Amino Acid Proximities in Two Sup35 Prion Strains Revealed by Chemical Cross-linking*

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Background: Different but currently uncharacterized chain folding patterns of the Sup35 protein determine the prion strain type.

Results: Strain-specific chemical cross-linking is obtained.

Conclusion: Chain topology constrained by cross-linking indicates that N-terminal residues of Sup35 are differently arranged in two prion strains.

Significance: Different Sup35 folding patterns are revealed by general methods readily applicable to other prions and pathological amyloids.

Strains of the yeast prion [PSI] are different folding patterns of the same Sup35 protein, which stacks up periodically to form a prion fiber. Chemical cross-linking is employed here to probe different fiber structures assembled with a mutant Sup35 fragment. The photo-reactive cross-linker, p-benzoyl-l-phenylalanine (pBpa), was biosynthetically incorporated into bacterially prepared recombinant Sup(1–61)-GFP, containing the first 61 residues of Sup35, followed by the green fluorescent protein. Four methionine substitutions and two alanine substitutions were introduced at fixed positions in Sup(1–61) to allow cyanogen bromide cleavage to facilitate subsequent mass spectrometry analysis. Amyloid fibers of pBpa and Met/Ala-substituted Sup(1–61)-GFP were nucleated from purified yeast prion particles of two different strains, namely VK and VL, and shown to faithfully transmit specific strain characteristics to yeast expressing the wild type Sup35 protein. Intra- and intermolecular cross-linking were distinguished by tandem mass spectrometry analysis on fibers seeded from solutions containing equal amounts of 14N- and 15N-labeled protein. Fibers propagating the VL strain type exhibited intra- and intermolecular cross-linking between amino acid residues 3 and 28, as well as intra- and intermolecular linking between residues 32 and 55. Inter- and intramolecular cross-linking between residues 32 and 55 were detected in fibers propagating the VK type strain. Adjacencies of amino acid residues in space revealed by cross-linking were used to constrain possible chain folds of different [PSI] strains.

Yeast [PSI] strains are different aggregation structures of the same Sup35 protein, a subunit of the heterodimeric translation termination factor (1). In the normal cellular conformation, the translation termination factor recognizes stop codons to release elongating polypeptides from the ribosome. In the prion state, Sup35 adopts a strain-specific aggregation structure, which self-perpetuates by capturing and templating the conversion of soluble Sup35 molecules into its like. As a result, the functional cellular Sup35 concentration is reduced, causing readthrough of stop codons. Different [PSI] strains can titrate out different amount of soluble Sup35, resulting in varied degrees in translation readthrough.

[PSI] aggregates can be isolated from yeast cells to seed prion fibers from bacterially prepared recombinant Sup35 protein or its N-terminal prion-forming fragments (2, 3). When introduced into yeast without the prion ([PSI−]), the fibers can give rise to the same [PSI] strain as the original seeds, indicating that the strain-specific conformation has been faithfully propagated in the test tube. Taking advantage of the ability to propagate specific [PSI] strain structures in vitro, we previously prepared infectious Sup35 fibers of four different prion strains, namely VH, VK, VL, and W8, which were identified earlier in the laboratory using high resolution strain-typing methods (3–6). The VH and W8 strains caused strong translation readthrough (thus they were often called “strong strains”), and the VK and VL strains exhibited weaker effects (weak strains). The minimally required length of a Sup35 fragment to propagate prion infectivity is strain-dependent. Forty amino acid residues suffice for the VK strain, but for the VH, VL, and W8 strains, the first 53 residues of the Sup35 protein are required (5, 7).

Electron diffraction studies revealed that fibers of the four strains all exhibited a 4.7 Å periodicity, characteristic of β-sheet-rich amyloid. Mass per length measurements on the fibers further established that there was approximately one Sup35 molecule for every 4.7 Å repeat length along the fiber axis (4, 5). Therefore, Sup35 monomers must adopt a distinct folding pattern for each strain, which then stacks up every 4.7 Å to form a fiber (Fig. 1). Recent solid state NMR measurements on fibers nucleated by a spontaneously formed Sup35 amyloid sample, which mostly induced strong translation readthrough in yeast, further suggested that the same amino acid residue in

