylene group in glutamate compared to aspartate results in a larger conformational space, which could favor solvent exposed conformations over buried conformations. Glutamate is also more frequently observed in α helices than aspartate [9] and is more helix stabilizing, because the carboxyl group is further from the backbone and thus imposes fewer restraints on the conformational space of the residues in the helix [10]. Transient helicity is frequently observed in IDPs, and a higher population of transient helicity in the free state has been linked to faster binding to a target [11, 12], which could be a structural and functional explanation to the observed enrichment of glutamate. However, the analysis of sequence composition of IDPs could be biased by factors that are not related to structure. Most of the proteins in the intrinsic disorder database DisProt [13-15] are eukaryotic and more than a third of the proteins are human, while the Protein Data Bank (PDB) [16] contains many bacterial proteins. A bias in sequence composition when comparing DisProt to the PDB could therefore be because the sequence composition from eukaryotes differs from bacteria or because the human protein sequence composition is distinct from the average sequence composition. Further, since the early studies by Uversky and co-workers [7, 8], the number of experimentally verified disordered sequences deposited in DisProt has increased five times, as of July 2022.

In this work we revisit the sequence enrichment profile of IDPs using a larger disorder data set and investigate if a compositional bias arising from other factors than structural properties exist, including species variability. The analyses confirm the previous observation of more glutamates than aspartates in IDPs, and we do not find significant differences across species. To address if glutamate is more disorder promoting than aspartate, we designed Glu/Asp variants of a model IDP. Deleted in split hand/split foot 1 (Dss1) from *S. pombe* was chosen because it is a well-studied IDP with a high content of negatively charged residues, many of which are fully conserved showing preferences for either Glu or Asp, Figure 1B. Dss1 is known to have multiple interaction partners [17] and is found in various complexes, including the 26S proteasome [18] [19]. Dss1 adapts different conformations when bound in different complexes but retains disordered regions upon binding [20]. The C-terminal region adapts an α-helical conformation when bound, which also exists transiently when free in solution [21]. Dss1 binds mono ubiquitin at two binding sites exploiting a disordered ubiquitin binding motif [22] (UBS) I from D38-D49 and UBS II from D16-N25, with UBS I having the strongest affinity for ubiquitin [23]. The transient C-terminal helix interacts dynamically with UBS I, and this fold-back mechanism may function to shield the binding site and thereby regulate binding partner interaction [17]. From alignments, the sequences of both UBS I, UBS II and the C-terminal helix are highly conserved within the family (Figure 1B). By designing variants of Dss1 containing only aspartate, only glutamate or Glu/Asp swaps, we addressed the impact of Glu/Asp variants of Dss1 on its function by growth assays in yeast and by measuring ubiquitin binding. Additionally, we investigated the conformational ensembles of the Dss1 Glu/Asp using nuclear magnetic resonances (NMR) spectroscopy, small-angle X-ray scattering (SAXS) and molecular dynamics (MD) simulation. We find that Glu/Asp variants do not

impair yeast growth and that the proteins have similar global dimensions and bind ubiquitin, but that the specific Glu/Asp pattern in UBS I influences binding affinity. Additionally, the length and population of the transient C-terminal helix was found to depend on the presence of N-terminal helix capping aspartates and the presence of a glutamate in the center of the helix. We conclude that glutamate and aspartate confer different functional and structural properties in an IDP acting on a local scale, which may contribute to the observed higher enrichment of glutamate in IDPs.

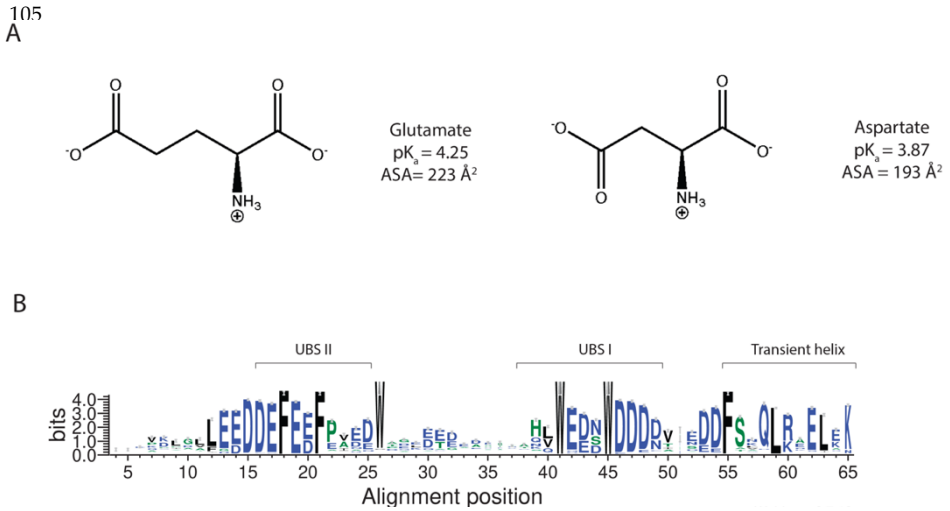

Figure 1. Glutamate and aspartate have similar chemical properties. (A) Chemical structure of the amino acids glutamate and aspartate, **(B)** graphical representation of multiple sequence alignment of the Pfam [24] Dss1/Sem1 family (PF05160), showing only the positions that are present in S. Pombe Dss1, with information content on the y-axis (for the full MSA, see supplementary Figure S1). The graphic was made using WebLogo [25].

2. Materials and Methods

Composition profiles were constructed using the web tool developed by Uversky et al, Composition Profiler [8], using 50,000 boot strap iterations and a statistical significance value of 0.05. The query dataset consisted of all non-redundant, non-ambiguous and non-obsolete sequences from DisProt v. 9.0.1 [13-15], and for the background dataset, the reviewed part of the database UniProt – UniParc (release 2022_02) [26] was used to obtain a non-redundant set of protein sequences with natural origin from the Protein Data Bank (PDB) [16].

We designed three variants of *S. pombe* Dss1 consisting of a variant with all aspartates substituted for glutamate (All-E), all glutamates substituted for aspartate (All-D), all glutamates substituted for aspartate and all aspartates substituted for glutamate (Swap) and included the wildtype (WT) for comparison. All variants carried a substitution at the C-terminal of asparagine for cysteine and codons were optimized for *E. coli* expression. Additionally, peptides corresponding to the transient helical region of Dss1 (residues 51-69) with substitutions at key positions were designed and purchased from Pepscan (US).

