Modulating the Intrinsic Disorder in the Cytoplasmic Domain Alters the Biological Activity of the N-Methyl-D-aspartate-sensitive Glutamate Receptor*

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Background: The NMDA-sensitive glutamate receptors contain disordered cytoplasmic domains that support isoform-specific signaling.

Results: Proline residues dictate the conformational dynamics in disordered proteins, which were used to affect NMDA receptor activity.

Conclusion: The intrinsically disordered cytoplasmic domain is involved in specific modes of NMDA receptor regulation.

Significance: The underlying dynamics of protein disorder contribute to allosteric regulation.

The NMDA-sensitive glutamate receptor is a ligand-gated ion channel that mediates excitatory synaptic transmission in the nervous system. Extracellular zinc allosterically regulates the NMDA receptor by binding to the extracellular N-terminal domain, which inhibits channel gating. Phosphorylation of the intrinsically disordered intracellular C-terminal domain alleviates inhibition by extracellular zinc. The mechanism for this functional effect is largely unknown. Proline is a hallmark of intrinsic disorder, so we used proline mutagenesis to modulate disorder in the cytoplasmic domain. Proline depletion selectively uncoupled zinc inhibition with little effect on receptor biogenesis, surface trafficking, or ligand-activated gating. Proline depletion also reduced the affinity for a PDZ domain involved in synaptic trafficking and affected small molecule binding. To understand the origin of these phenomena, we used single molecule fluorescence and ensemble biophysical methods to characterize the structural effects of proline mutagenesis. Proline depletion did not eliminate intrinsic disorder, but the underlying conformational dynamics were changed. Thus, we altered the form of intrinsic disorder, which appears sufficient to affect the biological activity. These findings suggest that conformational dynamics within the intrinsically disordered cytoplasmic domain are important for the allosteric regulation of NMDA receptor gating.

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2 The abbreviations used are: CTD, C-terminal domain; GluN, NMDA-sensitive receptor subunit; IDP, intrinsically disordered protein; bis-ANS, 4,4′-dianilino-1,1′-binapthyl-5,5′-disulfonic acid; SEC, size exclusion chromatography; ANS, anilinonaphthalene sulfonic acid; smFRET, single molecule, fluorescence resonance energy transfer.
IDPs are not densely packed like the core of folded proteins. IDPs range in compactness from swollen "random" coils to disordered globules (14). Proline is strongly associated with intrinsic disorder, and proline content correlates with polypeptide expansion (15). Proline’s cyclic structure renders the polypeptide more rigid than other amino acids. Additionally, although the proline side chain is hydrophobic, proline’s lack of an NH group amino acid renders polyproline much more water-soluble than polyglycine or polyalanine (16). We took advantage of these unique properties of proline as a means of modulating the intrinsic disorder by systematically replacing proline residues in the GluN2B CTD.

By altering the conformation of the disordered CTD with proline depletion, we were able to uncouple physiological regulation of the NMDA receptor by extracellular Zn\(^{2+}\) while preserving basic functions associated with gating, biogenesis, and trafficking. As predicted, biophysical measurements found a direct correlation between proline content and polypeptide compaction. Specific proline residues were necessary to prevent aggregation of this otherwise soluble protein. Thus, proline could modulate the attractive potential of short linear motifs. In accordance with this notion, we also observed reduced binding affinity to a PDZ domain from the scaffold protein PSD-95.

Our studies show that proline residues are critical regulators of the conformational dynamics in intrinsically disordered polypeptides. The same mutations affected the biological activity of the intact receptor. These findings suggest that the underlying dynamics of the disordered cytoplasmic domain mediate the allosteric regulation of ionotropic glutamate receptor gating.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—The C-terminal domain 2 (CTD2) of GluN2B (residues 1259–1482), which is C-terminal to the palmitylation sites (17), was cloned into pPROEX HTB (Invitrogen) and expressed in the Rosetta strain of *Escherichia coli* (EMD Biosciences, San Diego). The His\(_8\) fusion was purified under reducing and denaturing conditions as described (18, 19). The protein was exchanged to nondenaturating conditions by extensive washing while bound to nickel-nitrilotriacetic acid-agarose. The His\(_6\) tag was removed with tobacco etch virus protease, and the protein was further purified with cation exchange and size exclusion (GE Healthcare). Purity was greater than 95% as verified using SDS-PAGE. The PDZ2 domain from PSD-95 (155–249) was cloned into pPROEX HTB and purified on nickel-nitrilotriacetic acid-agarose. The His\(_6\) tag was removed with sequencing grade trypsin or chymotrypsin (Promega, Madison, WI). Purified proteins were injected at 20 nM in 10 mM sodium phosphate, pH 7.4, 50 mM NaCl, 0.5 mM DTT at 4 °C. Purified proteins were injected at 20 μM. The apparent molecular weight (*M*\(_a\)) of soluble CTD2 constructs was obtained by calibrating the column with gel filtration standards (Bio-Rad). The hydrodynamic radius (*R*\(_h\)) was obtained through Equation 2 (21).