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FIGURE 1. Structural model of Sup35 amyloids. Sup35 monomers, adopting a strain-specific chain folding pattern, stack up every 4.7 Å to form a fiber. The assembly scheme is based on data from electron diffraction and mass per length measurements; chain folding patterns, which are yet to be determined, are drawn more freely. Black and blue chains represent randomly mixed 15N- and 15N-labeled Sup35 polypeptides.

then combines the resulting radical to generate a covalent link between the ketone carbonyl carbon and the attacked methylene carbon. Chin et al. (15) extended the amino acid repertoire for cellular protein synthesis by engineered an orthogonal tRNA/aminocayl-tRNA synthetase pair that transferred pBpa at a uniquely engineered amber codon. Employing this technology, pBpa was biosynthetically incorporated into recombinant proteins, which were then converted into strain-specific prion conformations by nucleation. The cross-linking induced by UV irradiation was subsequently analyzed by tandem mass spectrometry.

Experimental Procedures

Plasmids Construction—The Escherichia coli T7 expression plasmid pSup(1–61)-GFP-Strep(II)-His6 was constructed by replacing the 1-kb Ndcl/NotI fragment of pHis-N1-GFP-Strep(II) (3) with a PCR fragment amplified by two primers (5’-gtacatatgtgcggattcaacagcgaaatctactaaggtggcgtatgatgttttccagctgcgtgcg; the Ndcl and NotI sites are underlined) using pHis-N1-GFP-Strep(II) as the template. p-Benzyoyl-L-phenylalanine (pBpa) substitutions (Asp5, Arg28, Tyr32, Tyr48, and Tyr53 and Tyr59 codons substituted with a TAG amber codon), four fixed methionine substitutions (Tyr13, Tyr29, Tyr35, and Tyr46 codons substituted with ATG), and S2A and S4A mutations were generated by QuikChange II site-directed mutagenesis kit (Agilent Stratagene). Plasmids were isolated using QIAprep spin miniprep kit (Qiagen), and the coding region for the mutant protein was sequenced.

Protein Expression and Purification—The plasmid pSup-BpaRS-6TRN, expressing the orthogonal tRNA_CUA/pBpa-tRNA synthetase pair (15), was co-transformed into E. coli strain OverExpress™ C41 (DE3) (Lucigen) together with mutant pSup(1–61)(+4M2A)-GFP-Strep(II)-His6 plasmids, which expressed pBpa-containing proteins. The transformants were grown in LB medium supplied with pBpa (270 mg/liter; Bachem AG), ampicillin (50 μg/ml), and chloramphenicol (17 μg/ml) until A600 reached 0.5. Isopropyl β-D-thiogalactopyranoside was added to a final concentration of 1 mM to induce protein expression at 37 °C for 3 h. The cells were then collected by centrifugation. Cell pellets were lysed by sonication in buffer A (20 mM Tris, 100 mM NaCl, 5 mM imidazole, pH 7.6) containing 1 mM PMSF and 1× complete EDTA-free protease inhibitor mixture (Roche). After centrifugation (12,000 × g, 30 min at 4 °C) to remove debris, lysate was loaded onto nickel-nitrilotriacetic acid column (Qiagen) pre-equilibrated with buffer A. Recombinant proteins were eluted in buffer B (20 mM Tris, 100 mM NaCl, 500 mM imidazole, pH 7.6) containing 1 mM PMSF and 1× complete EDTA-free protease inhibitor mixture (Roche). After centrifugation (12,000 × g, 30 min at 4 °C) to remove debris, lysate was loaded onto nickel-nitrilotriacetic acid column (Qiagen) pre-equilibrated with buffer A. Recombinant proteins were eluted in buffer B (20 mM Tris, 100 mM NaCl, 500 mM imidazole, pH 7.6) after washing with 10 column volumes of buffer A. The UV monitor was turned off during elution to minimize exposure. Eluted protein was subjected to ultracentrifugation at 200,000 × g on top of a 30% sucrose cushion (g/ml in buffer A) for 2 h at 4 °C. Soluble protein was aliquoted, snap frozen in liquid nitrogen, and stored at −80 °C. Immediately before seeding, protein was thawed on ice and loaded onto a StrepTactin column pre-equilibrated with buffer W (100 mM Tris, 1 mM EDTA, pH 8.0) at 4 °C. The column was washed with 5 column volumes of buffer W, and the protein was eluted with buffer E (buffer W containing 2.5 mM desthiobiotin). Protein concentrations were determined by