Yeast strains and techniques. The *dss1*Δ strain has been described before [21]. The pDUAL vector [27] was used for expression of *dss1*⁺ and the *dss1* variants carrying N-terminal HFG (6His, Flag, GFP) tags. Cloning and mutagenesis were performed by Genscript. The yeast strains were transformed using lithium acetate [28]. Growth assays were performed on Edinburgh minimal media (EMM2) (Sunrise Science Products) as described previously [21]. The preparation of cell lysate samples for SDS-PAGE was performed using trichloroacetic acid and glass beads as described previously [21]. The samples were separated by SDS-PAGE on 12.5% acrylamide gels and transferred to 0.2 µm pore-size nitrocellulose membranes (Advantec). The antibody was anti-GFP (1:1000,

Chromotek Cat# 3H9). Secondary horseradish peroxidase conjugated antibodies were from Dako Cytomation. Equal loading was checked using stain free imaging with 0.5% trichloroethanol (Sigma).

Protein purification. All four variants of *S. pombe* Dss1 were designed to encode an N-terminal His_6-SUMO tag to be cleaved with ubiquitin-like protein protease 1 (ULP1) following initial purification with a nickel column [29]. All four variants were purified with isotope labelling as described in previous work [30], resulting in lyophilized pure protein, ready for resuspension in the buffer of choice. His_6-SUMO Ubiquitin was purified for protein interaction studies with Dss1, similarly to what has been described in previous work [31].

NMR assignment. Assignments of Dss1 variants were done from a series of ^{1}H-^{15}N HSQC, HNCACB, HN(CO)CACB, HNCO, HN(CA)CO and HN(CA)NNH spectra, as described previously for WT Dss1 [17]. Spectra were recorded either on a Bruker Avance III HD 750 MHz spectrometer, with a Bruker proton-optimized triple-resonance 5mm TCI cryoprobe, or on a Bruker Avance Neo 800 MHz spectrometer, with a Bruker 5mm CPTXO cryoprobe. Spectra were processed in TopSpin version 3.6.2 (Bruker), transformed using qMDD version 3.2 [8] and processed through nmrDraw version 9.9 [32]. Analysis was done using CcpNmr Analysis version 2.5.0 [33]. Samples were transferred to single-use LabScape Essence 5 mm NMR Tubes (Bruker) and all spectra recorded at 10 °C, unless noted otherwise.

SCS analysis. Random coil chemical shifts were computed for variants using a predictor based on previous work [34, 35]. Conditions (10 °C and pH 7.4) were specified as input together with the sequence. From the observed assigned chemical shifts ($\delta_{observed}$) and the predicted random coil chemical shifts ($\delta_{random\ coil}$), the secondary chemical shifts (SCS or $\Delta\delta$) for different nuclei were calculated [36]:

$$SCS = \Delta\delta = \delta_{observed} - \delta_{random\ coil} \quad (1)$$

The resulting nuclei-specific datasets were used as an indication of local, structural properties in the different Dss1 variants.

Ubiquitin binding. Dss1 variant and ubiquitin were brought to the same buffer solution, with final concentration of 20 mM Na_2HPO_4/NaH_2PO_4 and 150 mM NaCl at pH 7.4 and added 10% (v/v) D_2O, 1% (v/v) DSS and 5 mM DTT and pH readjusted to 7.4. Ubiquitin and Dss1 variant were mixed, resulting in 1:1, 1:3, 1:6, 1:9, 1:20 and 1:40 molar equivalents of ubiquitin, keeping the Dss1 variant concentration at 50 μM in all titration points. ^{1}H-1D and ^{1}H-^{15}N HSQC spectra were recorded for all titration points as well as for a sample of 50 μM Dss1 variant with no ubiquitin present. The recorded spectra were referenced in TopSpin and analyzed in CcpNmr Analysis over the titration series. Chemical shift perturbations (CSPs) for the ^{1}H-^{15}N HSQC spectra were then exported, as calculated by [37]:

$$CSP = \sqrt{(\Delta\delta_H)^2 + (0.1 \cdot \Delta\delta_N)^2} \quad (2)$$

with $\Delta\delta_H$ representing the perturbation in the hydrogen dimension and $\Delta\delta_N$ the perturbation in the nitrogen dimension. The most perturbed residues in the Dss1 variant were used to fit the maximum perturbation expected at saturation of binding, $\Delta\delta_{max}$, as well as the dissociation constant, K_D, based on the following relationship [37]:

$$CSP = \Delta\delta_{max} \frac{[P_0]\cdot[L_0]\cdot K_D - \sqrt{([P_0]\cdot[L_0]\cdot K_D)^2 - 4\cdot[P_0]\cdot[L_0]}}{2\cdot[P_0]} \quad (3)$$

with $[P_0]$ and $[L_0]$ being the concentration of Dss1 variant and ubiquitin respectively.

Small angle X-ray scattering. SAXS data were collected at the DIAMOND beamline B21, London, UK, using a monochromatic (λ= 0.9524 Å) beam operating with a flux of 2 ×

10^4 photons/s. The detector was an EigerX 4M (Dectris). The detector to sample distance was set to 3.7 m. Samples were placed in a Ø = 1.5 mm capillary at 288 K during data acquisition. SAXS intensity profiles of the four proteins was measured at a temperature of 15 °C and a protein concentration of 3 mg/mL (WT, All-E) or 2 mg/mL (All-D, Swap). The average R_g was calculated from the SAXS profiles using ATSAS 3.0.1 [38], using the Guinier approximation with a q_{max} corresponding to $q_{max}*R_g = 0.9$, which is commonly used for IDPs [39].

Diffusion ordered NMR spectroscopy. Diffusion ordered spectroscopy (DOSY) experiments [40] were performed on the four Dss1 variants (50 µM) to determine hydrodynamic radii (R_H). Translational diffusion constants were calculated by fitting peak intensity decay within the methyl region (0.85-0.9 ppm), which was compared to the diffusion constant of the internal reference 1,4-dioxane (0.02% v/v) to estimate each protein's R_H as described [36]. Spectra were recorded (a total of 32 scans) on a Bruker 600 MHz 5mm QCI cryoprobe and Z-field gradient over gradients strengths from 2 to 98% and using a diffusion time (Δ) of 200 ms and gradient length of 3 ms (δ). Diffusion constants were fitted in GraphPad Prism v9.2.0.

