THE EFFECTS OF PRIMARY SEQUENCE PERTURBATION ON THE
STRUCTURE OF INTRINSICALLY DISORDERED PROTEINS

by

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DEDICATION

To my parents, Melissa and Richard Ricard, to whom I express extreme gratitude for their patience and support of both my scientific career and my personal life.
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<tr>
<td>2xYT</td>
<td>16 g/L Tryptone, 10 g/L Yeast Extract, 5.0g/L NaCl</td>
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<td>A</td>
<td>Alanine (amino acid sequences) or Adenine (DNA sequences)</td>
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<tr>
<td>Ampicillin-r</td>
<td>Ampicillin resistance gene, produces β-lactamase</td>
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<td>BME</td>
<td>2-mercaptoethanol</td>
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<td>CD</td>
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<td>D</td>
<td>Aspartate</td>
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<tr>
<td>DEAE</td>
<td>DEAE-Sepharose</td>
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<tr>
<td>df</td>
<td>Degrees of freedom</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DNP-Aspartate</td>
<td>N-2,4-DNP-L-aspartic acid</td>
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<tr>
<td>DOS</td>
<td>Density of States</td>
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<tr>
<td>E</td>
<td>Glutamate</td>
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<tr>
<td>E_r</td>
<td>Energy of general microstate $r$</td>
</tr>
<tr>
<td>F</td>
<td>F-statistic</td>
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<tr>
<td>F</td>
<td>Phenylalanine</td>
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<td>FASTA</td>
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G...............................................Glycine (amino acid sequences) or Guanine (DNA sequences)
GLY ........................................................................................................... Glycine
GuHCl ....................................................................................................... Guanidinium hydrochloride
GWAS ....................................................................................................... Genome-wide association studies
H.............................................................................................................. Histidine
HDM2 ...................................................................................................... E3 ubiquitin-protein ligase Mdm2
HIS ........ Histidine, or denoting the sequence of six histidines used for thrombin cleavage
I................................................................................................................... Isoleucine
IDP ........................................................................................................... Intrinsically Disordered Protein
IDR ........................................................................................................... Intrinsically Disordered Region
IPTG ................................................................. Isopropyl β-D-1-thiogalactopyranoside
K.................................................................................................................. Lysine
K_a .............................................................................................................. Number of non-synonymous mutations
k_b ........................................ Boltzmann’s Constant, 1.38064 · 10^{-23} \, \text{m}^2 \, \text{kg} \, \text{s}^{-2} \, \text{K}^{-1}
K_d ............................................................................................................ Retention factor for SEC
kDa ............................................................................................................. Kilodalton
K_s .............................................................................................................. Number of synonymous mutations
L.................................................................................................................. Leucine
lacI ............................................................................................................. Gene for lacI, the lac repressor protein
lacO ......................................................................................................... Binding site for lac repressor protein
lacO1 ................................................................. Binding site for lac repressor protein
LB ................................................................. Lysogeny broth
M ........................................................................ Methionine
M11p53 .................. p53(1-93) with 11 middle-most prolines substituted to glycine
MRE ................................................................. Molar residual ellipticity
ANOVA ............................................................... Analysis of variants
mRNA ................................................................. Messenger RNA
MS ........................................................................ Mean of squares
N ........................................................................ Asparagine
N11p53 .......... p53(1-93) with 11 prolines closest to N-terminus substituted to glycine
NaAc ................................................................. Sodium Acetate
NMR ................................................................. Nuclear Magnetic Resonance
Ori_pUC ............................................................. Bacterial origin of replication
P ................................................................. p-value
P ........................................................................ Proline
P_Amp ............................................................... Promoter for the Ampicillin gene
P_lacI ................................................................. Promoter of lacI gene
P_T5_Inducible ........ Promoter sequence for the T5 bacteriophage RNA polymerase
P_T7_Inducible ........ Promoter sequence for the T7 bacteriophage RNA polymerase
p53 ................................................................. Tumor suppressor protein p53
p53(1-93) ........................................................... First 93 residues of p53
p53(93-1) .................. p53(1-93) transcribed from C-terminus to N-terminus
PDB.................................................................Protein Data Bank

PP$_{II}$.........................................................Polyproline-II helix

Pr.............................................................Probability of microstate $r$

PRO.........................................................Proline

PrP$^c$.........................................................Human prion protein

PSI..........................................................Pounds per square inch

Q..............................................................Glutamine

R..............................................................Arginine

RBS.........................................................Ribosome binding sequence

RF11p53....................................................N11p53 transcribed from C-terminus to N-terminus

R$_h$...........................................................Hydrodynamic Radius

RL11p53.....................................................C11p53 transcribed from C-terminus to N-terminus

RM11p53.....................................................M11p53 transcribed from C-terminus to N-terminus

RNA..................................................................Ribonucleic Acid

RPM............................................................Rotations per minute

S.................................................................Serine

SDS............................................................sodium dodecyl sulfate

SDS...........................................................sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEC.............................................................Size Exclusion Chromatography

SOC............... 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl$_2$, 10 mM MgSO$_4$, and 20 mM glucose
SS ................................................................. Sum of squares
T .................................................. Threonine (amino acid sequences) or Thymine (DNA sequences)
TAD ........................................................................ Transactivation Domain
Term_bla ........................................................... transcription termination sequence for Ampicillin-r
Term_rpoC .......................................................... Transcription termination sequence for rpoC
Term_T7 .......................................................... Termination sequence for bacteriophage T7 polymerase
TGS ........................................................................ 25 mM TrisHCl, .192 M Glycine, 0.1% SDS
TP53 ........................................................................ Gene encoding p53
Tris-HCl .......................................................... Tris(hydroxymethyl)aminomethane hydrochloride
V ................................................................................. Valine
V₀ .............................................................................. Elution time of Blue Dextran
Vₑ .............................................................................. Elution time of sample
Vₜ .............................................................................. Elution time of DNP-Aspartate
W .............................................................................. Tryptophan
Y .............................................................................. Tyrosine
Z .............................................................................. Partition Function
β .............................................................................. 1/k_BT
ΔA .............................................................................. Differential Absorption
ABSTRACT

Intrinsically disordered proteins (IDPs) are a class of proteins that do not converge to a set of similar, energetically stable tertiary folds, but rapidly fluctuate between a wide variety of different accessible energy states. Previous work established the dependence of hydrodynamic radius ($R_h$) on intrinsic conformational propensity, particularly that of the polyproline-II ($PP_{II}$) helix, which is in turn dependent on the primary sequence. To explore this relationship between primary sequence and structure, six IDPs having the same number and type of each amino acid derived from the intrinsically disordered N-terminus of p53, were created by substituting 11 prolines of the total 22 prolines, which has a high propensity to form the $PP_{II}$ helix, to glycine, which has a low propensity to form the $PP_{II}$ helix, in different locations; the N-terminus, the C-terminus, and the middle of the protein, as well as their retro-transposed variants, i.e. translating from the original C-terminus to the original N-terminus. Additionally, the wild-type p53, as well as its retro-transposed variant, was isolated and purified to further test direct directional dependence of structure. The propensity to form the $PP_{II}$ helix is measured by circular dichroism spectroscopy, and $R_h$ is measured through size exclusion chromatography. Results showed a significant ($p < 0.05$) dependence of structure on directionality, as measured by differences in $R_h$; furthermore, circular dichroism spectra suggests that this collapse in structure is due to reduced occupancy of the $PP_{II}$ helix. Both directionality and location of amino acids appear to modulate the structure of IDPs, indicating local charge-driven effects.
I. INTRODUCTION

Overview

Understanding the structure and function of biological macromolecules is of importance in elucidating the life-sustaining mechanisms of the natural world and the molecular origins leading to diseased states. Proteins, a polymer made of amino acids linked together through peptide bonds, perform a variety of functions in biological organisms. Understanding the physical three-dimensional fold of proteins provides extensive information regarding their energetic landscape, which is a map of physical conformation to thermodynamic properties [1]. Such fundamental understanding of the generalized thermodynamic laws of proteins can be used in the creation of novel protein structures for a variety of industrial purposes, as well as open avenues for potential protein-targeted drug design. The traditional paradigm for understanding proteins, holds that the primary sequence dictates the secondary structure, and the secondary structure determines the tertiary structure [2]. However, this model fails to provide meaningful context for understanding the structure of intrinsically disordered proteins (IDPs), which are proteins that do not have stable tertiary folds. As a result, it is challenging to analyze structures that are not suited for crystallization via x-ray diffraction, which is the gold standard in structural determination of biological macromolecules [3].

Intrinsically disordered proteins are defined as proteins that rapidly fluctuate between a wide variety different microstates, or energetically accessible conformations, in contrast to folded proteins, which converge towards a system of more closely related microstates. Around 33% of all eukaryotic proteins are estimated to be disordered or contain intrinsically
disordered regions (IDRs) [4]. IDPs play diverse roles in cells, particularly in nucleic acid and protein recognition. Genome-wide association studies (GWAS) have shown that protein mutations involved in human cancers are statistically enriched with IDPs [5]. Prediction of intrinsic disorder can be made based solely on properties observed to be enriched in IDPs, such as the number of charged and hydrophilic amino acids, regardless of sequence position, as IDPs lack the extensive, site-specific tertiary contacts relative to folded proteins [6, 7]. Though the traditional structure-function paradigm of proteins holds that tertiary structure dictates the functionality of proteins, these disordered proteins do not contain a fixed tertiary structure and still retain functionality [8]. From a physical perspective, statistical-thermodynamic stability still exists within IDPs in the cellular environment [9]. Despite lack of a rigid structure, IDPs can adjust to a state of lower energy due to the system of dynamic equilibrium of distinct conformations. IDPs can adjust to a state of lower energy in a given environment than a protein more constrained to only accessing a smaller number of energetic states. [10]. An example of a common functional motif of IDPs that a folded protein cannot mimic is a coupled binding-folding mechanism, in which binding of a ligand by an IDP induces a conformation change stabilizing the IDP into a folded state [8]. This transition is analogous to, but fundamentally different than, binary structural changes such as the T to R transition in hemoglobin as a result of oxygen binding [11]. Instead of converting from one stable conformation to another, however, the IDP moves between a wide variety of ensembles. Many fundamental processes in nature depend heavily on the structure of IDPs and other amorphous polymers, and these flexible segments are uniquely able to compliment the certain roles of biological processes that structures with tertiary stability cannot [12].
From an experimental standpoint, direct understanding of the properties of both the general IDP and specific ones (in this study, the N-terminus of p53) provide a new avenue to target these biologically relevant polymers for treatment of disease, targeted drug design, antibody design, and other practical applications [13-15]. Furthermore, theoretical understanding of these IDPs pushes the boundary of current knowledge in many different fields, such as biophysics, structural biology, and evolutionary biology, as due to fundamentally different properties than traditionally studied folded proteins, IDPs open a new aperture for tying together interdisciplinary problems. For example, the evolution trajectories of IDPs have different properties than evolution of folded proteins, such as decreased sequence coevolution, increased positive selection pressures measured through $K_a/K_s$ ratio, the ratio of nonsynonymous ($K_a$) to synonymous ($K_s$) mutation rates, and functionary robustness to highly divergent sequence evolution in similarly functional proteins [16-18]. More directly, examination of physical properties of IDPs can further insight to the general rules that govern the structure of universal proteins. Current analysis of proteins, largely taking place without consideration to disordered structures is inherently incomplete, and a unification of the frameworks that govern both disordered and ordered protein structures is currently an underexplored area of molecular biology.