2 The abbreviations used are: pBpa, p-benzyoyl-L-phenylalanine; CID, collision-induced dissociation.
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A230 reading using theoretical molar extinction coefficients of 33,465 for Sup(1–61)(+4M2A) with a single tyrosine to pBpa substitution and 34,750 for D3pBpa and R28pBpa mutations (16). For 15N-isotope labeling, M9 minimal medium (3 g/liter KH2PO4, 6 g/liter Na2HPO4, 0.5 g/liter NaCl, 1 mM MgSO4, 0.5 mg/liter thiamine, 4 g/liter glucose) containing 1 g/liter 15NH4Cl (Cambridge Isotope Laboratories) as the sole nitrogen source was used in place of LB.

Sup35 Fibers and Yeast Infection—15N-Labeled recombinant proteins mixed in equal amount with unlabeled counterparts were nucleated by yeast particles of the VH, VK, and VL strain types to form fibers at 22 °C for 48 h. Yeast particles were purified as described (17). Briefly, VK particles labeled with Sup(1–61)-GFP, Sup(1–61)(G20D)-GFP, Sup(1–61)(Q23P)-GFP, and Sup(1–61)(Q23P, N27P)-GFP, as well as Sup35 Fibers and Yeast Infection—Sup35 Fibers and Yeast Infection—crosslinked Sup35 particles were purified from yeast using StrepTactin affinity chromatography (18). Fiber’s infectivity was assayed by co-precipitation with YCplac111 (LEU2) plasmid into 5V-H19 spheroplasts (MATa SLQ5 ade2-1(UAA) can1-100 leu2-3,112 ura3-52) (19) as described (5). [PSI+] transformants were strain-typed as described (5, 6, 17), by differential GFP labeling with Sup(1–61)-GFP, Sup(1–61)(G20D)-GFP, Sup(1–61)(Q23P)-GFP, and Sup(1–61)(Q23P, N27P)-GFP, as well as by characteristic colony color changes when Sup35 single mutants (G58D, G44R, S17R, and Q15R) were co-expressed. Each mutant fiber species was prepared at least twice with independently prepared prion particles.

Cross-linking and Cyanogen Bromide Digestion—Fibers were placed on top of a 30% sucrose cushion (g/ml in buffer W) to sediment at 200,000 × g for 2 h at 4 °C. The pellet was resuspended in 100 μl of buffer W to a concentration of 50–80 μM (monomer equivalent) and transferred to a 1.5-ml ice-cold microcentrifuge tube to be irradiated with UV light (365 nm, 0.9 J/cm2 intensity) in a Spectrolinker XL-1500 UV oven (Spectronics). One hundred microliters of 2× SDS sample buffer (125 mM Tris-HCl, pH 6.8, 50% (v/v) glycerol, 4% (g/ml) SDS, 0.02% (g/ml) bromphenol blue, 10% (v/v) β-mercaptoethanol, 10 μM urea) were added, and the sample was loaded onto a 12% Tris-glycine SDS-gel for electrophoresis. Gel bands corresponding to monomer (labeled as UV1) and dimer (labeled as UV2) species were excised, cut into small pieces, and destained in 100-fold molar excess to catalyze disulfide bond formation at 22 °C for 18 h and then removed from the sample with desalting column. The content of free thiol in the sample was assayed by reaction with 5,5′-dithio-bis(2-nitrobenzoic acid) (Sigma) (22), and the reading was normalized against that of the same protein, equally diluted, but not treated with oxidized DTT. Two buried cysteine residues in GFP were tested to be unreactive. Intermolecular disulfide linkage was negligible as judged by nonreducing SDS-PAGE. To form fibers, oxidized protein was nucleated overnight at room temperature with a catalytic amount of VK and VL particles purified from yeast. To rule out...
that fibers were exclusively assembled with the small amount of unoxidized protein left in the sample, we showed that much more than 10% of the protein monomers were incorporated into the fiber. Fibers were collected by centrifugation and dissolved in 8 M guanidine hydrochloride, and protein concentration was determined with a UV spectrophotometer (NanoDrop 2000C). Unseeded protein similarly processed and the prion seeds contributed negligibly to the sedimented material.