Dss1 peptide assignment. NMR resonances of Dss1 peptides ($Dss1_{51-69}$) were assigned using spectra recorded via Bruker Topspin v3.6.2 on a Bruker 800 MHz spectrometer equipped with a cryogenic probe and Z-field gradient using natural isotope abundance (peptide concentration 1.2 mM, 20 mM Na_2HPO_4/NaH_2PO_4, 150 mM NaCl, 2% (v/v) trifluoroethanol, 10% (v/v) D_2O, 0.25 mM DSS; pH 7.4), acquiring TOCSY, ROESY, ^{15}N-HSQC, and ^{13}C-HSQC for manual assignments. Spectra were transformed and referenced using TopSpin v3.6.2 (Bruker)" before being analyzed in CCPN Analysis v2.5 [33]. The fractional helicity of the peptides was calculated by averaging the Cα chemical shifts of residues D/E54 to G67, both included, and using 3.1 and 3.8 ppm as average reference chemical shifts for a fully formed helix (max helix), with the expression [41-43]

$$\frac{\sum_{i,j}(\Delta\delta\, C_i^{\alpha})}{\max helix} \quad (4)$$

Molecular dynamics simulations were performed with GROMACS v. 2019.6 [44-47] and plumed v. 2.6.1. [48-50] with the force field a99SB-*disp*[51]. Parallel bias metadynamics [52] with well-tempered bias potentials [53] was used for all simulations. The backbone dihedral angles and the R_g of the C^{α} atoms were chosen as collective variables for biasing the simulations of full-length Dss1. The R_g was biased within an interval of 1.3-4.0 nm. Only the backbone dihedral angles were biased for simulations of the Dss1 C-terminal region peptides. We used multiple walkers [54] with 20 replicas per variant for the simulation of full length Dss1. The gaussian hills were added to the bias potential at a frequency of 400 fs and a width determined using diffusion based adaptive gaussians [55]. The bias factor was set to 32. A dodecahedral box was used, and periodic boundary conditions were applied. The a99SB-*disp* water model was used and ions were added to a concentration of 150 mM. Simulations were run at a temperature of 283 K. Energy minimization was performed using a steepest descend algorithm followed by a conjugate gradient algorithm. The first equilibration was run for 2 ns with position restraints. The second equilibration was run for 2 ns without position restraints with pressure coupling using the Berendsen barostat. Constraints on bonds were applied with the LINCS algorithm [56]. For the equilibrations, constraints were applied to all bonds, and for the production, constraints were applied for bonds to hydrogen atoms. A cut-off of 0.9 nm was used for non-bonded interactions and PME [57] was used for electrostatic interactions. A leap-frog integrator was used for the equilibration and production runs. The Parrinello-Rahman barostat was used in the production runs and the velocity-rescale thermostat [58] was used for the equilibrations and production. Simulations of full length Dss1 and variants were run for 13 µs (0.65 µs per replica) and the peptides for 8 µs with a timestep of 2 fs. The

first 0.15 μs of each replica in the full length and 2 μs of the peptide simulations were discarded after calculating the accumulated bias for the entire trajectory, as the bias hereafter is considered static, allowing for reconstructing the un-biased probability distribution using reweighting [59]. The reweighted and un-biased trajectories were used for the subsequent data analysis. Errors on the R_g were estimated using block averaging [60, 61]. The block size used for error estimation of the average R_g of each simulation was the smallest block size in the plateaued region for the block error analysis of the free energy surface as a function of the R_g.

Theoretical small-angle X-ray scattering (SAXS) profiles for each of the full-length protein simulations were calculated for every 50th frame (0.5 ns) with Pepsi-SAXS [62]. The theoretical profiles were compared to experimental SAXS profiles by reduced $\chi 2$ statistics, which is $\chi 2$ statistic per the degrees of freedom. To account for uncertainty of the experimental errors, experimental errors were rescaled using a correction factor calculated with BayesApp [63, 64].

The secondary structure content of the MD simulated conformations of full length Dss1 and the helix region was calculated using the DSSP algorithm [65] v. 2.2.1. DSSP assigns secondary structure to a protein from the coordinates of the backbone atoms based on the possible hydrogen bonding patterns. Hydrogen bonding is defined by electrostatic interaction energy and the cut-off is set high, allowing the algorithm to pick up on hydrogen bonds that deviate from the ideal length and angle. Conformations classified by DSSP as α helix, 3_{10} helix or π helix were considered helical. DSSP was applied to all frames in the trajectories.

Contact maps for all simulations were calculated with the python package MDTraj [66]. Contacts between residues were defined with a distance cut-off of 8.5 Å between Cα atoms and calculated for every 10th frame in the trajectory. The contact maps were averaged and weighted by the metadynamics bias associated with each frame to arrive at a contact map representing the weighted fraction of simulation time that each residue pair is in contact. The difference contact maps between the wildtype and the variants were calculated as the log ratio of the fraction of contacts for each residue pair in the variant to those of the wildtype.

3. Results

3.1. The alphabet of disorder

We first examined whether the sequence composition of IDPs relative to folded proteins would depend on the chosen background dataset. Since the enrichment of amino acids in the IDP sequence composition profile is here defined as the difference to the background normalized to the frequency of the amino acid in the background dataset, variations within the background dataset will affect the calculated enrichment. We examined three background datasets with different criteria for folded proteins: 1) the standard Composition Profiler folded protein dataset with high quality X-ray structures, 2) a dataset with X-ray structures with low B-factors and thus presumably less dynamic proteins, and 3) a broadly defined dataset including proteins with low resolution (Figure S2). We found variations of amino acid frequencies depending on how we chose to define our dataset, and thus the enrichment profile was dependent on the chosen background dataset, in particular for amino acids of low frequency. We decided that a broader definition of folded proteins would give us a more representative sequence profile for IDPs, because restricting the folded dataset to high resolution globular proteins would also include the enrichment of amino acids in these structures (Figure S3). We chose a set of non-redundant and naturally occurring proteins that had an entry in the PDB as the best representation of folded proteins, which would include more diverse proteins than the standard Composition Profiler folded background dataset.

Next, we explored the composition profile in the DisProt database with the use of the selected background data. Here, we found that the recent growth of DisProt and the use of the larger and more diversely defined folded protein dataset available in 2022 resulted in some differences compared to the early enrichment profile described in Uversky et al 2013 [7] (Figure 2A). First, the main characteristics of the IDP sequence composition stands; a depletion in hydrophobic amino acids, an enrichment in polar and charged amino acids, and an enrichment of the structure disrupting amino acid proline. However, the most enriched and most depleted amino acids were now less extremely enriched or depleted. Glutamate thus appeared to be much less enriched compared to the original profile. A few amino acids shift from being depleted or slightly enriched to being more enriched in IDPs, including asparagine, threonine, glycine, and aspartate. From a structural viewpoint, the enrichment of glycine in IDPs can be explained by the rotational freedom from the lack of a sidechain, allowing for a larger conformational space. Although not as pronounced as previously, we still observed a larger enrichment of glutamate and glutamine compared to the similar amino acids, aspartate, and asparagine with one carbon shorter sidechain.

Next, we investigated whether the observed differences could be explained by species-specific amino acid frequencies as DisProt mainly contains eukaryotic proteins and mostly from human. To remove this potential bias, we created sequence profiles containing only eukaryotic or human sequences in both the folded and the disordered datasets. For all three sets, we observed a similar IDP composition profile (Figure 2B). However, we found a difference in the enrichment of glutamine in the species-specific profiles, indicating that the glutamine enrichment in IDPs can partly be explained by a depletion of glutamine in prokaryotes compared to eukaryotes. There was no substantial difference in enrichment of glutamate and aspartate in the species-specific composition profiles, and we could thus not explain the difference in glutamate and aspartate enrichment by a species-bias.