Many IDPs are involved in ligand recognition and binding [19]. Understanding the discrete influences of *global* (properties of protein chains that hold under permutation independent combinations of amino acids) and *local* (properties of protein chains that are dependent on the permutation of amino acids) regulation on IDP structure will provide understanding on the mechanisms that IDPs use to bind potential targets. Intriguing properties of DNA binding proteins, many of which are disordered, include sequence non-
specificity but DNA ligand global structural specificity, which highlights the importance of understanding the role that the global properties of architectural proteins play in DNA topology modulation [20]. Potential applications include a novel avenue for drug design in targeting specific ligands by differentially modulating global and local sequence effects.

**Structural Properties of IDPs and the Polyproline-II helix**

The $PP_{II}$ is an extended left-handed helix with dihedral angles $\varphi = -75^\circ$ and $\psi = +145^\circ$, commonly observed in fractional IDP ensembles, and is observed in IDPs to exhibit noncooperativity, due to the lack of native intramolecular tertiary contacts [21]. The polyproline-II helix conformation had been initially recognized in the structure of the collagen triple-helix [22]. Collagen represents one-third of the total protein in humans, and takes the fibrous structure of three parallel polyproline-II helices coiled into a right-handed helix [23]. The three individual strands of collagen repeat the motif X-Y-Gly, where X and Y can represent any amino acid, but is dominated by either proline (28%) or 4-hydroxyproline (38%). Later, it was discovered that the conformation could exist in non-fibrous proteins that did not consist of a statistical overrepresentation of proline, such as beta-melanocyte-stimulating hormone [24]. Due to the expanded polyproline-II helix conformation, proteins with higher $PP_{II}$ propensities tend to have larger hydrodynamic radius, or tumbling volume in water, than proteins with more canonical structures such as $\alpha$-helix and $\beta$-sheet, explaining why IDPs tend to be larger than others, with similar number of residues[25]. Previous work has shown structure perturbations in the hydrodynamic radius ($R_h$) can be predicted from
changes in $PP_{II}$ propensities, and these predictions show good agreement to experimentally measured values [25].

More recently, it has been shown that IDPs have significant intrinsic structural bias for the $PP_{II}$ helix, indicating that it is a significant structural element of many IDPs[22, 26, 27]. Evolutionary conservation of $PP_{II}$ bias has been observed in IDPs[28]. Previous work has established the extensive dependence of hydrodynamic radius ($R_h$) on sequence-dependent conformational propensity, particularly for the $PP_{II}$ and $\alpha$-helices, based on the $PP_{II}$ propensity scale proposed by Hilser et. al [25, 29]. The implications that IDPs can be predicted, with good agreement, based only on intrinsic $PP_{II}$ propensity indicate the statistical bias in the structure of IDPs, which are significantly larger than what would be predicted if IDPs took a random coil conformation, with relatively small correction for charge effects by measurement of charge groups laying within the Debye length of the given solvent [25].

The $R_h$ provides a simple yet physically meaningful metric to understand the structural compaction, inductively, the degree of solvent-chain interactions of IDPs and thus intrinsic conformational propensities, structural properties, and physicochemical features of IDPs. Prior work indicates the $R_h$ of IDPs is also responsive to changes in other global variables, particularly net charge density and temperature[25, 30]. Previous work established the following model from data obtained from 22 intrinsically disordered proteins, predicting the $R_h$ of IDPs based on the number of residues, propensity for the polyproline-II helix from Elam et. al and a linear net-charge correction term, given by[25]:

$$R_h = 2.16 N^{0.503-0.11 \ln (1-f_{ppii})} + 0.25 Q - 0.31 N^{0.5}$$
The model disregards sequence position, and instead considers $PP_{II}$ as an averaged sum of each fractional $PP_{II}$. The model indicates that the intrinsic conformational propensities are dependent on global factors alone. We directly test this hypothesis by creating six IDPs with identical inputs into the model for $R_h$.

**Protein Polymer Physics and Fractals**

Many of the properties, such as noncooperativity of $PP_{II}$ and the power-law model of $R_h$ of IDPs make them amenable for characterization through fractals. A fractal is an infinite iteration of self-similar portions, existing at every scale. A polymer is a chain of connected monomer building blocks; herein for example, amino acids are monomer subunits of a polymer protein chain. For a polymer, the scaling of hydrodynamic radius and peptide length follow a power-law scaling relationship of the form [31]

$$R_h \sim N^\nu$$

Where $R_h$ represents the hydrodynamic radius, $N$ represents the number of peptides, and $\nu$ represents a term sometimes referred to as the “Flory exponent” that is dependent on solvation conditions [32, 33]. An ideal polymer is a polymer that has equal chain-chain and chain-solvent interaction energies, also known as a $\Theta$ solvent. An excluded volume polymer, or a polymer in a “good” solvent, is a polymer-solvent system that favors higher chain-solvent over chain-chain interactions. Conversely, a “poor” solvent is a solvent that has
favorable chain-chain interactions, rather than chain-solvent interactions. An ideal solvent has a Flory exponent $\nu = \frac{1}{2}$, and Flory exponents are increased in “good” solvents, and decreased in “poor” solvents. Previous work has shown that IDPs can be modeled as excluded volume polymers with Flory exponents $\nu > \frac{1}{2}$ [25]. Physically, this quantity is represented by the observed larger hydrodynamic sizes for equivalent values of $N$ in IDPs and folded proteins. In “poor” solvent-polymer systems, intrachain interactions dominate over solvent-chain effects. Relevant examples of “poor” polymer-solvent interactions include that of the collapsed or globule state of the general folded protein [32].

**Self-similarity** is property of geometric objects where the larger scale structure of that object is similar to a smaller scale portion of the object. One relevant example of this behavior is a fractal. The property of a parameter to be similar at different scales is known as *scale-invariance*. The degree of a structure to follow a fractal pattern is given by the fractal dimension $D$, and is given by the reciprocal of the Flory exponent:

$$D = \frac{1}{\nu}$$

The fractal dimension $D$ represents the (possibly noninteger) degree to which a polymer changes as the scale changes [32]. Thus, an excluded volume polymer-solvent system has a higher $D$ than a “poor” solvent system. A decreasing fractal dimension implies less changing at different scales, i.e. the global structure is more similar to a smaller segment of the structure, while an increasing fractal dimension implies less similarity to a smaller segment [32]. Alternatively, a smaller fractal dimension implies that a polymer can more readily be
explained in terms of global structure, as opposed to local structure, as the details of local interactions are offset in comparison to global effects.

We can represent the accessible energetic states of proteins through a classical, discrete partition function of the form [34];

\[ Z = \sum_r e^{-\beta E_r} \]

Where \( \beta \) represents the reciprocal of the multiple of Boltzmann’s constant \( k_B \) and temperature in Kelvin, and \( E_r \) represents the energy iterating over each accessible microstate \( r \). The probability \( p_r \) of obtaining a state in a heat bath at constant temperature \( T \) from the partition function can be determined from [34];

\[ p_r = \frac{e^{-\beta E_r}}{Z} \]

From the probability and energy, we can obtain the average energy using the energetic population weighted averages [34];

\[ \langle E \rangle = \sum_r p_r E_r \]

The implication towards folded proteins can be understood in following manner. Folded proteins are not static (occupancy of a single energy state \( E_r \) with associated probability \( p_r = \)

8
1) but can be understood to occupy a finite collection of states energetically proximal to the lowest available energy state.

When discussing disordered structures, a few differences in the treatment of the partition function may be assumed. Compared to the finite number of accessible structures for folded proteins, the distribution of accessible energetic states disordered proteins are sufficiently more diffuse (their average density of states (DOS) is much lower). We introduce the concept of DOS to understand IDPs and provide justification for statistical treatment of IDPs.

Previous Characterization of Combinatorial Effects on Protein Structure

Two proteins are considered circular permutations of each other if the only difference between two proteins lies in the location of their N and C-termini [35]. For some folded proteins, a degree of similarity between primary sequences that are cyclic permutations of each other display similar three-dimensional (3-D) shape [36]. However, it is unclear if any structural conservation exists due to non-cyclic sequence permutation of proteins. Previous work has shown the large dependence of IDP structure based solely on global factors, particularly $PP_{II}$ conformational propensities and number of peptides $N$, with a small local charge correction term [25]. Collectively, these results indicate that there is at least some degree of structure that is dictated by global effects, or effects that depend on only combinations of amino acids, regardless of their permutation.

