

The Charge Distribution Patterns of IA₃ Impact Conformational Expansion and Hydration Diffusivity of the Disordered Ensemble

Katie M. Dunleavy,[§] Tianyan Li,[§] Eugene Milshcheyn,[§] Afnan M. Jaufer,[§] Shamon A. Walker,[‡] and Gail E. Fanucci^{§*}

[§] Department of Chemistry, University of Florida, P.O. Box 117200, Gainesville, Florida 32611, United States

[‡] Materials Research Laboratory, University of California, Santa Barbara, California 93106, United States

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ABSTRACT: IA₃ is a 68 amino acid natural peptide/protein inhibitor of yeast aspartic proteinase A (YPRA) that is intrinsically disordered in solution with induced N-terminal helicity when in the protein complex with YPRA. Based upon the intrinsically disordered proteins (IDPs) parameters of fractional net charge (*FNC*), of net charge density per residue (*NCPR*) and of charge patterning (κ), the two domains of IA₃ are defined to occupy different domains within conformationally based subclasses of IDPs; thus, making IA₃ a bimodal-domain IDP. Site-directed spin-labeling (SDSL) electron paramagnetic resonance (EPR) spectroscopy and low-field Overhauser dynamic nuclear polarization (ODNP) spectroscopy results show that these two domains possess different degrees of compaction and hydration diffusivity behavior. This work suggests that SDSL EPR line shapes – analyzed in terms of their local tumbling volume (V_L) – provide insight into the compaction of the unstructured IDP ensemble in solution and that protein sequence and net charge distribution patterns within a conformational subclass can impact bound water hydration dynamics; thus, possibly offering an alternative thermodynamic property that can encode conformational binding and behavior of IDPs and liquid-liquid phase separations.

INTRODUCTION

Intrinsically disordered proteins (IDPs) and proteins containing intrinsically disordered regions (IDRs) lack well-defined secondary/tertiary structure making up a class of proteins not defined by the standard structure to function paradigm.¹⁻⁷ Proteins containing disorder in 30 or more residues are defined as IDPs and IDRs with global or local disorder, respectively.^{3, 8-11} More than 15% of all eukaryotic proteins are determined to contain intrinsic disorder, signifying an important benefit of disorder.^{9, 12} Proteins containing local or global disorder under physiological conditions can benefit a cellular system by allowing for more dynamic linking regions, ease in finding a binding target due to overall flexibility, multiple binding targets, and conformational sampling.^{3, 9, 10, 12} Cellular signaling pathways and cellular regulation depends on these advantages, making IDPs highly associated to these processes.^{3, 13, 14} Key amino acid residues are known to promote disorder through charged and structure perturbing side chains, opposed to hydrophobic and aromatic residues that induce protein structure.^{1, 2, 4-7, 11, 15-20} Due to

the depletion of structure promoting residues and an increase in overall charged residues, IDPs adopt low mean hydrophobicity and overall high net charge.^{2, 9, 11}

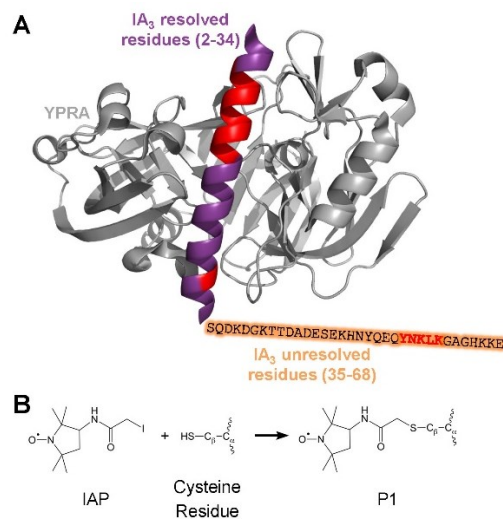


FIGURE 1. A) Ribbon diagram of IA₃ when bound to YPRA (PDB: 1DPJ)²¹ with helical N-terminal domain (NTD) shown in purple and disordered C-terminal domain (CTD) not resolved in the complex represented as an extended sequence highlighted in orange. P1 labeled cysteine variant sites are rendered in red (amino acid sequence given in Figure S1). B) Reaction scheme of IAP (3-(2-iodoacetamido)-PROXYL) attachment to a cysteine residue resulting in P1 labeled sidechain.

IA₃ is a 68 amino acid inhibitor of yeast aspartic proteinase A (YPRA).²¹ In general, proteases play important roles in many physiological, as well as pathological processes. As such, there is interest in the continued discovery, modification and development of novel protein-based protease inhibitors for use as versatile tools in the fields of medicine, agriculture and biotechnology.²²⁻²⁴ IA₃ is intrinsically disordered in solution (a.k.a. an IDP) and adopts an N-terminal domain (NTD) α -helix with an unresolved C-terminal domain (CTD) in the crystallographic complex when bound to YPRA (Figure 1A).²¹ Previous studies demonstrate that the NTD 32 residues of IA₃ are responsible for its inhibitory activity.²⁵⁻²⁷ In the absence of YPRA, helical structure throughout both the NTD and CTD of IA₃ can be induced by 2,2,2-trifluoroethanol (TFE).²⁸ The TFE-induced helical transitions in *S. cerevisiae* IA₃ have been studied using

circular dichroism (CD), 2D ^1H ^{15}N nuclear magnetic resonance (NMR), laser temperature-jump fluorescence and fluorescence resonance energy transfer (FRET), and site-directed spin labeling (SDSL) electron paramagnetic resonance (EPR) spectroscopies.²⁸⁻³⁵ The CTD of *S. cerevisiae* IA₃ does adopt helical structure in TFE, albeit to a lesser degree than the NTD.^{28, 29, 32, 33, 36}

SDSL is a sensitive method to interrogate the local micro-environment via probing conformational dynamics and changes in biomolecular and macromolecular systems through analysis of and changes therein of the nitroxide EPR lineshape, which is sensitive to molecular motions on the ns timescale.³⁷⁻⁴¹ Although commonly utilized for structured soluble and membrane proteins,^{42, 43} SDSL has found application to the study of IDPs.^{32-35, 44-49}

We have previously studied the TFE-induced helical transition in IA₃ with SDSL and CD spectroscopy.³²⁻³⁵ Results, consistent with earlier circular dichroism (CD) spectroscopy²⁸ and NMR investigations,²⁹ showed that the helical transition of the CTD was less cooperative than the NTD and that the CTD does not undergo the same extent of TFE-induced helical transition. We also demonstrated with SDSL EPR and CD that the degree of the TFE-induced helical transition of the NTD is mitigated by amino acid substitution that disrupt the hydrogen bonding network on the hydrophilic face of the helix³⁶ and that incorporation of the nitroxide spin label, 3-(2-Iodoacetimido)-PROXYL (IAP, termed P1 when chemically attached to a cysteine side-chain) (Figure 1B), generated constructs that possess greater than (S9P1, Q13P1), less than (V8P1, E10P1, I11P1, S15P1), or equivalent to (K7P1, F12P1, S14P1) the TFE-induced helical propensity as wild type (WT) IA₃.³³ CTD P1 variants Y57P1, N58P1, K59P1, L60P1 and K61P1 have similar TFE-induced helical propensity as WT.^{33, 50}