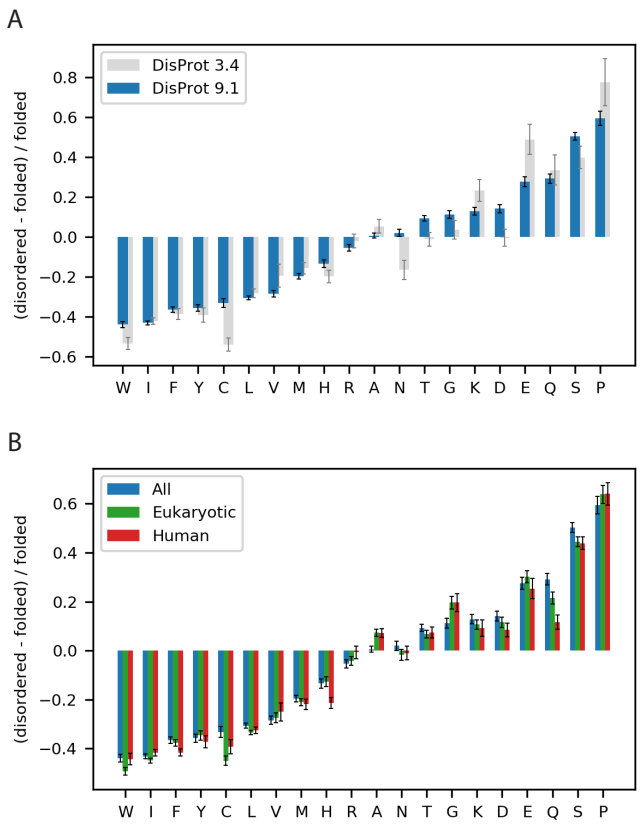

Figure 2. The disorder alphabet revisited. (A) Sequence enrichment profile of IDPs with the enrichment of amino acids in disordered proteins from the newest (9.1) and the older version (3.4) of DisProt [13-15] compared to folded proteins, defined respectively as non-redundant sequences in the PDB or the standard Composition profiler dataset PDBselect25. **(B)** Sequence enrichment profiles for eukaryotic and human IDPs from DisProt v. 9.1 using a background set of non-redundant sequences in the PDB. Error bars show the boot strap confidence intervals with a statistical significance value of 0.05.

3.2. Functional effect of aspartate and glutamate in Dss1

To investigate whether this apparent bias towards glutamate in IDPs would relate to functional effects, we used the small acidic IDP from *S. pombe,* Dss1, which is a component of several different protein complexes [17, 20], including the 26S proteasome [67-69] and which binds mono ubiquitin. Dss1 is overall highly negatively charged (-18) with a distributed content of both glutamates (9) and aspartates (14), totaling 23 negative charges. We designed three variants with different Glu/Asp ratios; an All-E variant, where all 23 acidic residues were glutamates, an All-D variant where all were aspartates and a swap variant where we exchanged glutamate for aspartate and *vice versa* (Swap). Together with the wildtype (WT) protein, we first assessed the functional effect of the aspartate-glutamate substitutions.

3.2.1 The Glu/Asp variants are functional *in vivo*

To investigate whether the Glu/Asp variants of Dss1 retained the function of WT Dss1, we tested the ability of overexpressed GFP-tagged versions of the Dss1 variants to rescue the temperature-sensitive growth defect of a Dss1 knockout strain (*dss1*Δ). First, we tested the expression of the recombinant Dss1 variants by analyzing whole-cell extracts by SDS-PAGE and western blotting. This revealed that all variants were expressed at roughly equal levels (Figure 3A). As shown before [21], the *dss1* null mutant is viable at 29 °C, but unable to form colonies at 37 °C (Figure 3B). In all cases, we observed that overexpression of the recombinant *dss1* variants suppressed this temperature-sensitive growth defect as efficiently as WT (Figure 3B). We conclude therefore that any changes in the conformational ensembles of the variants are too subtle to substantially impair the Dss1 function relevant for this phenotype *in vivo*.

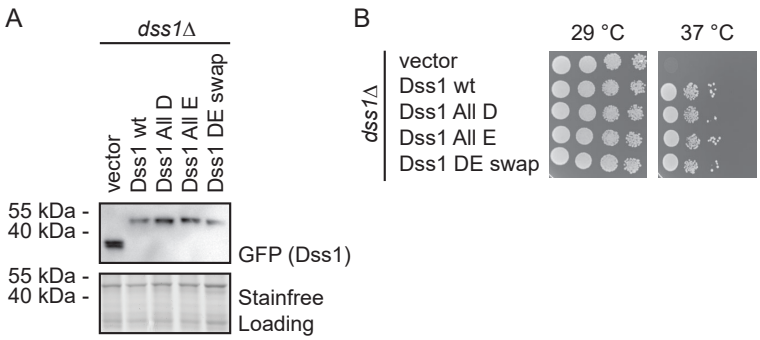

Figure 3. Growth effects of Asp and Glu substitutions in Dss1. (A) *S. pombe* cells deleted for Dss1 (*dss1*Δ) were transformed to express GFP-tagged wild-type (wt) Dss1 and the indicated Dss1 variants. Whole cell lysates were then compared by SDS-PAGE and western blotting using antibodies to GFP. Stain-free labelling was used as a loading control. **(B)** The growth of the strains from (A) was compared by serial dilution and incubation on solid media at 29 °C and 37 °C. Note that the temperature sensitive growth defect of the *dss1*Δ strain is rescued by all Dss1 variants.

3.2.2 Ubiquitin binding affinity, but not binding ability, depends on glutamate

Using NMR spectroscopy, we assessed if ubiquitin binding could be detected for all variants of Dss1. This was done from quantifying changes in the chemical shifts (chemical shift perturbation, CSP analysis) in a ^{15}N-HSQC NMR spectrum after addition of 40 molar excess of mono ubiquitin (Figure 4). To do this, the chemical shifts of each variant were first assigned using sets of triple resonance 3D NMR spectra. Overall, the same residues in the variants were affected by the addition of ubiquitin, confirming binding of ubiquitin to all four variants. However, binding to UBS I in Dss1 led to disappearence of peaks in the spectra of the WT and All-E variant, indicating exchange between free and bound states on an intermediate NMR time scale. This indicates that these variants have a higher affinity for ubiquitin than the other two variants. The sequence of UBS I in WT contains a central glutamate and is flanked by two aspartates on each side. The WT and All-E variant both have this central glutamate in common, while the two other variants have an aspartate in this position, suggesting that glutamate here increases binding affininty to ubiquitin compared to aspartate. Thus, in this case, a preference for glutamate in one specific position within the UBS I motif over aspartate increases affinity. Whether this relates to an increase in potential number of van der Waals' contact from the additional methylene group in glutamate was not adressed further.