\[
\log(*R*_h) = -0.204 + 0.357 \times \log(*M*_a) \tag{2}
\]

Limited proteolysis experiments were conducted by adding sequencing grade trypsin or chymotrypsin (Promega, Madison, WI) to purified CTD2 at a 1:100 ratio (proteinase to sample). For each time point, an aliquot from the reaction was inhibited by adding 0.5 mM PMSF followed by a 1:1 dilution with 2× SDS-PAGE loading buffer and flash-frozen. The samples were resolved on 15% acrylamide gels.

**Ensemble fluorescence spectra** were collected using an ISS PCI photon counting spectrofluorimeter (ISS Inc., Champaign, IL). For 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (bis-ANS; Invitrogen), excitation was at 385 nm. For endogenous fluorescence, excitation was at 250 nm. All slits were set to 2 nm.

**Circular dichroism spectra** were collected using a Chirascan spectrometer (Applied Photophysics, Leatherhead, UK). Proteins were at 15 μM in 10 mM sodium phosphate, pH 7.4, 50 mM NaCl, at 20 °C.

**Fluorescent Labeling**—CTD2 has three native cysteines. All constructs was obtained by calibrating the column with gel filtration standards (Bio-Rad). The hydrodynamic radius (*R*_\(_h\)) was obtained through Equation 2 (21).

\[
%\text{ inhibition} = 100 \times \frac{(I_{\text{control}} - I_{\text{Zn}})}{I_{\text{control}}} \tag{1}
\]

where *I*\(_{\text{control}}\) is the average of *I*\(_{\text{pre}}\) and *I*\(_{\text{post}}\). Results are presented as mean ± S.E. Student’s *t* test was used to define statistical differences. Significance was defined at *p* < 0.05.

**Analytical size exclusion chromatography (SEC)** measurements were performed on a Shodex KW-802.5 column in 20 mM Tris, pH 7.5, 150 mM NaCl, 0.5 mM DTT at 4 °C. Purified proteins were injected at 20 μM. The apparent molecular weight (*M*_\(_a\)) of soluble CTD2 constructs was obtained by calibrating the column with gel filtration standards (Bio-Rad). The hydrodynamic radius (*R*\(_h\)) was obtained through Equation 2 (21).

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tein (S1323C) using site-directed mutagenesis. All other cysteines were mutated to serine. For studies on conformational dynamics, CTD2 was randomly labeled with an equimolar ratio of Alexa Fluor 555 C6 maleimide and Alexa Fluor 647 C2 maleimide (Invitrogen) overnight at 4 °C in 25 mM HEPES, pH 7.4, 300 mM NaCl, 0.5 mM tris(2-carboxyethyl)phosphine. For binding studies, CTD2 was singly labeled at Cys-1453 with Alexa Fluor 647. PDZ2 contains no native cysteines so one was introduced (M159C) using site-directed mutagenesis and labeled with Alexa Fluor 555. In all cases, free dye was removed by desalting on a PD-10 column (GE Healthcare) followed by dialysis.

Single Molecule Microscopy—Detailed procedures for our single molecule fluorescence measurements were described previously (22). Briefly, for intramolecular FRET measurements, CTD2 was encapsulated during extrusion within 100 nm liposomes made from egg phosphatidylcholine containing 0.1 mol % of biotin-phosphatidylethanolamine (Avanti Polar Lipids, Alabaster, AL). Liposomes were immobilized on a quartz microscope slide passivated with biotinylated BSA. For binding studies, biotinylated CTD2 was directly immobilized to the passivated quartz slide by streptavidin. For inter-molecular binding assay, 50 nM of donor-labeled PDZ2 was added to the observation buffer. To eliminate nonspecific binding to the surface, we further passivated the BSA-coated surface with egg phosphatidylcholine liposomes. The FRET signals were recorded using a prism-type total internal reflection microscope, detected by an iXon EMCCD camera (Andor Technologies, Belfast, UK). Samples were illuminated with an alternating laser sequence of 532 and 635 nm to identify single donor and acceptor dyes. All measurements utilized an oxygen scavenger (20 units/ml glucose oxidase, 1000 units/ml catalase) and a triplet-state quencher (100 μM cyclooctatetraene) to delay photobleaching and prevent rapid blinking.