Having a series of spin labeled constructs with known WT behavior allows for continued investigations of differences in the local properties and behavior of the two domains of IA₃. Local hydration dynamics within 5-10Å of a nitroxide radical spin-probe can be probed with low-field ODNP. This method combines ~9.5-9.8 GHz X-band continuous wave-EPR (CW-EPR) and ~14.6-14.8 MHz nuclear magnetic resonance (NMR) techniques to amplify the ^1H NMR signal of local water molecules.⁵¹⁻⁵⁴ At 0.35T, the Overhauser effect transfers polarization from the electron spin to adjacent water molecules due to electron- ^1H dipolar coupling via cross-relaxation, enhancing the ^1H NMR signal through saturation of the electron spin, giving rise to local water diffusivity relative to the proximity of the electron spin probe.^{51, 55, 56} The time needed for water to diffuse through the 5-10 Å distance surrounding the spin label is defined as the translational diffusion correlation time (τ_c). Through measurements of the ^1H NMR signal enhancement via ODNP and T_1 relaxation time changes due to the presence of the spin-label, the contributions from local electron- ^1H cross-relaxation (k_σ) can be separated from local waters undergoing self-relaxation (k_ρ).⁵⁷ The coupling factor (ξ), a ratio of k_σ and k_ρ , is a parameter that describes water diffusivity, and has been separated into different environments such as bulk-like ($\xi > 0.153$), surface ($0.076 < \xi \leq 0.153$), intermediate ($0.043 < \xi \leq 0.076$), or buried ($\xi \leq 0.043$) (Figure 2) based upon experimental results of Han and coworkers for known spin label locations in macromolecules encompassing proteins, polymers and lipid dispersions.⁵²

The experimentally determined value of ξ , using the free hard sphere (FFHS) model of translational diffusion⁵⁸ to calculate the spectral density function for how the overall timescale of the dynamics modulate the dipolar interaction between the spin label and water, can be converted into a correlation time τ_c . The solid line in Figure 2 is a graphical representation of this equation (Eq. S3), which is used to show relationships between local water diffusivity properties and the measured value of ξ . The ratio of ξ/ξ_{bulk} (or $\tau_c/\tau_{c,\text{bulk}}$) can be used as a comparative parameter to describe the effective retardation in hydration diffusivity relative to bulk water.⁵² These experimentally determined parameters can further differentiate fast diffusing water (DW) on the picosecond timescale (k_σ), and slower diffusing or “bound” water (BW – waters that have rotational correlation times dictated by the macromolecule) on the millisecond-nanosecond timescale (k_{low}).^{55, 57, 59}

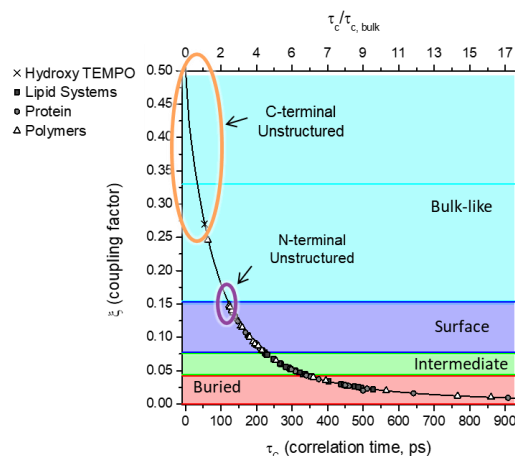


FIGURE 2: Plot of collective literature-based ξ values and their modeled relationship to τ_c and $\tau_c/\tau_{c,\text{bulk}}$ for spin label hydroxy-TEMPO (x), lipid systems (solid squares), proteins (grey circles), and polymers (open triangles).^{51-53, 60-64} Results discussed within for IA₃ NTD (purple oval) and CTD (orange oval) sites are graphically depicted to show that anomalously fast hydration dynamics was observed for CTD sites, with NTD region giving interfacial hydration dynamics. Figure modeled after those shown in Ref ⁵¹ by Han and co-workers.

Here, we extend our studies of IA₃ via SDSL EPR and low-field ODNP by characterizing the two domains (NTD and CTD) of IA₃ as separate conformational subclasses of IDPs. Our CW-EPR and ODNP results reveal differences in the average local tumbling volume (*i.e.*, compaction) and the hydration dynamics of the two disparate domains for the intrinsically disordered state of this IDP. This work adds to the growing interest in understanding how structural and dynamic properties of IDPs are controlled by charge and sequence patterning.^{1, 2, 4, 6, 7, 17-20, 65} A deeper comprehension of these effects can impact our fundamental understanding of physiological and pathological processes, as well as inform the rational design of IDPs as bio-inspired materials with biotechnological applications.

MATERIALS and METHODS

Materials. BL21-DE3-pLysS and XL-1 blue *E. coli* cells used in protein overexpression and DNA amplification, respectively,

were purchased from Invitrogen (Carlsbad, CA). pET-22b+ and 3-(2-Iodoacetamido)-PROXYL spin label were purchased from Millipore Sigma (Burlington, MA). Primers for cysteine residue site directed mutagenesis were purchased from Integrated DNA Technologies (Coralville, IA). Restriction enzymes (*Xho*I, *Nde*I), T4 ligase, Phusion HF DNA polymerase, and DpnI for cloning and mutagenesis were purchased from New England Biolabs (Ipswich, MA). A HiTrap Chelating HP and HiTrap 26/10 Desalting columns were purchased from GE Healthcare Life Sciences (Chicago, IL). 16.5% Tris-Tricine SDS PAGE gels were purchased from Bio-Rad Laboratories (Hercules, CA). The 0.60 i.d. x 0.84 o.d. quartz capillary tubes for CW-EPR and ODNP were purchased from Fiber Optic Center (New Bradford, MA). Cha-Seal was purchased from Kimble Chase Life Science (Rockwood, TN). Unless otherwise noted, all other products and reagents were purchased from Fisher Scientific (Pittsburgh, PA).

Protein Expression and Purification of IA₃ Mutants. The *E. coli* codon optimized gene of IA₃ (UniProt P01094) was cloned into a pET-22b+ vector (Invitrogen) containing a C-terminal 6 x His-tag, adding the sequence LEHHHHHH to the C-terminus (Figure 3 and Figure S1).³² Constructs K7C, V8C, S9C, E10C, I11C, F12C, Q13C, S14C, S15C, S27C, Y57C, N58C, K59C, L60C, and K61C were generated as described previously.³³ Wild type and cysteine substituted IA₃ protein variants were purified using nickel affinity chromatography and eluted in 50 mM sodium phosphate, 300 mM sodium chloride, pH 4.0 as described previously.³² To chelate residual nickel and to ensure cysteine reduction, ethylenediaminetetraacetic acid (EDTA) and dithiothreitol (DTT) were added to purified constructs to a concentration of 100 mM and 0.1 mM, respectively. Protein purity was assessed using a 16.5% mini-PROTEAN Tris-Tricine SDS-PAGE gel.

Spin Labeling. Purified IA₃ fractions were pooled, concentrated, and buffer exchanged into 50 mM sodium phosphate, 300 mM sodium chloride, pH 7.4 using a HiTrap 26/10 desalting column to remove residual nickel, EDTA, and DTT prior to spin-labeling. Upon buffer exchange, cysteine substituted constructs were immediately reacted with 5-10x molar excess IAP spin label dissolved in 50-100 μ L of 100% ethanol for 12 h at 4°C, in the dark. The labeling scheme is shown in Figure 1B. Excess spin label was removed through repeated buffer exchange as described above. If necessary, spin-labeled protein samples were concentrated to a final concentration of \sim 200 μ M using a Millipore 5,000 MWCO filter operated under gas pressure. IAP was chosen for these studies based upon the nature of the chemical bond formed upon reaction of the cysteine sulfhydryl with the iodoacetamide group.^{32, 33} Spin labeled construct purity and efficiency was verified through mass spectrometry mass analysis (University of Florida, Gainesville, FL).³³