Previous molecular dynamics simulations showed that $PP_{II}$ propensity is a reliable predictor of $R_n$, which indicates that the structural properties of IDPs rely on a sequence-combination dependent effect (global) as opposed to a sequence-permutation dependent
effect (local) [25]. Additionally, the thermal denaturation curve for IDPs exhibits noncooperativity as a linear trend between $R_h$ and temperature is observed for p53(1-93) [37]. It has shown that $PP_{II}$ propensity, measured through GLY $\rightarrow$ PRO substitutions in p53(1-93) for every proline, does significantly compact $R_h$, but it is uncertain if the location of these substitutions confer different effects, the hypothesis directly being tested here [30].

Introduction of Methods

The peptide backbone is canonically understood as the source of secondary structure in proteins, making circular dichroism spectroscopy (CD) a suitable method of analysis for understanding the extent of impact on $PP_{II}$ propensity. The spectra of CD reveals information on the secondary structure of proteins through differential absorbance of circularly polarized light, directly measuring in proteins the structure of the amide backbone. In quantitative terms, CD measures the absorbance difference $\Delta A$ between left and right-handed circularly polarized light for a given wavelength $\lambda$:

$$\Delta A = A_{L\lambda} - A_{R\lambda}$$

Where $A_{R\lambda}$ and $A_{L\lambda}$ represents the absorption of right and left circularly polarized light at wavelength $\lambda$. Differential absorbance can be used to derive the molar extinction coefficients $\varepsilon$ for right and left circularly polarized light for each wavelength $\lambda$ and, using Beer’s law, extinction coefficients can be converted to concentration normalized absorbance. CD instrumentation measures absorbance directly, using experimentally derived
concentration values and standardized cuvette path length, obtaining a value for $\Delta \varepsilon$, in terms of $M^{-1}cm^{-1}$. Conventionally, CD spectra has units of molar ellipticity, $[\theta]$, which is obtained from $\Delta \varepsilon$ by;

$$[\theta] = 3298 \Delta \varepsilon$$

Yielding the magnitude of the difference between right and left circularly polarized light absorption in concentration normalized units of deg$^1$cm$^2$dmol$^{-1}$. When successive chromophores, in this case, the amide backbone of proteins, are organized into a patterned array, such as a secondary structure motif, the result of CD is a characteristic, reproducible spectra. The $PP_{II}$ helix, previously shown to be an important structural element of disordered proteins, displays a similar spectra to that of random coil proteins, but has a characteristic peak around 220 nm, a wavelength not visible in the random coil spectra [38]. The use of CD on the p53(1-93) substitutions at a variety of temperatures and monitoring the changes in at 221 nm will indicate how the propensity for the $PP_{II}$ helix changes as a result of site-specific substitutions and of directionality.

Size exclusion chromatography (SEC) is a method that can measure the $R_h$ of a given peptide through determination of the length of time needed for a sample to elute through a matrix consisting of homogenously sized beads. By comparing the elution time for the sample to known protein standards, the average sizes can be determined. Due to the lack of stability and the large number of accessible states, the $R_h$ obtained from SEC provides a reproducible metric to empirically compare the effect of p53(1-93) variants and directionality on the average structure of these proteins, which is indicative of their intrinsic conformational propensities [25].
Project Goals

Recent work shows that the prediction of $R_h$ from number of residues and theoretical polyproline-II structure shows good agreement with Pearson’s correlation coefficient of 0.93 for 25 proteins experimentally derived $R_h$ through size exclusion chromatography (SEC). This result is not surprising since IDPs lack tertiary stability and thus structural features should be dominated by sequence properties. However, the model implies that the order of sequence does not hold influence in determination of $R_h$, and thus, sequence-dependent intrinsic conformational propensities.

Ferreon and Hilser performed binding studies on the SH-3 domain of SEM-5 to Sos, a peptide ligand which binds to SH-3 when the peptide is in the $PP_{II}$ conformation [39]. By substituting two different prolines on the 11 peptide ligand to glycine, they were able to determine that a single substitution to glycine was responsible for an average of a 26.7-fold increase in ensemble size over the non-substituted wild-type Sos peptide, as measured with conformational enthalpy of binding using isothermal calorimetry (ITC). This is consistent with the scale produced by Hilser et. al, using the same SH-3 domain SEM – Sos binding system [28], in which ITC determined that glycine has the lowest fractional propensity of all amino acids for the $PP_{II}$ helix (13% of proline) by measuring the change in enthalpy of binding as a result of different substitutions, causing IDPs to behave more like unbiased random coil ensembles. Because the substitution from proline to glycine should disrupt the $PP_{II}$ helix the most, we designed proline to glycine mutations at different regions of p53(1-
should yield the most insight on site-specific effects of intrinsic conformational propensities of IDPs.

The main objective of this project is to understand the degree of local and global sequence regulation in intrinsically disordered proteins. The specific hypothesis tested here is whether there is a significant difference between a collection of seven synthetic variants of the N-terminus of human p53, \( p53(1-93) \), which contains 22 proline residues. Tumor suppressor p53, coded by \( TP53 \), has been widely studied and characterized for its role in disease, and has been referred as the “guardian of the genome”, and is the most frequently mutated protein in human cancer [40]. The transactivation domain (TAD) at the amino terminus of p53 is an IDR responsible for binding to cell-signaling related proteins, such as HDM2 (also called MDM2) [41]. Here, the first 93 residues of p53, hereafter refered to as p53(1-93), is used as a model IDP due to 1.) being a relatively well-studied IDP; 2.) functional relevance towards human disease; 3.) highly negative net charge; 4.) high proline content and thus \( PP_H \) propensity; and 5.) established laboratory protocols for isolation and characterization. Three of the variants have conserved directionality, or retention of N- and C-termini; \( N11p53 \), created by the mutating the first 11 proline residues from the N-terminus to glycine; \( M11p53 \), created by mutating the middle 11 prolines to glycine; and \( C11p53 \), created by mutating the last 11 prolines from the N-terminus to glycine. Thus, the three constructed peptide permutations possess the same amino acid constituents as well as directionality, with alterations only in different positions of sequence. Next, the four variants are created similarly to the previous proteins, but with reverse directionality; \( p53(93-1) \), created from reversing the directionality of p53(1-93), such that the N-terminus and C-terminus are reversed; \( RF11p53 \), created by reversing the N-terminus and C-terminus of
N11p53; \textit{RM11p53}, created by reversing the N-terminus and C-terminus of M11p53; and \textit{RL11p53}, created by reversing the N-terminus and C-terminus of C11p53. These sites are chosen as they convey information on the impact of substitutions in regions with varying charged density; the N11p53/RF11p53 have 13 negatively charged residues between the first and last proline substitutions, and the M11p53/RM11p53 contain 10 negatively charged residues between the first and last proline substituted, while the C11p53/RL11p53 mutants contain only a single glutamate residue between the first and last proline substituted.

The $R_h$ of the isolated and purified peptides is measured using size-exclusion chromatography (SEC). This method is preferred as it is cheaper than other methods of $R_h$ determination (such as sedimentation and NMR spectroscopy) have been used previously for similar peptides, and results have been well characterized in the lab [25, 30, 37]. The null hypothesis is there will be no significant difference between peptide permutations at different locations, as measured with pairwise test on the $R_h$ of each peptide. Secondary structure perturbations are also measured using circular dichroism spectroscopy, as this method has shown success in detecting residual structure in the p53(1-93), and can be used to understand the influence of the $PP_{II}$ helix on compaction of $R_h$ [42]
II. MATERIALS AND METHODS