FRET Transition Analysis—To characterize dynamic FRET signals, we analyzed single molecule trajectories using hidden Markov modeling as implemented in HaMMY version 4.0 (23). This algorithm determines the most probable time sequence of FRET states sampled by each molecule. For analysis of stochastic conformational transitions, we used the initial guesses provided by the software for a three-state system (24, 25). To investigate the compositional biases in the CTD of GluN2B, we used the Composition Profiler (26), which calculates the normalized content of a given residue in solution, $C_{\text{order}}$, and ends with the equation $C_{\text{order}} = C_{\text{order}} - C_{\text{order}}^\text{wild type}$. The PONDR VLXT score, which calculates the normalized content of a given residue in typical folded proteins, is shown in Fig. 1A.

RESULTS

Prediction of Disorder in the GluN2B CTD—Intrinsically disordered proteins lack the “order-promoting” amino acids (Cys, Trp, Ile, Tyr, Phe, Leu, His, Val, and Asn), which form the hydrophobic core of folded proteins, and instead contain amino acids that promote disorder (Ala, Gly, Arg, Thr, Ser, Lys, Gln, Glu, and Pro) (24, 25). To investigate the compositional biases in the CTD of GluN2B, we used the Composition Profiler (26), which calculates the normalized content of a given residue in our query protein relative to that found in typical folded proteins (27). For reference, we include the composition profile for typical disordered proteins (28).

The CTD of the GluN2B subunit (838–1482) is depleted in order-promoting amino acids, which would define a low propensity for independent folding (Fig. 1A). Relative to disordered proteins, GluN2B is slightly enriched in aromatic residues (Tyr and Phe), which are normally associated with order. This could define sites of protein interaction with GluN2B or local regions of the CTD that could become ordered. The GluN2B CTD contains a palmitoylation site, which breaks the CTD into two subdomains CTD1(838–1258) and CTD2(1259–1482). The com-
positional profile for CTD2 is shown separately in Fig. 1A. CTD2 is enriched in proline with 19 proline residues distributed with regular spacing (11 ± 8 residues). Proline enrichment is typical of disordered proteins so the DisProt database is similarly enriched in proline.

Proline Is Necessary for Solubility—CTD2 contains both Src phosphorylation sites associated with alleviating Zn2+ inhibition. To modulate the conformation of this intrinsically disordered domain, we removed the proline residues from CTD2 by proline depletion. To modulate the conformation of this intrinsically disordered CTD2 insoluble (data not shown). The proline-depleted construct expressed well and remained soluble in 2 M urea. Below 2 M urea, the construct formed an irregular precipitate. Because we observed no differences between serine and alanine, subsequent studies focused on alanine substitution.

Based on the disorder predictor PONDR-VLXT (Fig. 1B), the first half of CTD2 is expected to be highly disordered, but this tendency decreases in the central 100 residues. The substitution of proline noticeably decreased the propensity of disorder in several regions. The most dramatic effect was linked to removal of prolines from the central region. Analysis with Zyggregator, which predicts protein aggregation (29), revealed that CTD2 contains aggregation-prone regions, but these are typically short (Fig. 1C). The aggregation propensity of the central region (residues 1356–1409) is most drastically affected by the proline depletion with the overall aggregation score approaching the value of 1.0. Thus, the central region has a higher potential to become ordered upon proline depletion and is likely to become aggregation-prone.

To experimentally assign the proline residues needed for solubility, we divided CTD2 into three regions with equal proline content as follows: N-terminal (residues 1259–1355, 7 prolines), central (residues 1356–1409, 6 prolines), and C-terminal (residues 1410–1482, 6 prolines) (Fig. 2A). We then depleted each region of prolines. To denote the region of proline depletion, we adopted a binary nomenclature, where 0 (or white) is used to represent the presence of endogenous prolines, and 1 (or black) is used to represent the substitution of all prolines in a region to alanine (Fig. 2A). Wild type is denoted by 000, and the completely proline-free CTD2 is denoted by 111. Five intermediate constructs were created designated 010, 001, 011, and 101.