CW EPR spectra. CW-EPR spectra utilized for calculating the local tumbling volume have been reported previously, and details for data collection are given in the Supporting Information.³³ It is known that the ensemble of IDPs can be impacted by temperature as observed in CD spectroscopy,^{66, 67} as such, we performed control studies to evaluate how sensitive the EPR lineshape of IA₃ was in terms of effects of temperature, TFE-induced viscosity and denaturation by urea are given in Supporting information (Figures S2-S4). Unless otherwise stated,

all data have been collected at 27°C because this was the easiest temperature for stability in our set up of \pm 0.5°C.⁶⁸ All samples are prepared with a final solution containing 2.5 mM sodium phosphate, 21.5 mM sodium chloride, pH 7.0

Local Tumbling Volume X-band EPR Data Analysis. From baseline corrected area-normalized spectra, the local tumbling volume parameter (V_L), was calculated for continuous wave (CW) X-band (\sim 9.5-9.8 GHz) EPR spectra in the fast limit ($\tau < 2$ ns) according to Equation 1, which gives the expression for V_L ,

$$V_L = \frac{kT\tau}{\eta}$$

(1)

where k is Boltzmann's constant, T is the absolute temperature, τ is the rotational correlation time, and η is the solvent viscosity.^{69, 70} Values of τ were determined from line shape simulations using the EWVoight program, which is based on Redfield theory, generously provided by Alex Smirnov (NCSU). All fits to spectra are shown in Figures S5-7. The EWVoight program simulation provides values for the Lorentzian line widths of the three transitions that are subsequently used in Equation (2),

$$T_2(M)^{-1} = A+BM+CM^2$$

(2)

where M is the nuclear spin quantum number of the M^{th} hyperfine line, $T_2(M)$ is the spin-spin relaxation time (which is inversely proportional to the homogeneous line width) of that line, and A , B , and C are the line shape parameters. In the fast motional regime, assumptions can be made and B and C alone can be used to determine rotational correlation time.⁷¹⁻⁷³ Values of τ were determined using Equation (3),

$$\tau = -1.22 \times 10^{-9}B(G)$$

(3)

where the value of B is obtained from solving Eq. (2) for the three hyperfine lines and are reported in Table S1.

Viscosity Measurements. The viscosity of the 0-40% (v/v) TFE solutions was determined using a Cannon-Fenske Viscometer (size 50) suspended in a water bath at 27.0 \pm 0.3 °C and are reported in Supporting Information Tables S2-S3. The measurements were repeated four times for reproducibility. The solutions were prepared in 5% increments by combining the appropriate amount of TFE and the pH 7.4 buffer described previously.^{33, 36} The measurements were collected in units of seconds and converted to kinematic viscosity using the conversion factor of the viscometer. The kinematic viscosity, which was expressed in units of centistokes (cS), was converted to dynamic viscosity (kg m⁻¹s⁻¹) using the densities of each of the solutions.

CW W-band Electron Paramagnetic Resonance. CW W-band EPR spectra were collected on a Bruker Elexsys 680 spectrometer equipped with a W-band ENDOR resonator at the National High Magnetic Field Lab (NHMFL) in Tallahassee, FL. Temperature was regulated by use of a CF935 Cryostat (Oxford Instruments). Samples were prepared by concentrating samples studied at X-band to approximately 2 mM spin-labeled protein.

Samples were loaded into 0.15 i.d. X 0.25 o.d. suprasil quartz capillary tubes (Vitrocom, Mountain Lakes, NJ) and sealed with X-Sealant®. The capillary tube with the sample was placed inside a quartz tube with a 0.50 i.d. X 0.90 o.d. that was sealed at one end. All experiments reported were performed at $27 \pm 1^\circ\text{C}$ unless otherwise stated. All spectra were collected as 180 Gauss (G) scans with 0.0042 mW incident microwave power. The 100 kHz field modulation amplitude and time constant of the detector were optimized to provide maximum signal-to-noise ratio with no line broadening. All spectra are reported as an average of ~ 15 scans. Spectra are given in the Supporting Information (Figure S8)

Multifrequency CW X- and W-band EPR Spectral Line Shapes Fitting. The EPR spectral line shapes of the CTD sites, Y57C, N58C, K59C, L60C, and K61C labeled with the IAP spin-label at both X- and W-band were simulated using the Labview program for fitting multi-component CW EPR spectra, written by Dr. Christian Altenbach from the laboratory of Dr. Wayne Hubbell at University of California, Los Angeles. The experimental line shapes were normalized and phased before being loaded into the program. All the EPR spectral line shapes could be fit with one component assuming isotropic motion. The g -tensor values for g_{xx} , g_{yy} , and g_{zz} were determined experimentally from the rigid limit spectrum of free IAP spin label at W-band at 150 K and are: 2.00821, 2.00641, and 2.00205; respectively.³⁵ The g -tensor values were held constant for each fit. The isotropic A -tensor (A_1), the isotropic rotational diffusion tensor (R_{bar}), and the isotropic Lorentzian linewidth tensor (W_1) were all allowed to vary. The definitions of the parameters were described by Budil et al.⁷⁴ Results from spectral fitting are given in Supporting Information Tables S4-S5. Similar results were obtained for correlation times from multifrequency X-band and W-band fitting and those obtained using the Redfield fast-limit simulation (EWVought) of X-band data.

ODNP sample preparation. The concentration of WT and spin labeled IA_3 constructs were calculated by measuring $A_{214\text{nm}}$ value at diluted volumes of each stock solution in triplicate. The extinction coefficient, $\epsilon_{214\text{nm}}$ for each construct was calculated as 140,794 $\text{M}^{-1}\text{cm}^{-1}$ (wild type), 140,976 $\text{M}^{-1}\text{cm}^{-1}$ (V8P1), 140,985 $\text{M}^{-1}\text{cm}^{-1}$ (S9P1), 140,985 $\text{M}^{-1}\text{cm}^{-1}$ (S14P1), 140,883 $\text{M}^{-1}\text{cm}^{-1}$ (N58P1), and 140,978 $\text{M}^{-1}\text{cm}^{-1}$ (K59P1).⁷⁵ Spin label concentration was verified using an IAP standard curve for S14P1 and was $>90\%$ comparable to $A_{214\text{nm}}$ measured protein concentration. Due to comparable spin label concentration and $>95\%$ spin labeling efficiency determined through mass spectrometry mass analysis, protein concentration was used for sample preparation and data analysis. X-band (9.5 GHz) CW-EPR absorption spectra for standard curve, spin count and verification of sample integrity before and after ODNP, were collected at room temperature using a Magnostech MiniScope MS-5000 benchtop or Bruker E500 spectrometer with a dielectric resonator. Spectra were reported as an average of 16 scans with 120 mT sweep width, 0.2 mT modulation amplitude, 100 kHz modulation frequency and 1 mW incident microwave power (2 mW incident microwave power on Bruker E500). Each sample was prepared to $\sim 150 \mu\text{M}$ protein concentration: 150 μL autoclaved nH_2O , and 350 μL of protein in buffer (as described for CW EPR sample preparation). This dilution factor was utilized to have the buffer ionic strength equivalent to those used during EPR and CD investigations with and without TFE addition.^{32,33}

³⁶ Approximately 3.5 μL was drawn into 0.6 mm I.D. x 0.84 mm O.D. quartz capillary tubes, via capillary action. The samples were sealed with a protective layer of Critoseal on the top and beeswax on the bottom (to protect samples from TFE evaporation).