Plasmid and Protein Sequences

The sequence of p53(1-93) was used as the template of this project to understand the effects of different substations on the intrinsic conformational propensities of IDPs, consisting of two charge rich transactivation domain regions (TAD1/TAD2) and a proline rich region (PRR). TAD1 represents residues 1-40, TAD2 represents residues 41-61, and the PRR represents residues from 62-91 [43].
Plasmid map for pJ404 plasmids. The N11p53 variant is illustrated. Figure obtained from ATUM (Newark, CA). Labels: $P_{\text{Amp}}$, a constitutively activated promoter that regulates the Ampicillin resistance gene; $Ampicillin-r$, a gene that confers ampicillin resistance by translating into $\beta$-lactamase; $P_{\text{lacI}}$, a constitutively active promoter that regulates the lacI gene; lacI, a gene which translates into the lacI protein, which binds lacO and LacO1 and inhibits protein synthesis until induced by IPTG; Ori$_{\text{pUC}}$, which contains the bacterial origin of replication; Term$_{\text{rpoC}}$, contains the transcriptional termination sequence for rpoC, a DNA-dependent RNA polymerase; Term$_{\text{bla}}$, contains the transcriptional termination sequence for $Ampicillin-r$ sequence, which promotes more efficient expression of $Ampicillin-r$ gene [44]; LacO1, binding site for lac repressor protein; $P_{\text{T5 Inducible}}$, contains the promoter for the T5 bacteriophage RNA polymerase; lacO, a secondary site of binding for lac repressor protein; strong RBS, a binding sequence which a ribosome binds to the mRNA in translation; HIS, a region encoding six histidines attached the protein of interest for purification; $p53(1\text{-}93)$ PRO to GLY first 11, the region containing the DNA for the protein of interest (either N11p53, M11p53, or C11p53).
Plasmid map for pJ414 plasmids. The RF11p53 variant is illustrated. Figure obtained from ATUM (Newark, CA). Labels: \textit{P\_Amp}, a constitutively activated promoter that regulates the Ampicillin resistance gene; \textit{Ampicillin-r}, a gene that confers ampicillin resistance by translating into \(\beta\)-lactamase; \textit{P\_lacI}, a constitutively activated promoter that regulates the \textit{lacI} gene; \textit{lacI}, a gene which translates into the \textit{lacI} protein, which binds to \textit{LacO1}, inhibiting protein synthesis until induced by IPTG; \textit{Ori\_pUC}, which contains the bacterial origin of replication; \textit{Term\_rpoC}, contains the transcriptional termination sequence for \textit{rpoC}, a DNA-dependent RNA polymerase; \textit{Term\_bla}, contains the transcription termination sequence for the \(\beta\)-lactamase sequence, which promotes more efficient expression of \textit{Ampicillin-r} gene \[44\]; \textit{P\_T7\_Inducible}, contains the promoter for the T7 bacteriophage RNA polymerase; \textit{LacO1}, binding site for lac repressor protein; \textit{strong RBS}, a binding sequence which a ribosome binds to the mRNA in translation; \textit{HIS}, a region encoding six histidines attached the protein of interest for purification; \textit{retro PtoG first 11}, the region containing the DNA for the protein of interest (either \textit{p53(1-93)}, \textit{p53(93-1)}, \textit{RF11}, \textit{RM11}, or \textit{RL11}); \textit{Term\_T7}, termination sequence for bacteriophage T7 polymerase.
FIG. 1. FASTA sequence of pJ414 plasmid containing p53(1-93). Sequences are highlighted according to the following legend: Ori pUC, Term rpoC, term bla, P T7 Inducible, T7/LacO1, RBS, HIS, p53(93-1), Term t7, AMP p, Ampicilin-r, P lac, lacI. This plasmid contains 4324 total base pairs
>retro wild type p53(93-1)
CCCTAAAGAAGCTACAGTTTCTTGGAGATCTCCCGAGTAAGTGCTGCTGCTTGCAAGAAAAAACACCCGGCTACCAGCGGTGGTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGCCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTAC
CAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCGAAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACCGGCTACTCTCCGCACAGTGCAGGACAGAAGACAGTATTTTAAGGCGCCGAGTGGTGTGGCGGTTTTTTTATGGGGGGAGTTTAGGGAAAGAGCATTTGTCAGAATATTAAAGGGCGCC
TGTCACTTTGCTTGATATA
TGAGAATTATTTAACCTTATAAATGAGAAAAAAGCAACGCACTTTAAATAAGATACGTTGCTTTTTCGATTGATGAACACCTATAATTAAACTATTCATCTATTATTTATGATTTTTTGTATATACAATATTTCTAGTTTGTTAAAGAGAATTAAGAAAATAAATCTCGAAAATAAATAAAGGGAAAATCAGTTTTTGATATCAAAATTATACATGTCAACGATA
ATACAAAATATAATACAAACTATAAGATGTTATCAGTATT
TATTATCATTTAAGGATTAATGTTTGATGCCTGGCAGTTCCCTACTCTCGCATGGGGAGTCCCCACACTACCATCGGCGCTACGGCGTTTCACTTCTGAGTTCGGCATGGGGTGTCAGGTGGGACCACCGCGCTACTGCCGCCAGGCAAACAAGGGGTGTTATGAGCCATATTCAGGTATAAATGGGCTCGCGATAATGTTCAGAATTGGTTAATTGGTTGTAACACTGACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCTGATAAATGCTTCAATAATATTGAAA
AAGGAAGAATATGAGTA
TTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGA
ACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCGCACAGAAC
GTTTGTGATGCCCAAGATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCGATGGCAACAACGTTGCGCAAACTATTAA
CTGGCGAACTACTTACTCTAGCTTCCGGCAACAATTAATAGAACTGAGGAGGCGGAAATTGCTGCACAGCAATCAGGGTGGTGAATATGAAACCAGTAACGTTATACGATGTCGCAGAGTATGCCGGTGTCTCTTATCAGACCGTTTCCCGCGTGGTGAA
CCAGGCCAGCCACG
TTTCTGCGAAAACGCGGGAAAAAGTGGAAGCGGCGATGGCGGAGCTGAATTACATTCCCAACCGCGTGGCACAA
CAACTGGCGGGCAAACAGTCGTTGCTGATTGGCGTTGCCACCTCCAGTCTGGCCCTGCACGCGCCGTCGCAAATTGTCGCGGCGATTA
AATCTCGCGCCGATCAACTGGGTGCCAGCGTGGTGGTGTCGATGGTAGAACGAAGCGGCGTCGAAGCCTGTAAAGCGGCGGTGCACAACTTCT
CGCGCAACGCGTCAGTGGGCTGATCATTAACTATCCGCTGGATGACCAGGATGCCATTGCTGTGGAAGCTGCCTGCACTAATGGTTCCGGCGTTATTTCTTGATGTCTCTGACCAGACACCCATCAACAGTATTATTTTCTCCCATGAGGACGGTACGCGACTGGGCGTGAGCATCTGGTCGCATTGGGTCACCAGCAAATCGCGCTGTTAGCGGGCCCATTAAGTTCTGTCTCGGCGCGTCTGCGTCTGGCTGGCTGCATAAATATCTCACTCGCAATCAAATTCAGCCGATAGCGGAACGGGAAGGCGACTGGAGTGCCATGTCCGGTTTTCAACAAACCATGCAAATGCTGAATGAGGGCATCGTTCCCACTGCGATGCTGGTTGCCAACGATCAGATGGCGCTGGGCGCAATGCGCGCCATTACCGAGTCGGGGCTGCGCGTTGGTGCGGATATCTCGGTAGTGGGATACGACGATACCGAAGATAGCTCATGTTATATCCCGCCGT
TAACCACCAT
CAGGATTTTCGCCTGCTGGGGCAAACCAGCGTGGACCGCTTGCTGCAACTCTCTCAGGGCCAGGCGGTGAAGGGCAATCAGCTGTTGCCAGTCTCACTGGTGAAAAGAAAAACCACCCTGGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGA
CTCATGACCAAAATCCCTTAACGTGAGTT
ACGCGCGCGTCGTTCCAC
TGAGCGTCAGAC

FIG. 2. FASTA sequence of pJ414 plasmid containing p53(93-1). Sequences are highlighted according to the following legend: Ori_pUC, Term_rpoC, term_bla, P_T7_Inducible, T7/LacO1, RBS, HIS, p53(93-1), Term_t7, AMP_p, Ampicilin_r, P_lacI, lac. This plasmid contains 4324 total base pairs.
FIG. 3. FASTA sequence of pJ414 plasmid containing RF11p53. Sequences are highlighted according to the following legend: Ori_pUC, Term rpoC, term bla, P_T7_Inducible, T7/LacO1, RBS, HIS, p53(93-1), Term t7, AMP_p, Ampicillin-, P_lacI, lacI. This plasmid contains 4327 total base pairs.
FIG. 4. FASTA sequence of pJ414 plasmid containing RM11p53. Sequences are highlighted according to the following legend: Ori pUC, Term rpoC, term bla, P T7 Inducible, T7/LacO1, RBS, HIS, P53(93-1), Term t7, AMP_p, Ampicilin-r, P lac, lacI. This plasmid contains 4327 total base pairs.
FIG. 5. FASTA sequence of pJ414 plasmid containing RL11p53. Sequences are highlighted according to the following legend: Ori_pUC, Term rpoC, term_bla, P_T7_Inducible, T7/LacO1, RBS, HIS, p53(93-1), Term_t7, AMP_p, Ampicillin-r, lac, lacI. This plasmid contains 4327 total base pairs.
FIG. 6. FASTA sequence of pJ40 plasmid containing N11p53. Sequences are highlighted according to the following legend: Ori puC, term rpoC, Term bla, LacO/I T5 Inducible, P T5 inducible, lacO/P T5 inducible, 5'UTR T7 10A, RBS, HIS, IMP, Ampicilin-r, lac, lac. This plasmid contains 4321 total base pairs.
Fig. 7. FASTA sequence of pJ404 plasmid containing M11b53. Sequences are highlighted according to the following legend: Ori _puC_, term _rpoc_, Term _bla_, LacO/P _T5_ Inducible, P _T5_ inducible, lacO/P _T5_ inducible, 5'UTR T7 10A, RBS, HIS, AMP_p, Ampicillin-r.

This plasmid contains 4321 total base pairs.
>p53(1-93) PRO to GLY last

FIG. 8. FASTA sequence of pJ404 plasmid containing C11p53. Sequences are highlighted according to the following legend: Ori_puC, term_rpos, Term_bla, LacO1/P T5_Inducible, P_T5_inducible, lacO/P_T5_inducible, 5'UTR_T7_10A, RBS, HIS, Amp_p, Ampicillin, lac, lacI. This plasmid contains 4321 total base pairs.

GCGTGCAGAC

25
FIG. 9. **Amino acid sequence of p53(1-93).** The portion in red indicates the histidine affinity tag used to purify and be subsequently cleaved by thrombin. The portion in blue is the remainder after thrombin cleavage.

FIG. 10. **Amino acid sequence of p53(93-1).** The portion in red indicates the histidine affinity tag used to purify and be subsequently cleaved by thrombin. The portion in blue is the remainder after thrombin cleavage.

FIG. 11. **Amino acid sequence of RF11p53.** The portion in red indicates the histidine affinity tag used to purify and be subsequently cleaved by thrombin. The portion in blue is the remainder after thrombin cleavage. The orange glycines indicate PRO → GLY substitution sites.
FIG. 12. **Amino acid sequence of RM11p53.** The portion in red indicates the histidine affinity tag used to purify and be subsequently cleaved by thrombin. The portion in blue is the remainder after thrombin cleavage. The orange glycines indicate PRO $\rightarrow$ GLY substitution sites.

FIG. 13. **Amino acid sequence of RL11p53.** The portion in red indicates the histidine affinity tag used to purify and be subsequently cleaved by thrombin. The portion in blue is the remainder after thrombin cleavage. The orange glycines indicate PRO $\rightarrow$ GLY substitution sites.
FIG. 14. **Amino acid sequence of N11p53.** The portion in red indicates the histidine affinity tag used to purify and be subsequently cleaved by thrombin. The portion in blue is the remainder after thrombin cleavage. The orange glycines indicate PRO $\rightarrow$ GLY substitution sites.

1 MRGSHHHHHH SSDLVPRGSM EEPQSDPSVE PPLSQETFSD LWKLLPENNV
60 LSGLGSQAMD DLMLSGDDIE QWFTEDGGGD EAERMEEAAG GVAGAGAAPT 110
PAAPAPAPPSW PL

FIG. 15. **Amino acid sequence of M11p53.** The portion in red indicates the histidine affinity tag used to purify and be subsequently cleaved by thrombin. The portion in blue is the remainder after thrombin cleavage. The orange glycines indicate PRO $\rightarrow$ GLY substitution sites.