On SDS-PAGE, wild type CTD2 shows anomalous electrophoretic mobility, migrating as a protein with larger molecular mass (Fig. 2B). Anomalously high electrophoretic mobility is typical for IDPs (30). The proline-depleted construct (111) showed increased electrophoretic mobility but still migrated slower than expected based on mass. Constructs 100, 101, and 111 showed increased mobility, and the other constructs showed mobility similar to that of wild type CTD2. Thus, electrophoretic mobility did not correlate with proline content. The electrophoretic mobility difference was only associated with proline depletions in the N-terminal third of the polypeptide. This suggests that specific proline residues affect the detergent-induced, denatured state of CTD2.

Each construct was tested for solubility by extracting the protein in physiological buffers followed by centrifugation to remove insoluble material. Solubility was assessed by comparing SDS-PAGE before and after sedimentation (data not shown). Interestingly, solubility did not directly correlate with the number of proline mutations (Fig. 2A, right column). The most depleted construct remained soluble (101,13 prolines replaced). In contrast, depletion of six prolines from the central region (010) was sufficient for insolubility, as predicted by Zyggregator. To narrow down the proline residues controlling aggregation, we mutated individual alanines in 010 back to prolines (Fig. 2C). Restoring only three proline residues, 1356, 1377, and 1387, was sufficient to restore partial solubility, although any additional proline completely restored solubility to wild type levels. To avoid complications with aggregation, all
subsequent experiments used mutants that were soluble in recombinant expression unless otherwise indicated.

**Proline Depletion Uncouples Biological Regulation of the Glutamate Receptor**—In NMDA receptors, the divalent cation Zn\(^{2+}\) inhibits the opening of the ion channel in response to glutamate (1, 5). Zn\(^{2+}\) acts at the extracellular N-terminal domain. Src kinase phosphorylation in CTD2 can alleviate Zn\(^{2+}\) inhibition (6). Previously, we showed that Src phosphorylation causes a uniform expansion of CTD2 (19). Thus, the conformational dynamics of CTD2 may be important in allosteric regulation of GluN2B-containing NMDA receptors.

To address whether the conformational dynamics of CTD2 contribute to channel gating, we introduced proline depletions into the full-length GluN2B subunit and recorded membrane currents in NMDA receptors using patch clamp electrophysiology. To avoid complications associated with aggregation, we used the soluble 101 construct, which lacks proline near both Src phosphorylation sites. NMDA receptors are obligate heteromultimers, requiring the GluN1 and GluN2 subunits to form functional receptors. We therefore co-expressed GluN1 with either wild type GluN2B or mutant GluN2B(101).

For wild type GluN1/GluN2B, glutamate application yielded large inward currents (Fig. 3A, black). In the presence of 1 \(\mu M\) Zn\(^{2+}\), glutamate-activated currents were reduced in amplitude (Fig. 3A, red), as expected (6). GluN1/GluN2B(101) receptors also showed prominent glutamate-activated currents (Fig. 3B, black). This confirms that proline depletion did not impede subunit oligomerization, trafficking to the membrane, or receptor gating.

In contrast to wild type GluN2B, the amplitude of glutamate-activated currents with GluN2B(101) was unaffected by Zn\(^{2+}\) (Fig. 3B, red). Overall, 1 \(\mu M\) Zn\(^{2+}\) reduced current amplitudes by 51.5 ± 2.4% in wild type GluN1/GluN2B, whereas in GluN1/GluN2B(101), the inhibitory effect of Zn\(^{2+}\) was significantly lessened to 15.0 ± 2.1% (Fig. 3C). This phenotype, where the receptor is less sensitive to extracellular Zn\(^{2+}\), is highly reminiscent of that associated with Src kinase phosphorylation of GluN2B (6). These results are consistent with the idea that the conformational dynamics of CTD2 can exert allosteric effects on NMDA receptor gating.

**Proline Depletion Affects Protein Interactions**—The NMDA receptor binds the scaffold protein PSD-95 through a short linker motif at the C terminus (residues 1476–1482) that interacts with the second PDZ domain (PDZ2) from PSD-95. We used the soluble 101 construct, which lacks proline near both Src phosphorylation sites. NMDA receptors are obligate heteromultimers, requiring the GluN1 and GluN2 subunits to form functional receptors. We therefore co-expressed GluN1 with either wild type GluN2B or mutant GluN2B(101).