ODNP Data Collection. Overhauser DNP was performed at a 0.35 T field using a Bruker EMX CW EPR spectrometer, a Bruker Avance NMR console, a Bruker ER4119HS microwave resonator, and a custom built 10 W 9.8 GHz microwave source system similar to the one described in.⁷⁶ The NMR probe and sample were inserted into a ventilated quartz tube inside the resonator. Building air was flown through the quartz tube for temperature control. All ODNP measurements were performed at room temperature with a deviation of $\pm 0.5^\circ\text{C}$. The microwave resonator was tuned to ~ 9.8 GHz, the rf tuning circuit was tuned to ~ 14.8 MHz, and the magnetic field was set on resonance with the central electron spin transition. The ^1H free induction decay signal and the $T_{1,1\text{H}}$ relaxation time for each sample were measured as a function of microwave power from 0 to ~ 200 mW. Both the $T_{1,1\text{H}}$ and $T_{1,0}$ (spin lattice relaxation of the sample without spin label) was measured using inversion recovery pulse sequences. Each ODNP experiment was repeated three times. ODNP data and analysis are given in the Supporting Information (Figures S9 and S10, Tables S6 and S7) and follow the procedures of Han and co-workers.^{55,57,77}

RESULTS AND DISCUSSION

IA_3 is a bimodal domain IDP. The global sequence of IDPs can be evaluated in terms of their amino acid sequences, specifically focusing on hydrophathy and fractional charge distribution of positive, f_+ and negative, f_- amino acids.^{1,2,4-6,17,20} The net fraction of charged residues of the global sequence is defined as $\text{FCR} = (f_+ + f_-)$. The global net charge per residue (NCPR) over the entire IDP sequence is defined as $|\text{NCPR}| = |f_+ - f_-|$.⁵ Using the software localCIDER (<http://pappulab.wustl.edu/CIDER>),⁴ these parameters can be readily calculated for amino acid sequences as a function of solution pH; thus, allowing for fractional charges and distributions to include partial charges on histidine residues based upon standard pKa values for amino acid side chains.

Five different conformationally based subclasses have been defined based upon global sequence values of FCR and $|\text{NCPR}|$.^{2,7} The graphical depiction of this “phase plot” has been termed a Das-Pappu plot (Figure 3A) with five regions, termed R1, R2, R3 and R4/R5 that differ in their FCR and $|\text{NCPR}|$ parameters, and which have predicted characteristics of their structural ensembles.^{4,5,17} More specifically, amino acid sequences with $\text{FCR} < 0.25$ & $\text{NCPR} < 0.25$ reside in region R1 and adopt globule conformations. This region is sampled by weak polyampholytes/polyelectrolytes and polar tracks. Regions R4/R5 represent the other extreme for $\text{FCR} > 0.35$ & $\text{NCPR} > 0.35$ containing acid versus base rich polyelectrolytes, respectively. Because of the electrostatic repulsions between charged side chains, these sequences sample semi-flexible worm-like conformations. Region 3, with $\text{FCR} > 0.35$ & $\text{NCPR} \leq 0.35$ is comprised of strong polyampholytes that can form either coils or hairpins depending upon the combination of FCR and their charge patterning. Thus, κ is a parameter that provides

a quantitative measure of the distribution of charges within a given amino acid sequence.² It is very useful to describe different distribution patterns of charged amino acids in IDPs with identical amino acid compositions. As charges are segregated within the linear sequence, $\kappa \rightarrow 1$ (Figure 3B), and hairpin conformations are favored. As charges are more interspersed, $\kappa \rightarrow 0$; stabilizing coil-like conformations. The mathematical calculation of κ has been described by Pappu and co-workers.² described more below). The remaining phase space, R2 given by $0.25 \leq FCR \leq 0.35$ & $NCPR \leq 0.35$, is populated with sequences that adopt chimeras of globules and coils and are oftentimes those sequences that undergo binding-induced conformational changes to a structured state. Overall, the types of conformational ensembles of IDRs/IDPs have been shown to be dictated by low hydrophathy and a combination of both κ and FCR .¹

Analysis of the IA₃ sequence according to these methods shows that the two domains of IA₃ reside within different conformational subclasses in the Das-Pappu plot and differ in their hydrophathy, FCR , $|NCPR|$ and κ parameters. The IA₃-NTD (1-32 a.a.; $FCR = 0.344$; $|NCPR| = 0.031$) falls within region R2: a globule to coil depending upon the context, whereas the IA₃-CTD (33-68 a.a.; $FCR = 0.417$; $|NCPR| = 0$) is characterized by R3, a coil to hairpin conformation (Figure 3A). These values were calculated with CIDER for the *S. cerevisiae* IA₃ sequence (Figure 3C) at pH 7.0. Our expression construct contains a HIS₆-tag (LEHHHHHH), which when included into the calculation slightly modifies the values for the CTD as depicted in Figure 3A. However, it is unknown if the presence of the HIS tag modulates the conformational properties of IA₃. Figure 3D plots the sequence dependent values of $NCPR$ over a stretch of 5 amino acids ($NCPR_5$),⁴ showing a differences in the range of $NCPR_5$ values between the two domains. Specifically values for the NTD range between $-1 \leq NCPR_5 \leq 1$, whereas values range between $-3 \leq NCPR_5 \leq 2$ for the CTD. Additionally, the charge patterning of the *S. cerevisiae* IA₃ CTD is more segregated (*i.e.*, $\kappa_{CTD}/\kappa_{NTD} \sim 3$) than its NTD sequence (Figure 3B).

The impact of these IDP conformation ensemble parameters on the two disparate domains of IA₃ have been seen in the crystallographic complex,²¹ as well as in SDSL EPR spectroscopy,^{32,33} NMR spectroscopy²⁹ and fluorescence spectroscopy investigations of IA₃.³¹ However, given many of these earlier investigations were performed prior to the emerging knowledge of how sequence impacts IDP conformation, the results were not discussed in terms of the sequence parameters of IA₃. It is noteworthy that our and biophysical experiments and IDP parameter analysis of IA₃ are performed at pH 7.0, NMR studies are performed at pH 5.5,²⁸ whereas the kinetic inhibition studies of YPRA by IA₃ are performed at pH 3 – 4.5;²⁷ likely reflective of the pH within the yeast vacuole. Future EPR and ODNP studies are planned to be performed on the *S. cerevisiae*³⁶ as well as other yeast IA₃ constructs without a HIS-tag and as a function of pH and concentration.

Local Tumbling Volume Reflects Local IDP Compaction. The CW X-band EPR spectra of P1 labeled *S. cerevisiae* IA₃ have been published previously.³²⁻³⁵ All CW X-band EPR spectra for IA₃ in solution are reflective of the motionally averaged regime, also referred to as the isotropic limit or fast limit ($\tau_c < 2$ ns). These motionally narrowed spectral line shapes can be

analyzed in terms of the local tumbling volume (V_L) parameter.^{32, 69, 70} From Eq. 1, the value of V_L is directly proportional to the spin-label correlation time, τ_c . Using V_L allows for comparison across the TFE titration because it corrects for changes in solution viscosity induced by increasing TFE. Correlation times were determined from simulation/fitting of CW X-band spectra for NTD labeled sites (Figures S5-S7, Table S1) and multifrequency CW X-band/W-band spectra for CTD labeled sites utilizing two different methods and software packages (Figure S8, Tables S4 & S5) The correlation time obtained from simulation of X-band EPR spectra of nitroxide comprises motional averaging from the tumbling of the macromolecule, fluctuations of the peptide backbone and internal rotational motions of the spin-label itself.^{78, 79} Thus, V_L represents the volume of correlated motion that the nitroxide spin label samples.