1 MRGSHHHHHH SSDLVPRGSM EEPQSDPSVE PPLSQETFSD LWKLLPENNV
60 LSPLPSQAMD DLMLSPDDIE QWFTEDPGPD EAERMGEAAAG GVAGAGAAGT 110
GAAGAGAGSW GL

FIG. 16. **Amino acid sequence of C11p53.** The portion in red indicates the histidine affinity tag used to purify and be subsequently cleaved by thrombin. The portion in blue is the remainder after thrombin cleavage. The orange glycines indicate PRO $\rightarrow$ GLY substitution sites.
Materials

All water for experiments was filtered and deionized using an EMD Millipore Milli-Q Integral 3 water purifier (Billerica, MA). Glassware and other equipment was sterilized using a Hirayama HICLAVE HV-50 autoclave (Kasukabe-Shi, Japan). All agar plates were obtained from Teknova (Hollister, CA). Sonication was performed using a Branson Sonifer S-450A (Danbury, CT). Incubation of bacteria cultures was performed using a Fisher Scientific MaxQ 500 floor-model shaker (Waltham, MA). Column chromatography was performed using a Bio-Rad Biologic LP chromatography system (Hercules, CA). The pH electrodes used were either the Beckman Coulter Theta 510 pH meter (Brea, CA) or a Thermo Scientific Orion Star A111 pH meter (Waltham, MA). Reagents were weighed using a A&D GH-200 analytical balance (Tokyo, Japan) or a PA84C Pioneer from Ohaus (Parsippany, NJ). A Welch DryFast 2032 Ultra Diaphragm Pump was used to degass column media and for vacuum filtration (Niles, IL). CD spectroscopy was performed using a Jasco J-710 spectropolarimeter with a Jasco PFD-425S Peltier (Easton, MD).

Transformation

The genes containing the sequences above were cloned into the pJ404 expression vector for the N11p53, M11p53, and C11p53 proteins and into the pJ414 expression vector for p53(1-93), p53(93-1), RF11p53, RM11p53, RL11p53, from DNA 2.0 (Menlo Park, CA). Glycerol stocks created by Dr. Steve Whitten were used for wild-type expression. The pJ404 expression vector contains a gene that confers ampicillin resistance as well as an IPTG-
inducible T5 promoter. The pJ414 expression vector contains an ampicillin resistance gene and an IPTG inducible T7 promoter. The plasmids were aliquoted and diluted with autoclaved, deionized water to a concentration of 20 ng/µL and frozen at -80 °C. The plasmids were then combined with competent BL21(DE3) E. coli cells by mixing 10 ng of DNA with 100 µL of bacteria.

Transformation was completed by allowing the mixture to incubate on ice for 30 minutes, subjecting to heat-shock at 42 °C for 2 minutes, and incubating on ice for 2 minutes. An outgrowth was prepared by adding 1 mL of either SOC or 2xYT broth, and allowed to incubate at 37 °C with 225 RPM shaking for 60 minutes. Cells were plated on LB agar containing ampicillin and LB agar without ampicillin as a control by taking 50 mL of outgrowth and spreading on the plate, and allowed to incubate overnight at 37 °C and shaking at 225 RPM.

Expression

A single colony was obtained from previously transformed plates and allowed to grow in 3 mL of 2xYT broth containing 100 µg/mL of ampicillin overnight at 37 °C. The following day, 1 mL of overnight growth culture was added to 1L of 2xYT media containing 100 µg/mL of ampicillin and incubated at 37 °C and 225 RPM until reaching an optical density ~ 0.6 - 0.7 at 600 nm to express during the log phase of bacterial growth. After reaching the log phase of growth, 0.5 mM IPTG was added to induce expression for 4 hours. The culture was centrifuged at 4 °C, 30,240 x g for 15 minutes in a F14-6x250y rotor. The supernatant was decanted and the precipitate cell pellets frozen at -80 °C.
Purification

Cell pellets were removed from the freezer and solubilized in 6 M GuHCl, 10 mM Tris-HCl, 100 mM Na$_2$PO$_4$, pH 8.0. The pellets were lysed through four cycles of sonication for two minutes each, and centrifuged at 4 °C, 30,240 x g for 1 hour. The samples were loaded onto a His-Select Nickel Affinity gel column from Sigma-Aldrich (St. Louis, MO) equilibrated in 6 M GuHCl, 10 mM Tris-HCl, 100 mM Na$_2$PO$_4$, pH 8.0. Column washes were performed using 1) 80 mL of 6 M GuHCl, 10 mM Tris-HCl, 100 mM Na$_2$PO$_4$, pH 8.0; 2) 80 mL 10 mM Tris-HCl, 100 mM Na$_2$PO$_4$, pH 8.0 3) 80 mL 10 mM Tris-HCl, 100 mM Na$_2$PO$_4$, 10 mM imidazole, pH 8.0; and eluted with 60 mL 10 mM Tris-HCl, 100 mM Na$_2$PO$_4$, 350 mM imidazole, pH 4.3. The eluent was dialyzed overnight in 4L 20 mM Tris-HCl, 100 mM NaCl, pH 8.

Following overnight dialysis, the eluent was treated with 10 units of thrombin from Sigma-Aldrich (St. Louis, MO) for either 4 hours (plasmids containing T5 promoter) or 16 hours (plasmids containing T7 promoter) to digest the poly-histidine tag. The samples were loaded onto degassed DEAE gel column from GE Healthcare (Little Chalfont, UK) equilibrated in 20 mM NaAc, 25 mM NaCl, pH 4.8. Column washes were performed using 1) 60 mL 20 mM NaAc, 25 mM NaCl, pH 4.8; 2) 60 mL 20 mM NaAc, 50 mM NaCl, pH 4.8; 3) 60 mL 20 mM NaAc, 150 mM NaCl, pH 4.8; and eluted with 60 mL 20 mM NaAc, 400 mM NaCl, pH 4.8. The eluent was dialyzed overnight in 4L 10 mM Na$_2$HPO$_4$, 100 mM NaCl, pH 7.

Gel Electrophoresis
Gel electrophoresis was performed to verify complete digestion of the histidine tag and show purity of product. Gels were 18 Well 4-20% Tris-HCl, 1.0 mm Criterion Precast Gels from Bio-Rad (Hercules, CA). Samples for loading were mixed with equal volumes of 19:1 ratio of 2x Laemmli Sample Buffer and β-mercaptoethanol. Sample/buffer mix of 20 µL was loaded into the gel buffered with TGS. Unused lanes were loaded with 10 µL of buffer mix. A ladder of 4 µL Precision Plus Protein™ All Blue Standards from Bio-Rad (Hercules, CA) was loaded for comparison to known standards. A voltage of 100 V was applied for 10 minutes, then voltage was increased to 200 V until the migration was complete. Gels were stained with 10% Acetic Acid, 40% Methanol, 0.05% Coomassie R-250 for approximately 2 hours, then destained with 10% Acetic Acid, 40% Methanol until bands began to appear, approximately 15 minutes. Gels were imaged using either a ChemiDoc™ XRS+ System from Bio-Rad (Hercules, CA). To estimate purity, gels were analyzed using the software ImageJ [45]. Images were first converted to 32-bit greyscale, then individual lanes were selected to generate a plot profile. The area underneath the image intensity plot profile is a semi-quantitative way to determine gel purity.

Circular Dichroism Spectroscopy

Circular Dichroism was performed using a J-710 Spectropolarimeter equipped with a PFT-425S Peltier from Jasco (Oklahoma City, OK). Samples were diluted to a concentration indicated in figure legends and a total volume of 300 µL into a J/0556 Cuvette from Jasco (Oklahoma City, OK). Nitrogen gas was used to purge the spectropolarimeter, applied at a
pressure of ~20 PSI. A total of eight wavelength scans between 195-245 nm were measured for 10° increments between 5 °C and 85 °C, and subtracted from a baseline measurement of 10 mM Na₂HPO₄, 100 mM NaCl, pH 7.

**Size Exclusion Chromatography**

To determine $R_h$, samples were measured through size exclusion chromatography using Sephadex G-75 Media from GE Healthcare (Little Chalfont, UK) hydrated in degassed 10 mM Na₂HPO₄, 100 mM NaCl, pH 7. The elution time of p53(1-93) samples were compared to the elution times of Blue Dextran from Sigma (St. Louis, MO) and DNP-Aspartate from Research Organics (Cleveland, Ohio). The relative elution times for each sample were calculated into $K_d$ ratios;

$$K_D = \frac{V_e - V_0}{V_t - V_0}$$

Where $V_e$ represents the elution time of the sample, $V_0$ represents the elution time of Blue Dextran, and $V_t$ represents the elution time of DNP-Aspartate. This was calculated for each of the p53 variants, as well as horse heart myoglobin (Sigma-Aldrich M1882), Bovine Carbonic Acid (Sigma-Aldrich C5023), *Staphylococcus* nuclease (Previously purified by Lance English, according to Whitten and Garcia-moreno 2000), and Chicken Egg Albumen (Thermo-Fisher Scientific Acros 40045), hydrated in 10 mM Na₂HPO₄, 100 mM NaCl, pH 7, which were run as standards. The $R_h$ for the SEC protein standards were determined by measuring the distance between the two furthest α-carbons of a single subunit in the protein.
crystal structure PDB file. This method has been shown to be a reasonable approximation for prediction of $R_h$ of folded proteins and for computationally generated IDP structures [25, 29]. The $R_h$ of protein standards are shown in Table 1.

Table 1. **Calculated protein crystallographic $R_h$**. Values were derived from PDB files, and calculating the largest distance between two alpha carbons located in a single subunit.