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**Proline Depletion Affects Protein Interactions**—The NMDA receptor binds the scaffold protein PSD-95 through a short linear motif at the C terminus (residues 1476–1482) that interacts with the second PDZ domain (PDZ2) from PSD-95. We used smFRET to measure this interaction. The palmitoylation sequence from CTD2 was replaced with a biotinylation site, which allowed optically resolved, single molecules to be attached to the microscope slide using streptavidin (Fig. 4A). The slide was passivated with biotinylated BSA and phospholipids, which prevents nonspecific binding but also surrounds CTD2 with proteins and lipids. Such directional attachment mimics the native geometry and environment of CTD2. CTD2 was labeled with an acceptor dye at the native cysteine Cys-1453. This labeling site is 23 residues away from the PDZ-binding motif so the dye should not impede PDZ binding, which involves the terminal six residues. The isolated PDZ2 domain from PSD-95 was labeled with the donor dye at an introduced cysteine chosen, based on the NMR structure (31), so as not to occlude the ligand-binding site. This labeling scheme gives rise to mid-FRET (~0.6) when the proteins are bound, and no FRET when the proteins are dissociated (data not shown).

Representative traces show stochastic bursts of FRET efficiency that correspond to individual protein binding events (Fig. 4B). We used hidden Markov modeling (23) to analyze the sequence of FRET events and extract the dwell times in the bound and unbound states. The individual dwell times were compiled into histograms, which were analyzed with nonlinear fitting to extract the kinetic rate constants for protein interactions, \(k_{on}\) and \(k_{off}\) (Fig. 4C).
Proline Depletion Affects Small Molecule Binding

To test the effect of proline depletion on this protein interaction, we used the soluble 001 construct, which lacked prolines in the C-terminal region that contains the PDZ ligand. None of the proline mutations directly affected residues involved in PDZ binding. Nonetheless, proline depletion reduced the measured $K_D$ by 18% to $388 \pm 12$ nM. Analysis of the rate constants showed that this effect arises solely from a significant increase in $k_{off}$ while $k_{on}$ remained constant (Fig. 4C). A similar reduction in $K_D$ through increased $k_{off}$ was also observed when CTD2 was phosphorylated by Src kinase (Fig. 4C). That $k_{on}$ was unaffected suggests that the exposure of the PDZ-interacting motif was not altered. The increase in $k_{off}$ is puzzling given that the actual ligand sequence is unaltered, but it suggests that conformational dynamics can affect ligand affinity. Hence, the bound state becomes less stable compared with the native protein.

Proline Depletion Affects Small Molecule Binding—IDPs can bind small molecules making them potential drug targets (34). The binding of the small molecule ANS has long been used to probe for protein folding (35). This environmentally sensitive dye undergoes an increase in fluorescence quantum yield as well as a blue-shift in the emission spectrum when bound to nonpolar cavities in proteins. In the presence of wild type CTD2, the fluorescence emission intensity of bis-ANS increased by 2.5-fold (relative to bis-ANS in buffer), and the peak in the emission spectrum ($\lambda_{max}$) was blue-shifted from 533 nm to near 490 nm (Fig. 4D). Thus, CTD2 has a nonpolar site that is capable of binding small molecules. Proline depletion further increased the bis-ANS fluorescence but did not alter the $\lambda_{max}$ relative to wild type CTD2. Proline depletion either causes tighter bis-ANS binding or protects bis-ANS from solvent exposure. A single proline-depleted region increased bis-ANS fluorescence by 7.0- and 9.8-fold (relative to buffer) for the 100 and 001, respectively. The 001 construct showed a 13.4-fold increase suggesting that the effects are additive. Thus, proline depletion affected both protein binding and small molecule binding.

Proline Depletion Causes Polypeptide Compaction—To characterize the structural effects of proline depletion, we measured the ensemble radius of hydration ($R_{H}$) using analytical SEC. Wild type CTD2 showed an $R_{H}$ of $3.49 \pm 0.1$ nm (Fig. 5A). Based on the published mass dependence of $R_{H}$ for proteins in different conformational states (21), our measured $R_{H}$ value is larger than expected for a folded protein of 24.4 kDa (2.3 nm). In 6 M guanidinium chloride, the $R_{H}$ value of CTD2 increased to $4.59 \pm 0.02$ nm (data not shown), which agrees well with the predicted value for a denatured protein (4.6 nm) or an extended random coil (4.1 nm). The $R_{H}$ under native conditions was close to that expected for a pre-molten globular protein (3.4 nm). Thus, $R_{H}$ for CTD2 is closer to a random coil than a folded state.

For the interaction of wild type CTD2 with PDZ2, the measured rates correspond to an equilibrium dissociation constant ($K_D$) of $270 \pm 24$ nM. This $K_D$ is slightly higher than published values based on ensemble measurement of peptide binding in solution, which report values near 1 $\mu$M (32, 33).