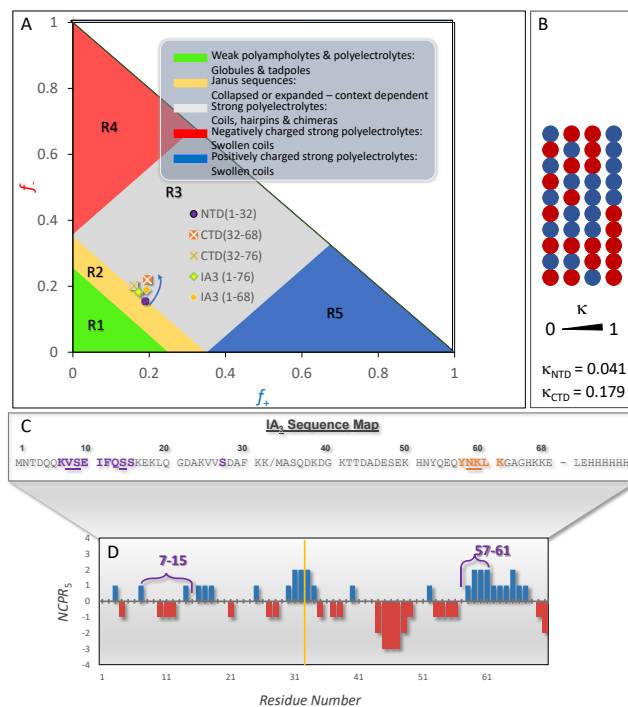


FIGURE 3. Sequence analysis of *S. cerevisiae* IA₃ NTD and CTD within (A) subclass in Pappu plot, (B) κ values, (C) amino acid sequence where the forward slash after residue K32 marks the end of the NTD as defined by the sequence that gives optimal inhibition of YPRA, and with NTD and CTD sites studied by SDSL EPR in purple and orange, respectively.²⁷ Underlined residues are those P1 labeled sites studied via low field ODNP. (D) $FCR/NCPR/\kappa$ analysis as a function of sequence with a blob size of 5 using CIDER. Sections A and B are modeled after Refs. 2 & 7.

Figure 4 plots values of V_L as a function of % (v/v) TFE for each P1 site (NTD data as squares; CTD as circles) during the transition from disordered-to- α -helix. Values of V_L are lowest at 0% TFE and increase with addition of TFE. Solid lines through the data points are linear fits to show that V_L increases linearly until the 20-25% TFE mark and then relatively levels off, with a slight up- or downslope attributable to errors in the simulation method utilized with the more anisotropic spectra.⁷⁰

The observed trends in the dependance of V_L as a function of % (v/v) TFE can be understood by considering the three sources of flexibility that contribute to the motional averaging of the CW X-band nitroxide EPR line shape.⁷⁹ When disordered in solution, although the overall tumbling of the unstructured 7.6 kD peptide may be > 1 ns, the backbone is likely undergoing large amplitude fluctuations resulting in locally high mobility of the spin-label with a small volume of space being sampled, *i.e.*, the local tumbling volume is small due to the flexibility of the protein backbone. As the α -helix is formed with an increasing TFE percentage, the rigidity of the helix causes the backbone fluctuations to become smaller, and the spin label motion becomes less isotropic as it is restricted by the formation of the helix and hence the “volume” that it samples increases due to both the local modes of motion of the spin-label and the larger volume occupied by the more rigid helix.^{69, 70} The relative leveling off of the local tumbling volume occurs because beyond 23% TFE the protein is fully α -helical and a higher percentage of TFE would not theoretically cause an increase to V_L .

The observed site-specific trends in values of V_L through the TFE-induced helical transition parallel three general trends that we reported earlier via $h_{(+1)}/h_{(0)}$ mobility parameter analysis of the EPR spectra.^{32, 33} First, results show more site-specific variation in the NTD compared to the CTD as a function of TFE show, second the difference in V_L between 0% and 40% TFE (a.k.a. the degree of transition) is on average less for the CTD ($\Delta V_{Lavg} \sim 400 \text{ \AA}^3$) than the NTD ($\Delta V_{Lavg} \sim 1000 \text{ \AA}^3$), and third the NTD undergoes a more cooperative TFE-induced transition than the CTD.

We previously discussed that the site-specific modulation in the EPR $h_{(+1)}/h_{(0)}$ mobility profiles as a function of % (v/v) TFE for NTD P1-sites tracks with changes with the TFE-induced helical propensity of the folded state upon P1 incorporation, as determined from CD analysis.³³ Meaning, that as P1 is moved through the amphipathic helix of the NTD of IA₃ substitutions alter the TFE-induced helical propensity, where the greater the helical content at 30-50% TFE, the less mobile the EPR line-shape. Consequently, a larger final value of V_L indicates a greater relative TFE-induced helical content of that variant. For example, the data for S9P1 in Figure 4 are representative of a P1 construct with $>$ WT helical propensity and V8P1 represents a construct with $<$ WT helical propensity. For comparisons made in this report when discussing the two domains, we will utilize data from NTD sites with WT TFE-induced helicity (K7P1, F12P1, S14P1). The TFE-induced conformational states of the two domains of IA₃ have different average values of compaction, with $V_{Lavg NTD} \sim 1900 \text{ \AA}^3 > V_{Lavg CTD} \sim 1700 \text{ \AA}^3$. This result demonstrates a strength of spin-labeling to interrogate site-specific behavior of the separate domains compared to CD, which averages over the entire protein sequence.

Table 1. Summary of V_L Parameters for *S. cerevisiae* IA₃.

	$V_{Lave} (\text{\AA}^3)$		ΔV_{Lave} (\AA^3)	d $V_{Lave}/d\%_{\text{TFE}}$ ($\text{\AA}^3/\%_{\text{TFE}}$)
	0% TFE	40% TFE		
NTD ^a	840 \pm 130	1900 \pm 40	1050 \pm 50	30 \pm 4
CTD	1260 \pm 60	1600 \pm 100	400 \pm 100	11 \pm 2

^a For the NTD calculations, only those constructs with WT TFE-induced helicity were averaged.

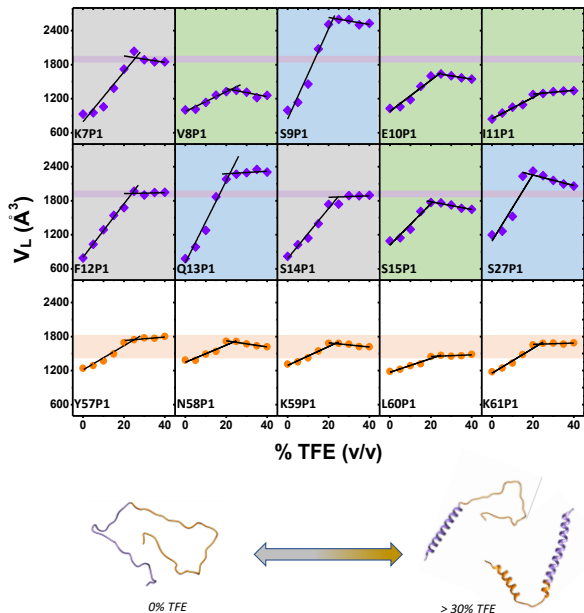


FIGURE 4. V_L analysis trends during TFE titration for NTD (purple squares) and CTD (orange circles) P1-IA₃ variants where solid lines are fits to the two linear regions as discussed within the text. Horizontal purple bars represent the average V_L values for P1-constructs with WT helicity in the TFE-folded state, with background shading of grey = WT helical propensity, green $<$ WT helical propensity, blue $>$ WT helical propensity. The light orange bar shows the similar average V_L behavior for CTD residues, and all CTD constructs studies here have overall total helicity similar to WT.³³ In all plots the size of the data points are larger than the error in the measurements. Data were collected at 27 °C in triplicate for each sample.