<table>
<thead>
<tr>
<th>PDB</th>
<th>NAME</th>
<th>RESIDUES</th>
<th>$R_h$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1OVA</td>
<td>Chicken Egg Albumen</td>
<td>386</td>
<td>35.92</td>
</tr>
<tr>
<td>1V9E</td>
<td>Bovine Carbonic Anyhydrase</td>
<td>259</td>
<td>27.40</td>
</tr>
<tr>
<td>1STN</td>
<td><em>Staphylococcal</em> Nuclease</td>
<td>149</td>
<td>21.20</td>
</tr>
<tr>
<td>2O58</td>
<td>Horse Heart Myoglobin</td>
<td>153</td>
<td>21.83</td>
</tr>
<tr>
<td>4R5S</td>
<td>Bovine Serum Albumin</td>
<td>583</td>
<td>42.07</td>
</tr>
</tbody>
</table>
III. RESULTS AND DISCUSSION

Nickel Affinity Chromatography

Proteins were successfully purified through nickel affinity chromatography, as indicated by the clear peak after elution buffers in the chromatograms, which indicates an absorbance of 280 nm, indicative of presence of a protein. An example of a nickel affinity chromatograph for the purification of p53(93-1) is displayed in Figure 17.

FIG. 17. Nickel affinity column chromatogram and conductivity readings for p53(93-1). The black line represents absorbance at 280 nm. The red line represents conductivity measurements. Buffer markings; Cells, clarified cell lysate from expression, T = 59.23 min; WB 1, wash buffer containing: 6 M GuHCl, 10 mM Tris-HCl, 100 mM Na₂PO₄, pH 8.0, T = 187.66 min; WB 2, wash buffer containing: 10 mM Tris-HCl, 100 mM Na₂PO₄, pH 8.0, T = 439.23 min; WB 3, wash buffer containing: 10 mM Tris-HCl, 100 mM Na₂PO₄, 10 mM imidazole, pH 8.0, T = 572.64 min; Elu, elution buffer containing: 20 mM NaAc, 400 mM NaCl, pH 4.8, T = 638.05 min. The peak after the addition of the elution buffer is the target protein.
Anion Exchange Chromatography

Following thrombin digestion of the poly-histidine tag and verifying complete digestion with a gel electrophoresis experiment, the samples were successfully run through a DEAE column, indicating a clear elution peak for each protein by measuring the absorption of light at 280 nm. An example of an anion exchange chromatograph for the purification of p53(93-1) is displayed in Figure 18.

FIG. 18. DEAE chromatogram and conductivity readings for p53(93-1). The black line represents absorbance at 280 nm. The red line represents conductivity measurements. Buffer markings; WB 1, wash buffer containing: 20 mM NaAc, 25 mM NaCl, pH 4.8, T = 24.05 min; WB 2, wash buffer containing: 20 mM NaAc, 50 mM NaCl, pH 4.8, T = 39.7 min; WB 3, wash buffer containing: 20 mM NaAc, 150 mM NaCl, pH 4.8, T = 56.36 min; Elu, elution buffer containing: 20 mM NaAc, 400 mM NaCl, pH 4.8, T = 82.26 min. The peak after the addition of the elution buffer is the target protein.
**Gel Electrophoresis**

The wild type p53(1-93), N11p53, M11p53, and C11p53 were purified and analyzed on a gel stained with Coomassie as shown in **Figure 19**. The retro variants, p53(93-1), RF11p53, RM11p53, and RL11p53 were also purified and run on a gel, as seen in **Figure 20**. Gel electrophoresis migration depends on electrophoretic mobility and hydrodynamic size, but protein standard ladders are indicated by apparent molecular weight. As expected, due to the higher molecular mass, the wild-type migrated the least, appearing around the standard indicated as 20 kDa. The C11p53 variant migrated the furthest, followed by M11p53, and N11p53 migrated the least out of the proline → glycine variants. The retro variants behaved similarly to their native isomers; the least migrated, as expected, was the retrop53(93-1), with the other retro variants following the same order of migration as their native isomers; RL11p53 migrated the furthest, followed by RM11p53, then RF11p53.

Purity analysis was performed on all variants. The purity analysis of gel electrophoresis of RM11 is shown in **Figure 21**. Gel electrophoresis results appear to display distinct hierarchy of electrophoretic mobility, where p53(1-93) and its retro variant migrate similarly just above an apparently molecular mass of 20 kDa, N11p53/RF11p53 migrate above M11p53/RM11p53 which migrates above C11p53/RL11p53. The purity analysis indicates that the proteins are pure at a level of 95%.
FIG. 19. SDS-PAGE gel of purified p53(1-93), N11p53, M11p53, and C11p53. Run on a 4-20% Tris-HCl Criterion Precast Gel with TGS, a tris-glycine running buffer. From left to right; Lane 1, Bio-Rad Precision Plus Protein Standards. Lane 2, wild-type p53. Lane 3, N11 p53. Lane 4, M11 p53. Lane 5, C11 p53.
FIG. 2. SDS-PAGE gel of purified p53(93-1), RF11p53, RM11p53, and RL11p53. Run on a 4-20% Tris-HCl Criterion Precast Gel with TGS, a tris-glycine running buffer. From left to right; Lane 1, Bio-Rad Precision Plus Protein Standards. Lane 2, retro p53(93-1). Lane 3, RF11p53. Lane 4, RM11p53. Lane 5, RL11 p53.
FIG. 21. **Protein purity calculations on RM11 using ImageJ software.** The boxed region in yellow indicates the region where the plot profile is measured. The lighter band at an apparent migration of around 25 kDa and the darker band at an apparent migration of around 17 kDa are represented by the smaller and larger peak, respectively. The peak area is proportional to the amount of protein present. The smaller peak has a relative area of 509.284 and the larger peak has a relative area of 8804.267, indicating a relative purity of 95%.
Circular Dichroism Spectroscopy

Each of the peptides were analyzed for secondary structure contribution using circular dichroism spectroscopy, as shown in Figures 22-30. As described by Schaub et. al, the peak at 220 nm indicates the presence of the $PP_{II}$ structure [42]. For all spectra, the linear correlation of $R^2 > 0.98$ indicates the noncooperativity of thermal unfolding, as observed previously by Schaub et. al. The peak was the highest with the wild-type, as suspected due to higher $PP_{II}$ propensity when calculated with the scale according to Elam et. al [28]. However, the N11p53 variant appears to display greater $PP_{II}$ structure than C11p53 and M11p53. Surprisingly, all native direction variants had significantly greater $PP_{II}$ structure than each of the retro counterparts, as indicated by the peaks at 220 nm. As predicted by all $PP_{II}$ scales, the retro p53 had more $PP_{II}$ structure than the other retro variants, but this is far less pronounced than the difference between the native direction wild type and variants. The RF11, RM11, and RL11 variants all displayed significantly diminished $PP_{II}$ structure relative to their natively oriented isomers.
FIG. 22. **Temperature dependent CD spectra for p53(1-93).** Buffer consisted of 10mM sodium phosphate and 100mM sodium chloride at pH 7. **Top:** Molar residual ellipticity (MRE) is measured in units of deg cm$^2$ dmol$^{-1}$ res$^{-1}$. Concentration of 16 µM for this peptide. Inset: Average value of MRE from 220-222 nm for all trials, subtracted from the measurement at 85°C. **Bottom:** The difference in MRE for each wavelength between 210 and 240 nm from 85°C spectrum.
FIG. 23. **Temperature dependent CD spectra for N11p53(1-93).** Buffer consisted of 10mM sodium phosphate and 100mM sodium chloride at pH 7. *Top:* Molar residual ellipticity (MRE) is measured in units of deg cm\(^2\) dmol\(^{-1}\) res\(^{-1}\). Concentration of 18 µM for this peptide. *Bottom:* The difference in MRE for each wavelength between 210 and 240 nm from 85°C spectrum.
FIG. 24. **Temperature dependent CD spectra for M11p53(1-93)**. Buffer consisted of 10mM sodium phosphate and 100mM sodium chloride at pH 7. *Top:* Molar residual ellipticity (MRE) is measured in units of deg cm$^2$ dmol$^{-1}$ res$^{-1}$. Concentration of 18 µM for this peptide. *Bottom:* The difference in MRE for each wavelength between 210 and 245 nm from 85°C spectrum, to show melting of PP$_{II}$ helix.
FIG. 25. **Temperature dependent CD spectra for C11p53(1-93).** Buffer consisted of 10mM sodium phosphate and 100mM sodium chloride at pH 7. *Top:* Molar residual ellipticity (MRE) is measured in units of deg cm$^2$ dmol$^{-1}$ res$^{-1}$. Concentration of 18 µM for this peptide. *Bottom:* The difference in MRE for each wavelength between 210 and 240 nm from 85°C spectrum, to show melting of $PPI_h$ helix.
FIG. 26. **Temperature dependent CD spectra for p53(93-1).** Buffer consisted of 10mM sodium phosphate and 100mM sodium chloride at pH 7. *Top:* Molar residual ellipticity (MRE) is measured in units of deg cm$^2$ dmol$^{-1}$ res$^{-1}$. Concentration of 17 µM for this peptide. *Bottom:* The difference in MRE for each wavelength between 210 and 240 nm from 85°C spectrum, to show melting of $PP_{II}$ helix.
**FIG. 27. Temperature dependent CD spectra for RF11p53.** Buffer consisted of 10mM sodium phosphate and 100mM sodium chloride at pH 7. *Top:* Molar residual ellipticity (MRE) is measured in units of deg cm$^2$ dmol$^{-1}$ res$^{-1}$. Concentration of 18 µM for this peptide. *Bottom:* The difference in MRE for each wavelength between 210 and 240 nm from 85°C spectrum, to show melting of $\beta$P$_{II}$ helix.
FIG. 28. **Temperature dependent CD spectra for RM11p53.** Buffer consisted of 10mM sodium phosphate and 100mM sodium chloride at pH 7. *Top:* Molar residual ellipticity (MRE) is measured in units of deg cm$^2$ dmol$^{-1}$ res$^{-1}$. Concentration of 18 µM for this peptide. *Bottom:* The difference in MRE for each wavelength between 210 and 240 nm from 85°C spectrum, to show melting of $PP_{II}$ helix.
FIG. 29. **Temperature dependent CD spectra for RL11p53.** Buffer consisted of 10mM sodium phosphate and 100mM sodium chloride at pH 7. *Top:* Molar residual ellipticity (MRE) is measured in units of deg cm$^2$ dmol$^{-1}$ res$^{-1}$. Concentration of 18 µM for this peptide. *Bottom:* The difference in MRE for each wavelength between 210 and 240 nm from 85°C spectrum, to show melting of $PP_{II}$ helix.
FIG. 30. **Average molar residual ellipticity difference from 85 °C from 220-222 nm.** The linearity of the plots indicates that the collapse of the structure of at this region is noncooperative, as expected for a $PP_{II}$ helix [21].
The structure of the $PP_{II}$ has been shown to represent the most efficient canonical secondary structure for bridging electron transport reactions, which may indicate why disrupting $PP_{II}$ structure at the charge dense region had a greater difference in terms of $PP_{II}$ as measured using CD [46]. The linear relationship of the molar residual ellipticity differences from 85 degrees at 221 nm supports that the hypothesis that the decrease in signal at 221 nm is indicative of melting of the $PP_{II}$ helix. The retro direction variants display lesser $PP_{II}$ as measured by the height of the molar residual ellipticity at 221 nm, seemingly regardless of proline content; the retro wild-type still had less ellipticity at 221 nm than each of the native direction proteins. However, no noticeable change in $PP_{II}$ occurred between variants of the same directionality. These results indicate that changing the direction of the variants reduced $PP_{II}$ structure in the protein variants, but not occurring in substitutions from the same direction.