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**FIGURE 4. Modulating intrinsic disorder affects PDZ interactions and small molecule binding.** A, schematic depicting the smFRET protein binding assay. CTD2 is biotinylated on the N terminus and surface-immobilized through biotin-streptavidin interactions on a passivated surface. CTD2 is labeled with an acceptor dye 23 residues away from the C-terminal PDZ ligand. The PDZ2 domain from PSD-95 is labeled with a donor dye and added in solution above the lipid bilayer. B, representative data showing individual protein binding events used for the measurement of dwell times. The presence of acceptor intensity defines the bound state. Gaps between binding events define the unbound state. A.U., arbitrary units. C, rate constants for PDZ2 binding ($k_{on}$, left axis, filled circles) and unbinding ($k_{off}$, right axis, open circles). Construct 001 is proline depleted in the region preceding the PDZ-binding site. Phosphorylation with Src kinase is indicated above the panel. Data are presented as mean ± S.E. * indicates values statistically different from both proline-depleted and phosphorylated CTD2 ($p < 0.05$, Student’s t test). D, fluorescence emission spectra of bis-ANS in the presence of CTD2 and the proline-free constructs. Spectra were collected using 1 $\mu$M bis-ANS with 0.1 $\mu$M protein. Protein-free buffer (yellow); wild type CTD2 (orange); Chimera 100 (red); Chimera 001 (blue); Chimera 101 (purple).
Only the soluble proline-depleted constructs could be analyzed with SEC under native conditions. All three soluble constructs (100, 001, and 101) had a longer elution time in SEC than wild type indicating that proline depletion caused a decrease in RH. Constructs 100 and 001 showed a similar RH of 3.42 ± 0.01 and 3.40 ± 0.03 nm, respectively, with 101 showing a further reduction in RH to 3.33 ± 0.01 nm (Fig. 5A). Thus, there was a direct linear correlation between RH and the number of prolines removed.

Conformational Effects of Proline Depletion—Proline is often thought of as a “structure breaker.” Thus, proline depletion could result in the formation of regular secondary structure. To examine this possibility, we measured circular dichroism for the four soluble constructs WT, 100, 001, and 101. All four spectra showed a single minimum at 200 nm, which is characteristic of disordered polypeptides (Fig. 5B). Thus, proline depletion did not induce detectable changes in secondary structure in CTD2.

Limited proteolysis can assess the exposure of protease cleavage sites, which provides another measure of how proline depletion altered the conformation. We compared the soluble construct 101 to wild type CTD2. Trypsin cleaves at lysine or arginine residues, except those followed by proline, of which there are two. For wild type, trypsin digestion was largely complete within 5 min (Fig. 5C, top row). Proline depletion had no effect on the kinetics of trypsin digestion but did result in more protected intermediates and a low molecular weight product that was protected for 30 min before disappearing. Chymotrypsin cleaves at large hydrophobic or aromatic amino acids but also disfavors residues followed by proline, of which there is one. Proline depletion slightly slowed the kinetics of chymotrypsin digestion, but the differences were not statistically significant (Fig. 5C, bottom row). However, the digestion pattern did change for 101. There were fewer protected intermediates with only two major species in 101 as compared with four major bands in wild type. Thus, proline depletion does not decrease accessibility of the aromatic residues, which remain accessible to chymotrypsin. In agreement with this notion, the endogenous fluorescence for both constructs was readily quenched by acrylamide with nearly identical concentration dependence (data not shown).

FIGURE 5. Conformational effects of proline depletion. A, changes in analytical size exclusion chromatography of CTD2 upon proline depletion. Data are plotted as hydrodynamic radius (RH) (left axis, filled circles), RH was determined from the apparent molecular weight in reference to globular protein standards (21). For reference, data are also plotted as elution time normalized by the void volume of the column (Ve/V0) (right axis, open circles). B, circular dichroism spectra for WT CTD2 (solid circles) along with soluble proline-depleted CTD2 constructs 100 (open circles), 001 (open squares), and 101 (open triangles). C, limited proteolysis of wild type (left column) and the proline-depleted construct 101 (middle column). Equal concentrations of both proteins were used in the reaction. The graph (right column) shows the kinetics of the disappearance of the full-length protein. The mean intensity at the position of full-length CTD2 was determined using ImageJ for wild type (red) and 101 (black). Lines show the fit to a mono-exponential decay. The top row shows proteolysis by trypsin. The bottom row shows proteolysis by chymotrypsin. At the indicated time points, a sample of the reaction was removed and inhibited to stop further degradation. Molecular mass markers are indicated to the left of the panel.
To probe the conformational dynamics of individual CTD2 molecules, we used intramolecular smFRET. Donor and acceptor fluorophores were attached to a single CTD2 polypeptide with a contour length of 38 or 30 nm between labeling sites. Proteins were encapsulated inside 100 nm liposomes, which could be surface-tethered while maintaining free diffusion of the encapsulated polypeptide. The two insoluble constructs, 010 and 011, were maintained in urea during purification and could be surface-tethered while maintaining free diffusion of the polypeptide, which can be observed as a shift to low FRET values upon phosphorylation.