Millhauser *et al.*, has shown that the degree of cooperativity of an unstructured to helical transition is reflected by the magnitude of the change in V_L as a function of temperature (a.k.a. the magnitude of the slope) for spin-labeled 16-mer and 17-mer peptides.^{69, 70} Hence, we can extend that analysis and conclude that the slope of our graphs reflect the relative cooperativity of the TFE-induced transition of IA₃. Slopes for the four CTD sites are similar in value, and they are less in magnitude than those for NTD sites with WT helical propensity (K7P1, F12P1, S14P1), indicating the NTD undergoes a more cooperative folding in TFE (Table 1) These differences in the degree of cooperativity of the transition were also seen via $h_{(+1)}/h_{(0)}$ mobility parameter analysis reported earlier, where data were fit with a sigmoidal curve and with the FWHM of the derivative of that curve reflecting the cooperativity of the transition.^{32, 33}

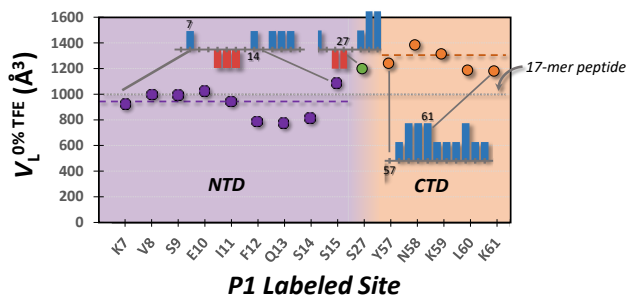


FIGURE 5. Graphical representation of V_L values for NTD (purple) and CTD (orange) P1 labeled sites of the intrinsically disordered state of IA₃. Dashed lines represent V_L average values of each domain, with $V_{Lavg(NTD)} = 840 \pm 40 \text{ \AA}^3$ and $V_{Lavg(CTD)} = 1260 \pm 60 \text{ \AA}^3$. Data for S27 (green) was not included in either the NTD or CTD calculations and is defined here as a linker region based upon the distribution of the $NCPR_5$ values (shown as inserts). There is a statistically significantly smaller local tumbling volume of the NTD residues compared to the CTD. The dark grey dotted line at 1000 \AA^3 represents a value obtained for a 17-mer alanine peptide.^{69, 70}

Another noticeable difference between V_L of the NTD and CTD are the **initial values** in the intrinsically disordered state. Figure 5 plots V_L values for P1 labeled amino acid locations in the disordered state (0% TFE), revealing a trend that $V_{Lavg(CTD)} (1260 \pm 60 \text{ \AA}^3) > V_{Lavg(NTD)} (840 \pm 40 \text{ \AA}^3)$. These volumes were calculated from average correlation times of $\tau_c = 0.19 \pm 0.02$ ns and 0.27 ± 0.03 ns; respectively. To increase our confidence in the values of correlation times obtained from X-band data simulations, W-band EPR spectra were collected for all IA₃ CTD constructs (Spectra and analysis in Supporting information Figures S8/Tables S6-7). These results imply that in the disordered conformational ensemble over the Y57 to K61 IA₃-CTD sequence, which has local high positive charge density, is more expanded than the K-7 to S15 IA₃-NTD. This finding suggests that the local tumbling volume may be reflective of local expansion/compaction dictated by a local charge density that influences the fluctuations and collapse or expansion of the protein backbone. Additional experiments are under way to further evaluate this hypothesis.

It is noteworthy that $V_{Lavg(CTD)} > V_{Lavg(NTD)}$ in the disordered state even with the presence of the HIS-tag; which has been predicted to strongly increase overall compaction of IDPs.¹⁷ The EPR results for the disordered state of IA₃ indicate *less compaction* of the CTD compared to the NTD, even in the presence of the C-terminal HIS₆-tag. Because of this, we feel confident that the presence of the HIS₆-tag is not obfuscating the V_L trends observed with our data. Nevertheless, future investigations will be made on constructs not containing a HIS-tag.

Work by Millhauser, *et al.*, on spin-labeled 17-mer alanine peptides [Ac-A-E-A-A-A-K-E-A-C(MTSSL)-A-K-E-A-A-A-K-A-NH₂; MTSSL is methanethiosulfonate spin label] gave local tumbling volumes of 1000 \AA^3 when unstructured, which is larger than IA₃ $V_{Lavg(NTD)} \sim 840 \text{ \AA}^3$ even though IA₃ is a 4.5 x larger protein. We interpret the similarity in the size of the local tumbling volume to reflect a parameter of compaction of the peptide backbone in the disordered ensembles. For further comparison, $V_L = 500 \text{ \AA}^3$ was obtained for spin-labeled

glutathione.^{69, 70} The larger V_L values observed for the IA₃ CTD ($\sim 1300 \text{ \AA}^3$) are consistent with a more extended/rod-like structure as predicted for R3 IDP conformational sequences² and sequences with higher overall net local charge.¹⁷ Thus, the higher local tumbling volume represents less compaction at those local sites within of the protein backbone in the disordered ensemble. These results may also suggest that those residues destined to become more helical are actually more compacted in the unfolded state; whereas those sites that are not as cooperatively ordered are more expanded in the disordered state. Further, the results demonstrate the capability of SDSL to provide information about mobility/compaction of the microenvironment in IDPs.

It is important to note that the correlation times obtained for IA₃ in the TFE-induced helical state represent motional averaging dominated by the correlation times of the internal modes of motion of the spin-label and do not directly reflect the global tumbling volume of a cylindrical IA₃. Millhauser predicts $V_L \sim 2000 \text{ \AA}^3$ for a perfectly helical 17-mer peptide at 10°C. For their 17-mer peptide, where helicity was induced by lowering the temperature, they obtained $V_L \sim 1800 \text{ \AA}^3$. For IA₃, the TFE-induced helical transition of the separate NTD and CTD are $V_L \sim 1900 \text{ \AA}^3$ ($\tau_c = 0.79 \pm 0.02$ ns for wild-type like constructs) and $V_L \sim 1600 \text{ \AA}^3$ ($\tau_c = 0.66 \pm 0.02$ ns); respectively. Our values for IA₃ (a 68-mer peptide with ~ 34 amino acids per domain) approach an average limiting value over both domains of $V_L < 2000 \text{ \AA}^3$, again, similar to values observed for a 17-mer peptide, indicating that the observed upper limit of V_L is likely indicated by the flexibility the spin-label utilized. It is also possible that IA₃ does not form a single helical coil in TFE.

Hydration Dynamics is influenced by Charge Distribution. Here, low-field ODNP was performed on 3 spin-labeled variants of IA₃ that reside within the NTD (V8P1, S9P1 and S14P1) and 2 spin-labeled variants within the CTD (N57P1 and K58P1). ODNP results reveal dramatic differences in the NTD and CTD surface hydration diffusivity (Table 1, Figure 2). In the unstructured-state of IA₃ in solution, P1-N-terminal sites give *surface-like* hydration diffusivity, with $\xi_{avg} = 0.15 \pm 0.03$. On the other hand, the two C-terminal P1-sites, have ξ values of 0.5 ± 0.2 (N57P1) and 0.4 ± 0.2 (K58P1), indicating *bulk-like* hydration diffusivity. The CTD ξ values are unusually large. In fact, they are greater than values for a spin-label in solution ($\xi_{bulk} = 0.27$,⁵²) and for sites on double stranded DNA ($\xi = 0.23$ - 0.26 ,⁵⁹), which were described as anomalously high compared to protein surfaces. The origin of these differences is described below.