**Size Exclusion Chromatography**

An example of a size exclusion chromatogram analysis of p53(93-1) is displayed in **Figure 31**.

SEC was performed on three different columns. The first, using G-100 media, measured the *R*ₜ for N11p53, M11p53, C11p53, RM11p53, and RL11p53, according to Table 2.
Table 2. **SEC using G-100 media.** $R_h$ calculated from standard curve of $K_d$ and crystallographic $R_h$, **Figure 32.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>$K_d$, Average</th>
<th>$K_d$, $\sigma$</th>
<th>$R_h$, Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>N11p53</td>
<td>4</td>
<td>0.2861</td>
<td>0.001</td>
<td>29.68</td>
</tr>
<tr>
<td>M11p53</td>
<td>4</td>
<td>0.2887</td>
<td>0.001</td>
<td>29.55</td>
</tr>
<tr>
<td>C11p53</td>
<td>4</td>
<td>0.2943</td>
<td>0.003</td>
<td>29.25</td>
</tr>
<tr>
<td>RM11p53</td>
<td>4</td>
<td>0.3034</td>
<td>0.005</td>
<td>28.77</td>
</tr>
<tr>
<td>RL11p53</td>
<td>5</td>
<td>0.2929</td>
<td>0.006</td>
<td>29.32</td>
</tr>
</tbody>
</table>
FIG. 32. **Standard curve of SEC proteins on G-100 media.** Linear regression indicates a relationship between $K_d$ and $R_h$ following $R_h = -1.525K_d + 4.9246$, with an $R^2 = 0.959$. 
FIG. 3. **Box and whisker plot of SEC results from G-100 media.** Sample sizes; N11, 4; M11, 4; C11, 4; RF11, 4; RL11, 5. The lower and upper limits of the box represent the lower and upper quartile cutoffs, meaning that the regions bounded by the whisker below and above the box represent the lowest and highest quartile of the data, respectively. Outliers are represented as points either below or above 3/2 times the lower or upper quartile, respectively.

Two different SEC runs were obtained on G-75 media. The first compared all proteins, and results are shown in Table 3.
Table 3. **First trial of SEC results on G-75 media.** $R_h$ calculated from standard curve of $K_d$ and crystallographic $R_h$, **Figure 34.** Two more trials were performed on the p53(1-93) using a purification by another student of the lab, but did not match previous laboratory measurements (laboratory average: 31.81 Å from 3 students; Lance English, Romel Perez, and Erin Tilton).

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>$K_d$, Average</th>
<th>$K_d$, $\sigma$</th>
<th>$R_h$, Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53(1-93)</td>
<td>1</td>
<td>0.137</td>
<td>-</td>
<td>31.82</td>
</tr>
<tr>
<td>N11p53</td>
<td>3</td>
<td>0.205</td>
<td>0.003</td>
<td>29.18</td>
</tr>
<tr>
<td>M11p53</td>
<td>2</td>
<td>0.220</td>
<td>0.007</td>
<td>28.61</td>
</tr>
<tr>
<td>C11p53</td>
<td>2</td>
<td>0.213</td>
<td>0.011</td>
<td>28.91</td>
</tr>
<tr>
<td>p53(93-1)</td>
<td>4</td>
<td>0.177</td>
<td>0.027</td>
<td>30.23</td>
</tr>
<tr>
<td>RF11p53</td>
<td>4</td>
<td>0.217</td>
<td>0.004</td>
<td>28.76</td>
</tr>
<tr>
<td>RM11p53</td>
<td>4</td>
<td>0.221</td>
<td>0.005</td>
<td>28.59</td>
</tr>
<tr>
<td>RL11p53</td>
<td>2</td>
<td>0.224</td>
<td>0.012</td>
<td>28.75</td>
</tr>
</tbody>
</table>
FIG. 34. **Standard curve of SEC proteins on the first trial of G-75 media.** Linear regression indicates a relationship between $K_d$ and $R_h$ following $R_h = -40.046K_d + 36.903$, with an $R^2 = 0.964$. Linear regression without *Staphylococcal* Nuclease: $R_h = -37.163K_d + 36.804$, with an $R^2 = 0.995$. 
FIG. 35. **Box and whisker plot of SEC results from first trial of G-75 media.** Sample sizes; N11, 3; M11, 2; C11, 2; Retro, 4; RF11, 4; RM11, 3; RL11, 2. The lower and upper limits of the box represent the lower and upper quartile cutoffs, meaning that the regions bounded by the whisker below and above the box represent the lowest and highest quartile of the data, respectively. Outliers are represented as points either below or above 3/2 times the lower or upper quartile, respectively.
The apparent difference of retro p53 and wild type contradicted preliminary experiments in the lab. In order to directly confirm that the wild type and the retro variant were different sizes, as well as measure the RF11p53 variant on another column, a second G-75 column was run, as shown in Table 4.

Table 4. **Second trial of SEC results on G-75 media.** $R_h$ calculated from standard curve of $K_d$ and crystallographic $R_h$ without *Staphylococcal* Nuclease, **Figure 36.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>$K_d$, Average</th>
<th>$K_d$, $\sigma$</th>
<th>$R_h$, Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53(1-93)</td>
<td>6</td>
<td>0.196</td>
<td>0.02</td>
<td>31.93</td>
</tr>
<tr>
<td>p53(93-1)</td>
<td>5</td>
<td>0.172</td>
<td>0.02</td>
<td>31.01</td>
</tr>
<tr>
<td>RF11p53</td>
<td>4</td>
<td>0.222</td>
<td>0.02</td>
<td>29.43</td>
</tr>
</tbody>
</table>
FIG. 36. **Standard curve of SEC proteins on the second trial of G-75 media.** Linear regression indicates a relationship between $K_d$ and $R_h$ following $R_h = -48.435K_d + 36.878$, with an $R^2 = 0.956$. Linear regression without *Staphylococcal Nuclease*: $R_h = -42.15K_d + 38.719$, with an $R^2 = 0.998$. 
FIG. 37. **Box and whisker plot of SEC results from second trial of G-75 media.** Sample sizes; *Retro*, 6; *Wild Type*, 5, RF11, 4. The lower and upper limits of the box represent the lower and upper quartile cutoffs, meaning that the regions bounded by the whisker below and above the box represent the lowest and highest quartile of the data, respectively. Outliers are represented as points either below or above 3/2 times the lower or upper quartile, respectively.
Size exclusion chromatography (SEC) was performed on each of the purified variants to measure hydrodynamic radius to quantitate differences in intrinsic conformational propensities from various permutations of sequence, depicted in Table 5. For all variants, standardized $R_h$ was decreased in retro sequences relative to their non-retro isomers, indicating the importance of directionality of sequence on the hydrodynamic preferences of p53(1-93). Mutations in the charge rich domain of p53 (N11p53 and RF11p53) were larger than mutations in the other regions, regardless of primary sequence directionality, similar to the trend observed through CD signal peak of $\Delta220$ nm as well as the apparent size through gel electrophoresis.