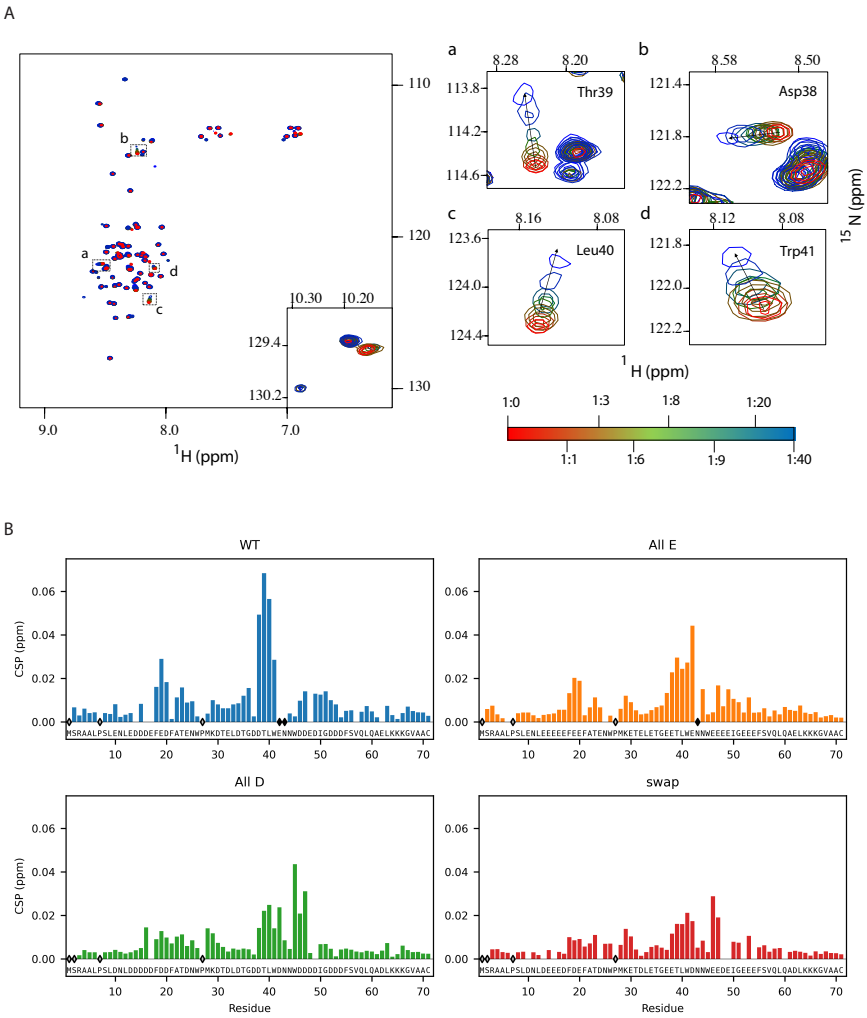

Figure 4. Ubiquitin binding ability is supported by both glutamate and aspartate. (A) HSQC spectra with varying concentrations of His$_6$-ubiquitin, and 50 µM Dss1 WT, **(B)** CSP for Dss1 WT and Glu/Asp variants binding to ubiquitin (2 mM) and quantified by combined amide chemical shifts perturbation analyses. Empty diamonds indicate non-assigned residues and filled diamonds indicate disappearance of peaks upon ubiquitin titration.

3.3 *Global compaction does depend on Glu vs Asp ratio*

To assess chain compaction of the four variants, we determined the hydrodynamic radius, R_H of the four Glu/Asp Dss1 variants by pulse field gradient NMR and the radius of gyration, R_g by SAXS. In parallel, we performed all-atom molecular dynamics (MD) simulations of each of the four Dss1 variants, applying enhanced sampling techniques to push the simulations to explore more conformations in the ensemble, Figure 5A. We used parallel bias metadynamics [52] and chose the R_g and the dihedral angles as collective variables, to increase sampling of backbone conformation without directly biasing the simulations towards a helical conformation. We calculated the average R_g from the MD simulations of each variant by calculating the R_g from the coordinates of the atoms in each frame. We then compared the dimensions of the four variants (Figure 5B). First, we found that the global dimensions of Dss1 as extracted by NMR diffusion and SAXS were overall similar for all Glu/Asp variants of Dss1, although we note that the R_H for WT Dss1 was slightly larger (by 12%). Second, we compared the R_g calculated from the MD ensembles to the R_g measured by SAXS and found no significant differences between the variants nor between simulation or experiment, except for the simulation of the swap variant, which showed a slightly more expanded chain by MD. Additionally, we calculated the theoretical SAXS intensity profiles from the MD ensembles using Pepsi-SAXS and compared the profiles directly to the experimental SAXS intensity profiles, which showed a similar agreement between simulation and experiment (Figure S4). From the simulations, we also calculated average contacts between C^{α} residues within the protein (Figure 5C) and were able to observe contacts between the C-terminal region and the UBS I, in agreement with conclusions from a study using paramagnetic relaxation enhancement NMR [17]. In our MD simulations, we observed that this interaction primarily took place between the last part of the helix, where there are three consecutive lysines, and the UBS I. We also observed that interactions between the C-terminal region and the UBS I were frequent in the WT and All-D, but nearly absent in the All-E variant. This implies that the aspartates on both sides of the binding site facilitate this interaction, which could, as proposed in [17], be a mechanism to regulate the accessibility of the binding site.