As described previously (18), single molecules of wild type CTD2 showed stochastic switching of FRET efficiency on the second time scale (Fig. 6A). The origin of this phenomenon is not clear as it could involve changes in both dye separation and photophysical parameters. Either way, it is caused by the polypeptide (18). Proline cis-trans isomerization also occurs on the second time scale so we initially assumed that proline was contributing to the phenomenon. Interestingly, proline depletion reduced the number of molecules that showed any stochastic FRET transitions. Thus, half of the molecules show steady FRET levels indicating a loss of slow time scale transitioning (Fig. 6B). Because of our time resolution, the stable FRET could arise from time averaging of rapid dynamics or attainment of an ordered state, which are not distinguishable with this measurement. However, for those molecules possessing FRET transitions, the proline-depleted constructs showed similar transition rates to the wild type (1.05 ± 0.01 and 1.07 ± 0.09 s⁻¹, respectively). Although all proline depletion constructs showed reduced transitioning, the insoluble constructs, 010 and 011, showed more transitions making them closer to the wild type (Fig. 6B).

Data from all single molecules were compiled into histograms that show the distribution of FRET states sampled by CTD2 (Fig. 6C). The histograms for all constructs were well fit by two Gaussian functions (data not shown). For wild type, these peaks were centered at FRET values of 0.36 ± 0.06 and 0.61 ± 0.03 with the major population being the low FRET state (60 ± 15% versus 40 ± 15%, respectively). The FRET distributions for the proline-depleted constructs were shifted to higher FRET efficiency with peaks at 0.42 ± 0.03 and 0.70 ± 0.01 with the major population being the high FRET state (40 ± 5% versus 60 ± 5%, respectively). The shift toward higher FRET is consistent with an increase in compactness. However, unlike the SEC results, the magnitude of the smFRET shift was constant and did not depend on the number prolines replaced. All proline-depleted constructs showed a very similar distribution of FRET states.

Src phosphorylation of CTD2 led to a general expansion of the polypeptide, which can be observed as a shift to low FRET

**FIGURE 6. Single molecule FRET measurements of conformation and dynamics.** A, representative data for a CTD2 molecule undergoing stochastic FRET transitions. Bar above the panel shows the sequence of laser illumination used to identify acceptor fluorophores (red) and excite donor fluorophores for the measurement of FRET (green). Panel shows acceptor (magenta) and donor (cyan) emission over time. The acceptor photobleaches at 12 s with an anticorrelated increase in donor fluorescence followed by donor photobleaching at 20 s. A.U., arbitrary units. B, fraction of single molecules that showed stochastic FRET transitions. The construct measured is indicated below each bar. Data are presented as mean ± S.E. * indicates significant difference from wild type (p value < 0.05, two-tailed t test). C, histogram of all FRET states visited by CTD2 molecules. The histogram contains both static and dynamic molecules. Constructs: wild type (black); 100 (red); 010 (brown); 001 (blue); 011 (purple); 101 (dashed line). D, conformational effects of Src phosphorylation on the proline-depleted construct 101. Protein was doubly labeled with donor and acceptor dye and phosphorylated by Src kinase in solution before encapsulation. Histogram shows all FRET values sampled by individual molecules confirmed to have a single donor-acceptor pair. As with the wild type protein (19), there is a shift to lower FRET values upon phosphorylation.
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...efficiency (19). The proline-depleted construct 101 also showed an increased sampling of lower FRET states (Fig. 6D). Unlike wild type CTD2 (19), the higher FRET state was still predominant even after phosphorylation. Proline depletion does not prevent the polypeptide expansion induced by Src phosphorylation.