There was no significant difference between the hydrodynamic radii C11p53 and RL11p53, indicating that changing the proline content (and inductively, $PP_{II}$ propensity) only had significant effects on the hydrodynamic size when altered in an area of high charge (the N11p53/RF11p53 have 13 negatively charged residues between the first and last proline substitutions, and the M11p53/RM11p53 contain 10 negatively charged residues). The existence of unfavorable interactions of charges being ameliorated through the $PP_{II}$ helix has been proposed by various computational studies and may explain the lack of noticeable compaction within the native and retro direction substitutions at the non-charge dense region (C11 and RL11) [25, 47, 48]. Because the $PP_{II}$ may be a mechanism to distribute charges, the N11 and RF11 may be consistently bigger than the other substitutions due to the need to relieve unfavorable charge based interactions through extension of structure through the $PP_{II}$ helix.
The statistical design of the experiment limited the impact of variability of each individual column and allowed for more confident direct comparison. Each protein was run at least for two trials on at least two different columns to ensure consistency of measurement. The average pair-wise difference between any two protein $R_h$ between the three columns was 0.272 Å, representing an error term of 0.92% from the average of all trials ($R_h = 29.67$ Å), indicating that the column-to-column variation has been sufficiently corrected for in through the measurements of the protein standards. Previously, we established that IDPs occupy a relatively large number of states (average DOS for occupied states is very small, the number of possible states $N >> 1$). In this system, the Central Limit Theorem applies, and that the sampling of distribution of IDPs also follow a normal distribution centered around a mean $R_h$. The $R_h$ for each peptide was calculated by using the linear regression fit for $K_d$ to calculated $R_h$ standards without *Staphylococcal* Nuclease. *Staphylococcal* Nuclease was not used in determination of the $R_h$ because it 1) consistently underfit the standard curve and 2) was below the detection cutoff for sufficient resolution in the G-100 media. The resulting $R_h$ are shown in Table 5. For comparison, the p53(1-93) protein had been run before by three different students (Lance English, Romel Perez, Erin Tilton) and had an average $R_h$ of 31.82 Å when measured through SEC, compared to 31.91 Å in this experiment.
Table 5. **Calculated \( R_h \) from SEC of p53(1-93) variants.** For each column, \( K_D \) values were converted to \( R_h \) values by protein standard relationships without using *Staphylococcal* Nuclease.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>AVERAGE ( R_h ), Å</th>
<th>ST. DEV.</th>
</tr>
</thead>
<tbody>
<tr>
<td>P53(1-93)</td>
<td>6</td>
<td>31.91</td>
<td>0.7</td>
</tr>
<tr>
<td>N11P53</td>
<td>7</td>
<td>29.47</td>
<td>0.3</td>
</tr>
<tr>
<td>M11P53</td>
<td>6</td>
<td>29.23</td>
<td>0.5</td>
</tr>
<tr>
<td>C11P53</td>
<td>6</td>
<td>29.13</td>
<td>0.4</td>
</tr>
<tr>
<td>P53(93-1)</td>
<td>10</td>
<td>30.64</td>
<td>0.9</td>
</tr>
<tr>
<td>RF11</td>
<td>8</td>
<td>29.09</td>
<td>0.6</td>
</tr>
<tr>
<td>RM11</td>
<td>9</td>
<td>28.69</td>
<td>0.2</td>
</tr>
<tr>
<td>RL11</td>
<td>7</td>
<td>29.08</td>
<td>0.5</td>
</tr>
</tbody>
</table>
FIG. 38. **Box and whisker plot of calculated $R_h$ of all variants.** $R_h$ is calculated using column standards from protein standard regression without *Staphylococcal* Nuclease. Data from Table 5. The bar located at the center of each box represents the median value. The lower and upper limits of the box represent the lower and upper quartile cutoffs, meaning that the regions bounded by the whisker below and above the box represent the lowest and highest quartile of the data, respectively. Outliers are represented as points either below or above 3/2 times the lower or upper quartile, respectively.
To understand the effects of substitution, a 3x2 ANOVA analysis was performed on the SEC data of the six combinatorically identical variants, as indicated in Table 6-7, using the substation site (columns) and directionality (rows) as factors.

Table 6. **ANOVA design for p53 variants.** *Rows,* direction factor; *Columns,* position of proline to glycine mutations.

<table>
<thead>
<tr>
<th></th>
<th>Substitution Site 1</th>
<th>Substitution Site 2</th>
<th>Substitution Site 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Direction</td>
<td>N11p53</td>
<td>M11p53</td>
<td>C11p53</td>
</tr>
<tr>
<td>Reverse Direction</td>
<td>RF11p53</td>
<td>RM11p53</td>
<td>RL11p53</td>
</tr>
</tbody>
</table>

Table 7. **ANOVA summary table.** *SS,* sum of squares; *df,* degrees of freedom; *MS,* mean of squares; *F,* F-statistic; *P,* p-value.

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rows</td>
<td>1.3</td>
<td>1</td>
<td>1.3</td>
<td>6.08</td>
<td>0.0184</td>
</tr>
<tr>
<td>Columns</td>
<td>0.98</td>
<td>2</td>
<td>0.49</td>
<td>2.29</td>
<td>0.1154</td>
</tr>
<tr>
<td>R x C</td>
<td>0.29</td>
<td>2</td>
<td>0.14</td>
<td>0.68</td>
<td>0.5128</td>
</tr>
<tr>
<td>Error</td>
<td>7.91</td>
<td>37</td>
<td>0.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>10.48</td>
<td>42</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The columns represent the different sites of substitution (either the N-terminus, middle of peptide, or C-terminus) and the rows either represent the normal directionality or the reverse directionality. The results indicate that there is a significant difference that can be attributed to the directionality factor \( p = 0.02 \) but not for sequence position \( p = 0.12 \). There is still a significant \( p < 0.05 \) difference between directionality when reducing the compared samples to the first G-75 trial and the G-100 trial, where each protein was run at least for two trials on each, except for RF11, which was run on the first G-75 trial only, indicating that differences are still significant when decreasing the variance in the measurement for RF11p53, which contained the highest standard deviation of the proteins tested.

To further test the hypothesis that directionality a significant effect on the structure of IDPs, were compared using a Welsh’s t-test of unequal variances and sample sizes performed on the \( K_d \) of p53(1-93) and p53(93-1) from the second G-75 media, giving a \( p \)-value of \( p = 0.05 \). This result indicates a significant pair-wise difference between p53(1-93) and p53(93-1), when run on the same column.

Collectively, both the ANOVA test performed between the six combinatorically equivalent proteins (N11p53, M11p53, C11p53, RF11p53, RM11p53, RL11p53) and the pair-wise Welch’s t-test between the retro variant and the wild type indicate a significant directional dependence of \( R_h \), but is not significantly dependent on site of substitution. However, it is important to note that it appears that the substitutions in the region with lower charge density (C11p53 and RL11p53) appear to not differ in directionality. The differences between the native and retro direction variants are displayed in Table 8.
Table 8. **Differences in measured $R_h$ of directional variants through SEC.** The average $R_h$ of p53(1-93) minus the average of p53(93-1), N11p53 minus the average RF11p53, M11p53 minus the average RM11p53, and C11p53 minus the average RL11p53.

<table>
<thead>
<tr>
<th>DIFFERENCE, $R_h$, Å</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>P53(1-93)</td>
<td>1.27</td>
</tr>
<tr>
<td>N11P53</td>
<td>0.37</td>
</tr>
<tr>
<td>M11P53</td>
<td>0.55</td>
</tr>
<tr>
<td>C11P53</td>
<td>0.05</td>
</tr>
</tbody>
</table>

There is a higher difference in p53(1-93) and its directional variant than any other pair. Almost no difference (0.06 Å) can be observed in the C11p53 and its isomer. These differences indicate that, according to the Flory model of polymer physics, that the reverse in directionality causes less preferential interaction with the solvent, and facilitates an increase in chain-chain interactions. Alternatively, the fractal dimension is reduced due to directionality, indicating the preference of the native directions to be more globally determined, rather than locally determined.

**Conclusion**

One of the most fundamental ideas in biochemistry is the dependence of a protein’s structure on the sequence of amino acids. Anfinsen’s “Thermodynamic Hypothesis” states that three-dimensional structure in a given environment is ultimately deterministic to the native state of the lowest Gibbs Free Energy [49]. This was postulated after experiments on ribonuclease A, which returned to its biologically active, native structure after being
completely denatured with urea and having disulfide bridges reduced by 2-mercaptoethanol, but only when allowed to re-oxidize in non-denaturing conditions.

Intrinsically disordered proteins provide a useful system to understand the generalized primary structure of proteins, as they do not occupy a small set of states, but distribute over a larger number of accessible energy states. For folded proteins, primary structure, combined with some sufficiently static environment, eventually leads to a smaller set of structures near some energetic minimum. For IDPs, however, the shift in their distributions of states towards a lower energy provide a more general approach for understanding the properties of proteins as it relates to their primary structure.

Reversing the sequence of p53(1-93) dramatically reduced $PP_{II}$ occupancy and $R_b$ regardless of net amino acid content (and any combinatorically based propensity calculation), as indicated by the decrease of signal at 221 nm in the native isomers over the retro isomers. According to the “thermodynamic hypothesis”, the protein seeks a lower energy state, and this either implies that 1) the energy cost required to form a $PP_{II}$ helix is increased past the point of being energetically favorable when translated in the opposite direction; 2) another viable conformation becomes more energetically favorable than forming a $PP_{II}$ helix; or potentially both. This finding suggests that determination of $PP_{II}$ helix propensity may be amicable to study through a permutation sensitive model.

Evidence presented here suggests that the hydrodynamic size and $PP_{II}$ propensity depended on the sequence directionality appear to have a charge dependent effect. Because proline causes steric constrictions towards the previous amino acid residue through binding of the side chain to the amine backbone, data was gathered from 1535 unique IDPs in the DISPROT database to compare the number of amino acids that were located proximally to
proline[50, 51]. The number of pre- and post-proline amino acids were compared to the
native direction and reverse direction variants of p53(1-93) used in this experiment to obtain
an explanation for the collapse of structure indicated by CD and SEC. The percentage of
amino acids that occur before and after proline residues in the native and reverse variants of
N-terminus p53 and the proteins in the DISPROT database are shown in Table 9. There is an
increased prevalence of alanine proximal to proline in p53(1-93), with 36% of prolines being
preceded by alanine and 23% of prolines being succeeded by alanine, compared to 11% and
10% of prolines, respectively, in the DISPROT database. The biggest difference in the
p53(93-1) relative to p53(1-93) and the prolines in the DISPROT database is the number of
prolines after leucine and serine, both of which occur before 14% of all prolines. In the
DISPROT database, 6% of prolines are preceded by Leucine, and 9% of prolines are
preceded by Serine, while 9% of prolines are preceded by both serine and leucine in p53(1-93).
Collectively, this analysis may indicate that the collapse of structure in the retro variants
may be attributable to differences in amino acids that precede proline.
Table 9. **Percentage of pre- and post-proline amino acids for p53(1-93) and DISPROT.** 1535 unique intrinsically disordered proteins were analyzed in the DISPROT database. The number of amino acids occurring before and after proline in p53(1-93) is the number that occurs after and before, respectively, in p53(93-1). *None* indicates that proline was the first or last residue in the protein for pre- and post calculations respectively.

<table>
<thead>
<tr>
<th></th>
<th>p53(1-93) Pre</th>
<th>p53(93-1) Pre</th>
<th>DISPROT Pre</th>
<th>DISPROT Post</th>
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<tbody>
<tr>
<td>A</td>
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<td>11%</td>
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Hydrodynamic sizes were significantly altered in retro variants over native direction variants. Altering the positional locations of proline to glycine substitutions did not produce a significant change in hydrodynamic sizes. These results indicate that the dynamic structure of p53, and perhaps of the generalized IDP, is highly dependent on sequence directionality, the extent of which depends highly on the location of charge dense regions.
REFERENCES