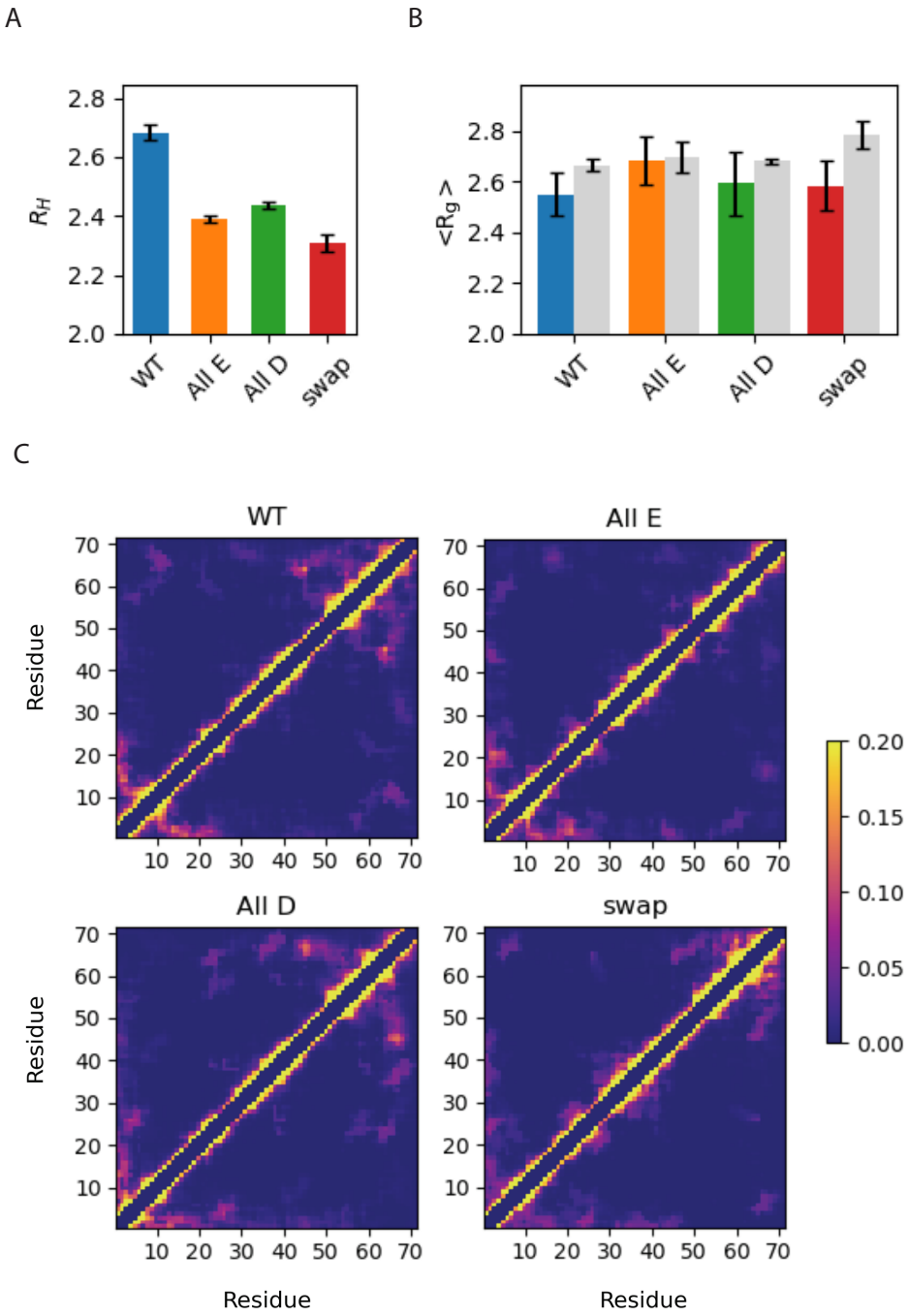

Figure 5. Global chain properties of Dss1 WT, All-E, All-D and Swap. (**A**) hydrodynamic radius (nm) determined by diffusion NMR; (**B**) average radius of gyration (nm) determined experimentally by SAXS (color) or by MD simulations (grey); **(C)** Contact maps showing the frequency of contacts between each of the 71 residues in the four Dss1 variants in the MD simulations, where yellow corresponds to >20% of the simulation time.

3.4 *Local structural changes in Dss1 depending on Glu/Asp variants*

Since the global properties of Dss1 appeared to be essentially indifferent to either glutamate or aspartate, but with changes in contacts between the C-terminal and the UBS

I, we asked if the glutamate bias would be explained by effects on local structure formation in Dss1. To answer this question, we analyzed the secondary chemical shifts (SCS) of the backbone atoms C^{α} and C^{β} in all four Dss1 variants (Figure 6). Additionally, we analyzed the local structure in the MD ensembles by calculating the most likely hydrogen-bond patterning based on the distances between atoms in each frame with DSSP and simulated the C-terminal region from residue 50-71 of the four proteins alone, allowing us to run longer simulations and observe formation and unfolding of the helix in a single trajectory. All four Dss1 variants showed formation of the transient C-terminal α helix, which was evident both from NMR SCSs and from the MD simulations of both full length Dss1 and the C-terminal helical region (Figure 6). From the NMR secondary chemical shifts, we found that the largest difference between the Glu/Asp variants and WT Dss1 was in the population of the transient C-terminal α helix, where all variants have a smaller α helix population compared to wild type, as well as in a region capping the N-terminal side of the helix (Figure 6). Here, WT and All-D had a short stretch of negative SCSs indicating an extended structure, which was absent in the Swap and All-E variant. The extended structures of the DisUBM-sites were maintained, but it appeared that consecutive aspartates increased the extendedness compared to consecutive glutamates. Thus, locally, there appears to be an effect of Glu/Asp variation where aspartate may better support local structure of extended characters.

While the MD-simulations do capture the formation and unfolding of the C-terminal transiently populated α helix in all variants, we did not observe a larger population in WT Dss1. In the simulations of the C-terminal regions, we observe that the formation and unfolding of the α helix is a slow process even when applying a bias against already visited conformations, where the process takes around 1 μs (Figure S5 and S6). Further, the full eleven residue helix rarely forms, while either one or the other half of the helix forms more frequently (Figure S7). Likely, the simulations of full length Dss1 of 10 μs do therefore not capture the C-terminal α helix population precisely. However, the populations of the C-terminal α helix are similar in both the simulations of the C-terminal peptides and the full length Dss1 variants, indicating that the true population can be expected to be in between these populations. However, like the conformations indicated by the NMR SCSs, we observed that glutamates (residue 52-54) positioned before the helix in the All-E and Swap variants to be more frequently helical than the aspartates in the wildtype and All-D variants. This indicates that glutamate supports a helix conformation better than aspartate, or that aspartate more frequently is found in a helix capping conformation and thus does not have a helical geometry.