DISCUSSION

The disordered cytoplasmic domains in NMDA receptors enable isoform-specific trafficking and signal transduction despite the conservation of their ordered domains (3). This supports distinct pharmacological selectivity. GluN2B imparts sensitivity to inhibition by extracellular zinc, which binds to the extracellular N-terminal domain and alters gating of the transmembrane ion channel. The mechanism for this allosteric modulation remains unknown. Tyrosine phosphorylation of CTD2 by Src kinase is known to alleviate Zn$^{2+}$ inhibition, but nothing is known about how the CTD modulates gating. We showed that Src phosphorylation increased $R_{1p}$, indicating polypeptide expansion (19). Thus, we reasoned that alterations in the underlying dynamics of this disordered region were involved in allosteric regulation of GluN2B-containing receptors.

The Src phosphorylation sites (Tyr-1336 and Tyr-1472) are in CTD2, which is enriched in proline (Fig. 1A). Meta-analysis of naturally occurring IDPs suggested that proline residues favor more extended states (15), so we used proline mutagenesis to modulate the conformational dynamics of CTD2. Depleting prolines near the Src phosphorylation sites eliminated Zn$^{2+}$ inhibition without deleterious effects on receptor biogenesis, surface trafficking, or ligand-induced gating. Mutation of 13 prolines to alanine prevented allosteric regulation of receptor gating by Zn$^{2+}$, which is the same effect as Src phosphorylation.

Proline depletion, as well as Src phosphorylation, decreased the affinity of GluN2B for a biological ligand, the PDZ2 domain of PSD-95. PDZ domains recognize short linear motifs of 4–10 residues, typically at the C terminus (36). Our nearest proline mutation was more than 20 residues from the PDZ-binding motif. If the effects arose from compaction and fly-casting, then we should have observed changes in $k_{on}$ rather than decreased kinetic stability (i.e. $k_{off}$). The PDZ ligand adopts an extended β-sheet conformation in the bound state. Global differences in the conformational dynamics of GluN2B may be altering the energetics of this transition. The reduced affinity for biological binding partners could have a direct effect on synaptic targeting.

We used a variety of biophysical methods to understand the changes in conformational dynamics induced by proline depletion in CTD2. Statistical, computational, and experimental results have all demonstrated that polypeptide expansion is driven by amino acid properties such as net charge and hydrophobicity. Here, we provide direct experimental confirmation, using several distinct methods, that compaction in a single IDP directly correlated with the number of proline residues in the polypeptide.

Proline was not necessary to maintain the disordered state. Proline depletion did not cause CTD2 to fold as evidenced by circular dichroism and limited proteolysis, which still proceeded rapidly with kinetics similar to the wild type protein. The proline-depleted constructs remain disordered, but the underlying dynamics have changed leading to global rearrangements. Single molecule fluorescence showed that proline depletion caused a loss of stochastic transitions. This implies that the dynamic time scale of disordered motions has increased.

Proline was required for polypeptide solubility. Proline has a hydrogen bond acceptor but no donor, which makes sequestering proline from solvent energetically unfavorable. Proline is more frequently on protein surfaces than valine, which also has three aliphatic carbons (37). CTD2 solubility was context-dependent rather than related to overall proline content. Specific proline residues have similarly been linked to aggregation in α-synuclein, a disordered protein linked to Parkinson disease (38). The induction of novel intermolecular aggregation pathways suggests that proline can control the attractive potential of internal linear motifs.

Src phosphorylation drives CTD2 toward a more extended state but had no effect on stochastic transitions (19). Proline depletion had the opposite effect on the conformation, but both manipulations had the same effect on receptor gating and PDZ binding. This suggests that the “ground state” conformation may be optimized for these functions. Our results suggest that the time scale and extent of dynamic motions in the intrinsically disordered regions are modulating NMDA receptor function. Proline residues can be used to manipulate this dynamic behavior and at the same time manipulate biological activity. Although we cannot rule out a folded state for CTD2 in vivo, these findings suggest that conformational dynamics are linked to biological activity.

Intrinsic disorder is genetically encoded by a lack of hydrophobic and order-promoting amino acids. Disordered proteins do not “fail” to fold. The dynamic nature of intrinsically disordered proteins was a recent evolutionary adaptation that is associated with mammalian signal transduction and multiprotein interactions (8). Disordered proteins can interact with multiple ligands by adopting different structures (11). However, not all disorder proteins function through a transition to an ordered state.

The conformational dynamics in disordered regions result in the polypeptide sampling a much larger volume than a folded protein of the same mass. The increased volume allows IDPs to function as entropic barriers in the nuclear pore (39). Disordered linkers can modulate supertertiary structure in multidomain proteins (40) and have been proposed to enable fly-casting (41), wherein IDPs “search” a large volume for potential ligands. In such instances, variation in compaction could regulate the biological activity at the level of individual proteins without invoking a folded state.

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