Regarding the NTD, we find that the water diffusivity within the 5-10 Å shell of P1 does not differ with amino acid substitutions that alter the TFE-induced helical propensity (V8P1 < WT, V9P1 > WT, S14P1 \approx WT), and by extension, folded state local tumbling volume.³³ The helical propensity of the TFE-induced state of V8P1 is similar to or less than CTD constructs studied here (with similar local tumbling volume), yet the hydration dynamics of the unstructured state of V8P1 are similar to the other NTD P1 constructs and not the CTD sites (for which it has similar TFE-induced behavior). Thus, suggesting that the hydration dynamics in the NTD are encoded by a parameter of the unfolded ensemble and not sequence features that stabilize the folded state. V8P1 gives a nice comparison to

the CTD as disrupts the TFE-induced helical transition and provides very similar local tumbling volumes to those seen for CTD sites. Yet, the hydration dynamics for V8P1 are nearly identical to those obtained for S9P1 and S19P1. Therefore, we interpret these results to suggest that it is not structure inducing features that dictate hydration, but rather the local charge density, NCPR_5 of the sequence that encodes for the hydration behavior.

The origin of the difference in ξ values of the two domains can be gleaned by separately evaluating k_σ and k_{low} , which represent different time scales of diffusing water as DW and BW; respectively.^{55, 57} Values of k_σ (Table 2) are relatively invariant ($k_{\sigma, \text{avg}} = 55 \pm 2 \text{ s}^{-1}\text{M}^{-1}$) across P1-labeled constructs of both domains of IA₃. This finding indicates that picosecond DW dynamics within the 5-10 Å shell layer around the IA₃ surface are not impacted by amino acid sequence parameters such as κ or NCPR . In contrast, $k_{\text{low, avg}}$ values are $510 \pm 20 \text{ s}^{-1}\text{M}^{-1}$ for the NTD and $100 \pm 40 \text{ s}^{-1}\text{M}^{-1}$ for the CTD, indicating that the slower bound water interactions differ leading to differences in ¹H-¹H relaxation.

By referencing to bulk water, $k_{\sigma, \text{bulk}}$, a scaled parameter for DW can be calculated as in Equation (4)

$$k_{\sigma, \text{scaled}} = \left(\frac{k_{\sigma, \text{site}}}{k_{\sigma, \text{bulk}}} \right)^{-1} \quad (4).$$

This value quantifies the marginal degree to which the surface diffusive water layer has been slowed compared to those waters interacting with TEMPO in water. We obtained an average $k_{\sigma, \text{scaled}} = 1.7 \pm 0.04$, over the narrow range of 1.6-1.7 for all 5 sites in IA₃. Comparison to literature (Figure 6) reveals that DW of IA₃ are less hindered compared to DW of structured proteins, lipid bilayers or membrane proteins, which have $k_{\sigma, \text{scaled}}$ values ranging up to > 6 .^{57, 65, 80} Interestingly, the DW behavior for both domains of IA₃ is similar, on average, to those previously seen for polyanionic DNA surfaces (1.4±0.2-fold scaling).⁵⁹

As described by Han and co-workers, the scaling “of DW dynamics can be viewed as reflecting on the enthalpic cost for disrupting and displacing the hydration water that is collectively strengthened by the attractive interactions between the protein surface and the hydrogen-bonds of the local water network.”⁵⁷ The narrow distribution of DW dynamics within and between the two domains of IA₃ indicate approximately equal enthalpic costs for displacing the local hydration water. The low scaling values indicate these waters are more readily removed from the surface of IA₃ than most of those on structured proteins and the 5-mer peptide fragments of CheY.⁵⁷

Table 2. Summary of ODNP results.

Site	ξ	k_σ ($\text{s}^{-1}\text{M}^{-1}$)	k_ρ ($\text{s}^{-1}\text{M}^{-1}$)	k_{low} ($\text{s}^{-1}\text{M}^{-1}$)	τ_c (ps)	
V8P1	0.153±0.019	58 ± 2	380±45	500±75	130±30	
NTD	S9P1	0.140±0.008	56 ± 1	400±20	530±35	130±13
	S14P1	0.145±0.014	55 ± 5	380±16	500±30	126±5
	N57P1	0.47 ± 0.14	57 ± 3	120±40	70±60	16±11
CTD	K58P1	0.36 ± 0.11	55 ± 11	150±40	130±70	38±20
	TEMPO ^a	0.27	94	353	366	54.1
bulk	IAP ^b	0.22 ± 0.01	71 ± 3	325 ± 12	376 ± 40	75±3

^a Data Taken from Refs. ^{51, 52, 55} ^b Values determined as described in Ref. ⁵² Errors represent standard deviations from replicate experiments.

Previously, Han and co-workers characterized the DW dynamics in 5 amino acids peptide fragments of the protein CheY (with spin-label in the center) and synthetic polyproline with lysine or aspartic acid (2-7 amino acids long with spin-labels on the N-termini) and found no correlation between the grand average hydropathy (GRAVY) values and DW dynamics. DW scaling for 5-mer CheY peptides and 5-mer to 10-mer polyproline peptides clustered between 1.6-2.7; marginally higher than what we observe for IA₃ (1.6-1.7).

Additionally, average $k_{\sigma, \text{scaled}}$ for IDPs α -synuclein^{57, 62, 81} and Δ tau-187^{57, 63, 82} spread over values of 1.1 – 4.0 and cluster near 2.5, respectively. Han and co-workers concluded that variation in local charges on disordered 5-mer small peptides of CheY surface do not modulate DW scaling (span values of 1.7 to 2.5). Here, with IA₃, we demonstrate this conclusion holds true in a larger IDP systems of up to ~ 34 amino acids that span both R2 and R3 conformational space of a Pappu plot. The α -synuclein and Δ tau-187 peptides in their study have sequences within the R1 and R2 Pappu phase space, respectively.

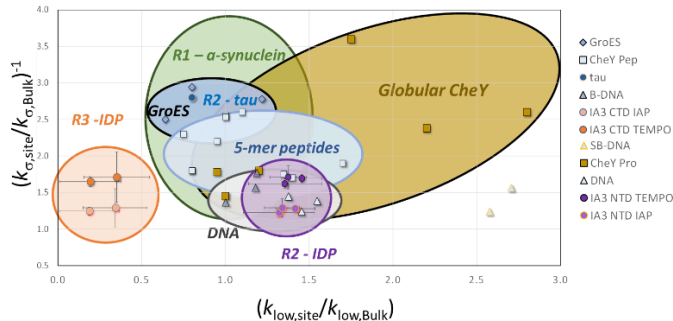


FIGURE 6: Scaled k_σ DW parameter as a function of scaled k_{low} for NTD (purple) and CTD (orange) IA₃-P1 constructs referenced to either TEMPO or IAP in bulk solution. Data for DNA;⁵⁹ GroES;⁸² globular CheY and 5-mer CheY peptides, tau and α -synuclein⁵⁷ taken from respective literature references with scaling to TEMPO.⁵²

If values of k_σ for IA₃ do not vary across the two domains (*i.e.*, as a function of NCR or κ), then the observed differences in NTD and CTD hydration diffusivity, ξ , must arise from alterations in the self-relaxation (k_ρ) of local waters, *i.e.*, those that “tumble” or move with the protein surface. NTD sites have values of $k_\rho \sim 385 \pm 10 \text{ s}^{-1}\text{M}^{-1}$, which are **slightly faster than** the self-relaxation rates obtained with TEMPO in water ($353 \text{ s}^{-1}\text{M}^{-1}$).⁵² In contrast, CTD P1 sites in IA₃ have $k_\rho \sim 140 \pm 20 \text{ s}^{-1}\text{M}^{-1}$, which is a **slower rate than** that observed for waters solvating TEMPO in solution (Table 2). In efforts to understand the relatively low k_ρ values of the IA₃CTD P1 sites, we performed ODNP of the nitroxide IAP in buffer (Table 2). Scaling to IAP bulk values changed k_σ slightly; whereas k_ρ values of TEMPO and IAP were similar (Table 2); hence we can conclude that these lowered k_{low} values do not arise from the IAP spin label and must then reflect properties of the amino acid sequence and structure that impact water relaxation.