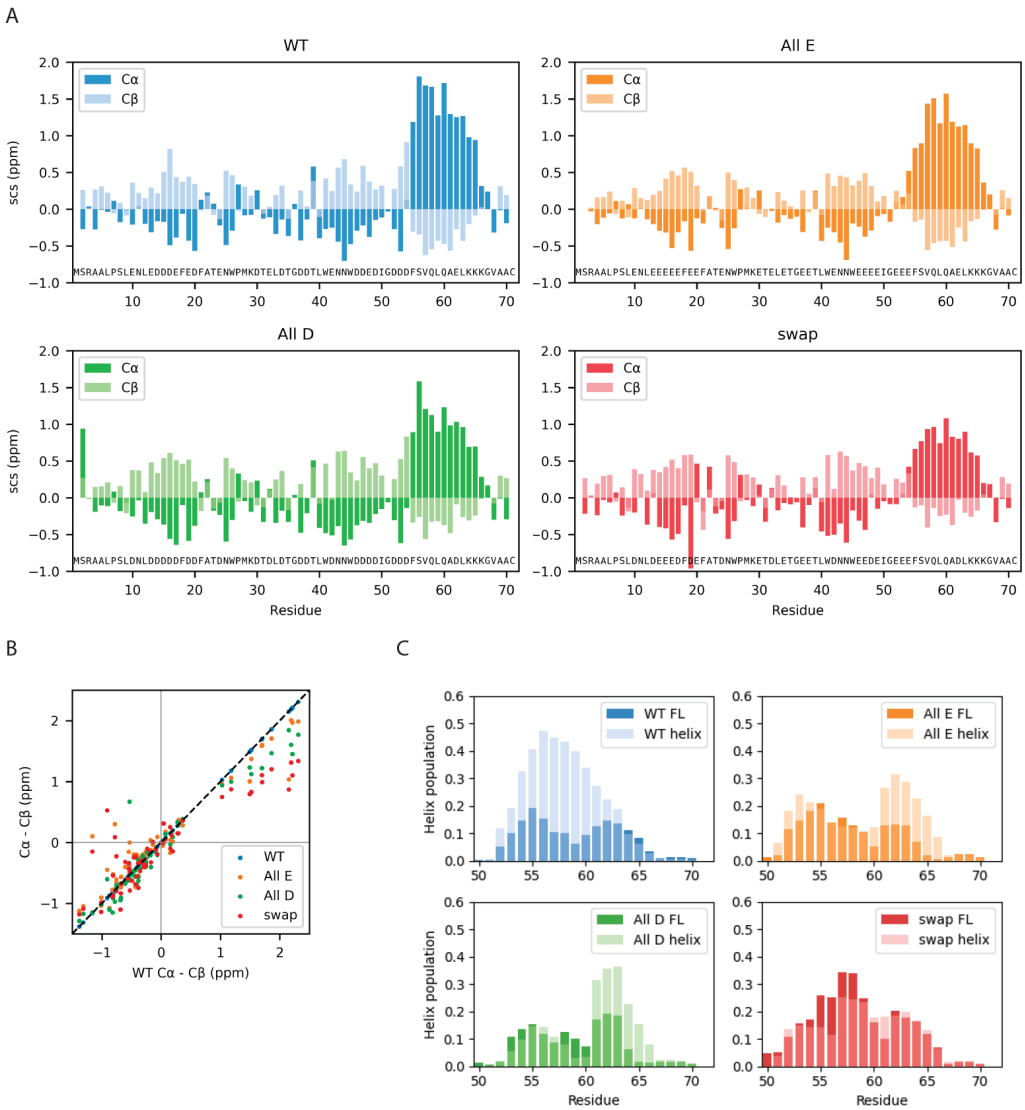

Figure 6. Local chain properties of Dss1 WT, All-E, All-D and Swap. (A) NMR secondary chemical shifts (C^{α} and C^{β}) for Dss1 wildtype and the three variants; **(B)** Correlation between SCSs of WT Dss1 and the three variants. **(C)** Helix population of the C-terminal region from MD simulations of full-length (FL) Dss1 overlayed with the helix population from the simulations of the helix region alone.

The most pronounced effect of Glu/Asp variations on the local structural levels was observed for the helix population, which changed by just minor alterations to the amino acid composition. We therefore decided to examine specific single amino acid substitutions that we hypothesized would either increase or decrease the helix population. To be able to capture these minor changes in population in an otherwise disordered chain we analyzed the effects using peptides corresponding to the helical 19-residue C-terminal region of Dss1, $_{51}$GDDDFSVQLQAELKKKGVA$_{69}$. From the MD-simulations of the full length Dss1 proteins, we observed that the lysines K65 and K66 often formed salt bridges with E62 in the WT and All-E variant more frequently than the aspartate E62D in the Swap and All-D variant, which formed salt bridges with K64 more often (Figure S8). When the residues are in an α-helical conformation, the sidechain of D62 will be in proximity of the residues K65 and K66, while K64 would be on the other side of the helix. Salt bridges between E62 and the K65 and K66 thus likely form stabilizing interactions, while salt

bridges between E62 and K64 are unlikely α-helical. We thus speculated that the interaction between K65 and E62 could stabilize the helix, and that the side chain of aspartate is likely too short to support this interaction. Further, the NMR chemical shifts and the secondary structure calculated by MD simulation indicate possible helix capping function of D52-D54, which could stabilize the helix. This would explain why the population of the helix is largest in the WT, as it has both glutamate at position 62 and aspartate at positions 52-54. Based on these observations, we designed the following peptides (residue 51-69): WT (12% helicity predicted by Agadir) and two helix-modulating D/E-swap variants, D54E (26% helicity) and E62D (9% helicity). Using NMR spectroscopy, we extracted helicity from the SCS for the C^{α} atoms (Figure 7). Here, we found that although not reaching the full effect, the predicted effects of the substitutions were captured experimentally, with the D54E variant increasing in helicity, mostly at the substitution site, and the E62D losing helicity. Thus, the D54E gained 6%-point in helicity and the E62D lost 28%-point. This suggests that substitution of aspartate for glutamate in the N-terminal of the helix increases helicity and removing a glutamate in the middle of the helix destabilizes it. Thus, these data support that glutamate is preferred for stabilization of this transient helix.

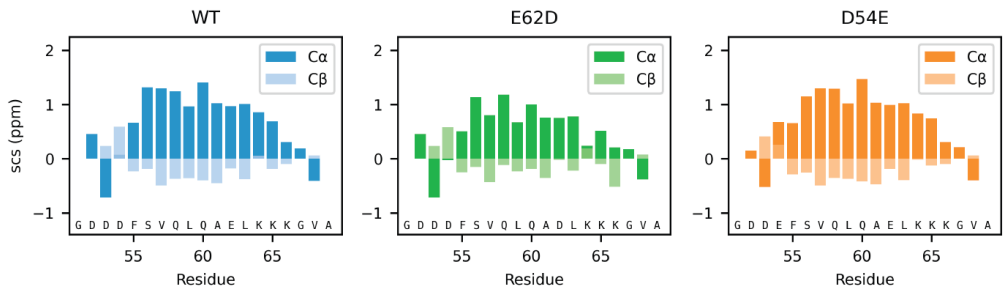

Figure 7. Local effects of Asp and Glu in helix stabilization. The SCS of the Cα and Cβ atoms of the three peptides WT, D54E and E62D analyzed in the peptides $Dss1_{51\text{-}69}$ frame.

4. Discussion

The enrichment of glutamate over aspartate in IDPs has been known since the early 2000s, but to our knowledge no systematic attempts to explain this bias have been performed so far. Since then, current databases on IDPs have expanded and additional proteomes have become available enabling revisiting the basis for this bias. Using a disorder dataset containing five times as many sequences and comparing to a more diverse dataset of folded proteins, found that while glutamate is indeed enriched in IDPs, the difference is less pronounced than in the original work. We also exclude the possibility that this difference is due to an underlying bias caused by the dominance of human proteins in the disorder database.