We note that the errors reported here for k_ρ in Table 2 are all similar because the value of k_ρ , as given in Equation 5,

$$k_\rho = \frac{1}{C_{SL}} \left(\frac{1}{T_{1(0)}} - \frac{1}{T_{1,0}} \right) \quad (5)$$

is dependent upon the inverse spin labeled protein concentration, C_{SL} as well as differences in $T_{1(0)}$ and $T_{1,0}$, which represent the relaxation times obtained for a spin-labeled sample and non-spin labeled sample; respectively. For IA₃CTD sites, the experimentally determined relaxation times for spin-labeled protein exhibited small changes in relaxation times compared to the non-spin-labeled sample (Table S7), leading to larger propagation of error in calculating values of k_ρ that originates from the error in the non-spin labeled $T_{1,0}$ measurement. Although, according to Franck et al., (47) greater certainty in $T_{1(0)}$ can be obtained by increasing protein concentrations, all values reported within were acquired for ~ 150 μ M protein samples.

For IA₃-NTD sites, k_{low} enhancements are approximately ~ 1.4 (defined as the ratio to k_{low} of TEMPO in water), which is similar to that obtained for the IDPs α -synuclein,^{80, 81} Δ tau-187^{63, 82} and 5- residue peptides⁵⁷ studied by Han and co-workers (Figure 6). Both the number of bound water molecules and values of the local correlation time modulate k_{low} enhancements. [53] For correlation times > 1 ns, k_{low} enhancements > 1 are reflective of a population of bound water molecules.⁵⁷ Accordingly, this implies that the IA₃-NTD, which has k_{low} enhancements ~ 1.4 has a sparse to no BW population; if we assume that the unstructured ensemble of IA₃ (7.6 kD) has $\tau_{rot} > 1$ ns. Rotational correlation times of semi-rigid globular proteins can be predicted to be and have been shown to be > 2 ns for proteins as small as 3 kD.⁸³ Han and co-workers concluded that a marked difference in hydration behavior of IDPs is the lack of BW harbored on the surface. This conclusion is based upon assuming $\tau_{rot} > 1$ ns for α -synuclein and Δ tau-187 proteins. The finding of similar ODNP results between IA₃-NTD and α -synuclein and Δ tau-187 may not be surprising given the sequence characteristics of these IDPs reside within in subclasses R1 and R2. It is noteworthy that k_{low} enhancements for the 5-mer CheY peptides,⁵⁷ double stranded DNA (12 bp and 24 bp)⁵⁹ and the folding chaperone GroES⁸² are similar to those of the IA₃-NTD (Figure 6).

In contrast, for CTD sites of IA₃ we observe k_{low} with fractional enhancements, meaning values of 0.2-0.3 were obtained. We currently cannot explain the molecular basis of these lowered enhancements. One possible explanation is that for IA₃ the assumption of $\tau_{rot} > 1$ ns may be incorrect. However, spectral simulation of CW X- and W-Band nitroxide line shapes of individual spin-label spectra within the IA₃ NTD and CTD demonstrate that the coupled spin-label and local backbone motion of IA₃CTD spin-labeled sites (a.a. 57-61) is **slower than** those observed over the NTD region (a.a. 7-15); in other words, CTD $\tau_c = 0.27 \pm 0.03$ ns $>$ NTD $\tau_c = 0.19 \pm 0.02$ ns. Therefore, if we assume $\tau_{rot} > 1$ for the NTD, then it follows this would also be true for the CTD. Additionally, the local correlation time of P1 sites in IA₃ are all greater than those for TEMPO in water, suggesting the enhancement values for k_{low} cannot be explained by the protein motion being faster than the small molecule or

that the dynamics of the CTD are too fast for the ODNP analysis of k_{low} . Our observation that k_{low} C-termini IA₃ $<$ k_{low} TEMPO in water along with k_{σ} C-termini IA₃ \approx k_{σ} N-termini IA₃ $<$ k_{σ} IAP is, to our knowledge, a unique finding in low-field ODNP studies of macromolecules.

Because k_{low} is obtained from the differences between the local electron-¹H cross-relaxation (k_σ) and local waters undergoing self-relaxation (k_ρ) as shown in Equation 6,

$$k_{low} = \frac{5}{3}k_\rho - \frac{7}{3}k_\sigma \quad (6)$$

k_{low} errors are also larger for CTD sites. Nevertheless, the data indicate ¹H-¹H self-relaxation within the CTD that is only 20-40% that of a spin-label in water (Figure 6).

Although the origin of the lowered self-relaxation (k_ρ) of local waters of the CTD are currently unknown, it might be possible that “bound water” interacts differently in a highly charged local environment than with a nitroxide in water. Perhaps the local electrostatic fields generated by the high local *NCPR* in the CTD (ranging between -3 to + 2) of IA₃ impact the slow-bound water relaxation properties differently than the bulk water-TEMPO interactions in solvent. Alternatively, the local dielectric constant may differ near the highly charged surface which can impact the organization of water hydrogen bonds with charged side chains that overcome the possible hydrophobic contacts between water and the spin-probe leading to a lessened effect on water self-relaxation. Hence, we postulate that a reason for the different values of ξ in the two domains in IA₃ arises from the way the “slower bound water molecules” interact with the highly charged surface of the CTD of IA₃ and that sequence charge distribution may modulate this behavior and be a way to “encode” IDP conformational ensemble and function. Future studies are underway to test this concept on additional R3, R4 and R5 sequences.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/1>. IA₃ sequence alignments, helical wheel projections, figures showing side-chain interactions in crystal complex, mass spectrometry results and summary of CD parameters discussed within the text.

Accession Codes

IA₃: P01094

AUTHOR INFORMATION

Corresponding Author

Gail E. Fanucci – Department of Chemistry, University of Florida, P.O. Box 117200, Gainesville, Florida 32611; Email: fanucci@chem.ufl.edu

Authors

Katie M. Dunleavy – Department of Chemistry, University of Florida, P.O. Box 117200, Gainesville, Florida 32611; Email: kmd@umn.edu

Tianyan Li – Department of Chemistry, University of Florida, P.O. Box 117200, Gainesville, Florida 32611; Email: tianyan.li@chem.ufl.edu

Afnan M. Jauffer – Department of Chemistry, University of Florida, P.O. Box 117200, Gainesville, Florida 32611; Email: m.mo-hamedjauffer@chem.ufl.edu

Shamon A. Walker - Materials Research Laboratory, University of California, Santa Barbara, California 93106, United States; Email: shamonwalker@ucsb.edu

Eugene Milshteyn – Department of Chemistry, University of Florida, P.O. Box 117200, Gainesville, Florida 32611; Email: eu-gene17uf@gmail.com

Author Contributions

K.M.D., E.M., A.M.J. performed mutagenesis, protein expression, purification, and spin-labeling of IA₃ for this work. K.M.D and S.W. performed and analyzed all low-field ODNP data. K.M.D. wrote the initial manuscript draft with E.M. E.M. performed CW EPR experiments, viscosity measurements and calculations of local tumbling volumes. T.L. performed all IA₃ sequence analysis. A.M.J. performed structural analyses, helped generate structural figures, calculated parameters related to tumbling volume changes and proofread final manuscript. K.M.D., A.M.J and G.E.F edited the final manuscript. All authors have read and approved of the manuscript.

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ABBREVIATIONS

TFE, 2, 2, 2-trifluoroethanol; ODNP, Overhauser dynamic nuclear polarization; IDP, intrinsically disordered proteins; NMR, nuclear magnetic resonance; CD, circular dichroism spectroscopy; MD, molecular dynamic simulations; CW-EPR, continuous wave electron paramagnetic resonance; WT, wild type; YPRA, yeast proteinase A inhibitor; DW, diffusive water; BW, bound water.

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TOC Graphic