We sought a functional explanation for the enrichment of glutamate over aspartate in IDPs, but found that in our model system, all tested Dss1 variants were able to complement the growth defect of a *dss1* deletion mutant, irrespective of the type of anionic side chain. Since previous studies have shown that the temperature sensitive phenotype of the *dss1*Δ strain is tightly connected with the incorporation of Dss1 into the 26S proteasome [21, 67-69], it is likely that the structural and dynamic effects of the Asp/Glu substitutions are not sufficiently pronounced to disrupt its interaction with the 26S proteasome *in vivo*. However, we note that the Dss1 variants were overexpressed, and that Dss1 is also engaged in many other cellular processes and is often found as a subunit in larger complexes [17]. Since the effects of these functions of Dss1 and more subtle effects on proteasome incorporation may not be captured by our growth assays, we cannot rule out that other Dss1 functions are not affected by the Asp/Glu substitutions.

As also suggested by the growth assays, we observed that all variants were capable of binding mono-ubiquitin. Although exchanging the anionic amino acids did not impair the ability of Dss1 to bind to ubiquitin, variants with glutamate at the center of UBS I bound ubiquitin stronger. Whether the glutamate supports binding because of its additional methylene group, adding van der Waals' interactions within this disordered motif is not clear. In a study on Dss1, Schenstrøm et al found that the C-terminal of Dss1 bends back and shields the UBS I and suggested that this may be a mechanism for regulating ubiquitin binding [17]. In our MD simulations, we were able to observe this interaction for all but the All-E variant. Interestingly, we find that the more aspartates the variants contain in the UBS I, the more frequent are the interactions between the C-terminal region and the UBS I. Similarly, in a recent study, Zeng et al. find that aspartate in IDPs forms hydrogen bonds more frequently than glutamate, likely stabilizing observed local chain compaction [70]. These results indicate that both the position and the amount of glutamate in the UBS I may determine the availability of the binding site and thus the strength of the binding and hence the interactome.

No large differences in R_H or average R_g in the simulated conformational ensemble and SAXS experiment were observed, and glutamate was therefore not found to promote more extended ensembles than aspartate. Only minor differences in local structure were observed by NMR, where consecutive aspartates better support extended structure than consecutive glutamates, and positional effects of having a Glu or an Asp can influence the population of transient helices. Although these small population changes were detectable by NMR, the effects were likely too small to be manifested and detectable in the R_H or R_g measurements. Thus, having glutamate instead of aspartate was thus not found to influence the dimensions of the conformational ensemble of Dss1 and the bias toward IDPs having more glutamates in IDPs than aspartate is likely not rooted in this parameter. Likely, for Dss1, which is already highly charged, changing the type of anionic side chain will have little effect on the net charge per residue and hence may not affect chain collapse [71].

Instead, we observed that local structural effects and binding strength could be modulated by exchanging Glu/Asp preferences. For Dss1, the transient helix in the C-terminal was populated differently in the conformational ensembles of the Glu/Asp variants. Using peptide variants, we could establish that glutamate stabilized the transient helix both when positioned in the N-terminal and when inserted into the helix at positions that enable salt bridge formation with positively charged side chains positioned +3 C-terminal to it. This is consistent with previous work on folded proteins, where glutamate is more frequently observed in α helices than aspartate [9] and where glutamate in the center of a helix is generally more stabilizing than aspartate, because the carboxyl group is more distant to the backbone. Thus, glutamate imposes less restraints on the conformational space of the residues in the helix [10].

Likely, the transition from random coil to an eleven-residue helix does not happen instantaneously but could be initiated by either end of the helix forming the first turn. Therefore, it is not unlikely that there would be an equilibrium between random coil and some intermediate state as observed in the simulations, which would contribute to the positivity of the C^{α} SCS in the NMR experiments. Alternating between two partially formed helix conformations could be a mechanism to facilitate binding to different binding partners through conformational selection and thus ensure specificity. The expected stabilizing effects of glutamate on the fully formed helix may not be entirely captured while Dss1 is free in solution but could play a larger role in the ability of Dss1 to bind to binding partners, where it adapts the 11-residue helix.

This work linked the Glu/Asp bias in IDPs to local structure and functional effect. A question remains as to when DPs would prefer glutamate over aspartate? Recent work has shown that higher helicity in the free state of an IDP may lead to higher affinity for a partner protein [12, 72, 73]. Combined with the preference for glutamate seen here to stabilize transient helices, this could suggest that glutamate would be a preferred residue for

highly populated transient helices in IDPs. Preformation of highly populated helices could be important in folding-upon-binding reactions, where increased helicity has been shown to affect affinity through effects on both *on*- and *off*-rate constants [12, 72, 73]. However, aspartate is a known helix N-capping residue [74], and has been shown in several IDPs to initiate helices, even at several positions within the same helical stretch [75, 76], but a quantitative comparison of the two amino acids for this property has not been done. Finally, in a recent work studying the interactions between IDPs and calcium ions, a preference for aspartate over glutamate in the so-called Escaliber-like motif was noted [30]. Thus, another possible reason for supressing the use of aspartate in IDPs would be to minimize binding of divalent cations.

While our results point towards a bias arising from the function of the anionic amino acids in IDPs, the difference in enrichment of glutamate and aspartate could also arise through other multiple local effects, explanations to which need exploring. The subtle differences in the choice of amino acid at specific positions may be able to shift the equilibrium populations of the conformational ensemble in IDPs and thus impact their function and interactome.

5. Conclusions

In this work we have addressed the compositional bias in IDPs, which have preference for glutamates over aspartates; a phenomenon pointed out already in the early 2000s [4, 6-8]. We find the dimension of the disordered chain is largely indifferent to the difference in these anionic amino acids, whereas highly local effects both on the populations of transient structures as well as on binding affinity were seen. We hypothesize that stabilizing local transient helix structure through capping effects and intra helix salt bridges, as well as adding binding strength through the additional methylene group, may be important reasons for the preference of glutamates over aspartates in IDPs. Finally, functional biases towards glutamates in regions undergoing helical folding-and-binding and towards aspartates in transactivation domains and calcium binding regions are likely just one of several functional reasons for selection of glutamate or aspartate in specific IDPs.

Supplementary Materials: The following supporting information can be downloaded at: XXX, Table S1: Sequences of S. pombe Dss1 wildtype and Glu/Asp variants; Figure S1: MSA logo for the Dss1_Sem1 family; Figure S2: Influence of background dataset on composition profile; Figure S3: Composition profile of high resolution X-ray crystal structures; Figure S4: Comparing experimental and theoretical SAXS profiles; Figure S5: Helix formation time series, peptides; Figure S6: Helix formation time series; Figure S7: Helix population, simulations; Figure S7: Salt bridge frequency, simulations.

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Data Availability Statement: MD simulations and SAXS data will be available via https://github.com/KULL-Centre/papers/tree/main/2022/DSS1-Roesgaard-Lundsgaard-et-al. Chemical shifts for all four Dss1 variants are available at BMRB, IDs:27618, 51551, 51552, 51557.

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