**Plasmid Shuffle**—Plasmid shuffle was performed with a derivative of the 74-D-694 background (MATa ade1-14(URA) his3-11 leu2-3 trp1 ura3-52) (23), whose SUP35 gene was deleted from the chromosome and was instead carried on a centromere-based plasmid YCp33-I-SupF, containing a URA3 marker. Another centromere-based plasmid, YCp111-KanMXs-I-SupF, expressing the full-length Sup35 (SupF) under a 1.2-kb native promoter, containing LEU2 and G418-selectable KanMX markers, was used in mutagenesis to obtain mutant plasmids expressing the Sup35(D38R) and Sup35(R28D) proteins, respectively. The mutant plasmids, as well as the wild type parent (used as control), were transformed into the 74-D-694 derivative harboring the VK and VL strain. Five transformants each were randomly selected on solid synthetic complete media lacking uracil and leucine (SC-Leu, Ura), transferred to and grown on rich YPD plates, and replica-plated to solid synthetic media containing 5-fluoroorotic acid to counterselect cells that had lost the YCp33-I-SupF (URA3) plasmid. After growth at 30 °C for 2 days, the yeast colonies, now expressing only mutant Sup35, were further replicated to YPD plates to observe changes in colony color (by comparison with the wild type control). The [PSI] strain type of each colony was determined as described above. Experiments for each [PSI] strain were performed with two independent 74-D-694ΔSUP35 [YCp33-I-SupF] isolates.

**Results**

**Infectious Fibers**—Experiments were performed with fibers derived from mutant Sup(1–61)-GFPs, containing the first 61 amino acid residues of Sup35 followed by the GFP, which enhanced solubility and facilitated experimental observation. Sup(1–61) was sufficient to propagate strain-specific infectivities of VH, VK, and VL in vitro (5, 7). Its short length would simplify subsequent analysis. Two affinity purification tags—a Strep(II) tag followed by a six-histidine tag—were added to the C terminus of GFP to facilitate protein purification.

Point mutations were introduced as described. (i) Routine tandem mass analysis of protein requires the sample to be fragmented to specific pieces of smaller masses. This is commonly achieved by the application of proteases and peptidases. Unfortunately, the Sup35 fusion protein was prone to aggregation in buffers optimal for peptidease activities and thus became resistant to enzymatic digestion. Moreover, the simple amino acid composition of the Sup(1–61) prion domain offered few peptidease recognition sites. A chemical digestion approach was therefore adopted. The amino acid residues Tyr13, Tyr29, Tyr35, and Tyr46 were replaced with methionine residues, which reacted with CNBr under acidic conditions to form homoserine lactone and freed the C-terminal portion of the polypeptide (24). Formic acid was used as the solvent, which readily dissolved Sup35 aggregates. (ii) The sole methionine residue in the native Sup(1–61) sequence is the initiating methionine, which is followed by a serine residue. CNBr reacts with Met-Ser dipeptide to yield two products (25) whose different masses split signals and complicate tandem mass analysis. Ser3 and Ser4 were thus replaced with alanine residues. The resulting mutant protein containing four methionine and two alanine substitutions is designated as Sup(1–61)(+4M2A)-GFP. (iii) Three derivatives of Sup(1–61)(+4M2A)-GFP were initially prepared, each of which contained a single pBpa substitution for cross-linking. The substitution was at Asp3, Tyr32, and Tyr52.

We first tested whether the pBpa-substituted mutants could still propagate strain-specific conformations in vitro, as judged by their fibers’ specific infectivity to yeast. Solutions of pBpa-containing proteins were nucleated with prion particles purified from yeast harboring VH, VK, and VL strains. Fibers appeared in all nucleated solutions but not unseeded controls. They were fragmented by sonic disruption and then introduced into [psi+] yeast cells, which expressed the wild type Sup35, to assay for strain-specific infectivity. VK and VL infectivity was propagated by all pBpa substitutions (Table 1); none of the fibers nucleated by VH particles were infectious. Subsequent cross-linking analysis was performed with fibers seeded by VK and VL.

**Photo Cross-linking**—To distinguish intra- and intermolecular linkage, 15N-labeled proteins were prepared from E. coli grown in minimal medium containing [15N]ammonium chloride as the sole nitrogen source. Labeled proteins were mixed with natural 14N preparations in equal molar ratio and then nucleated with yeast prion particles. Fibers were irradiated with UV light to activate cross-linking reactions and then dissolved in a SDS-containing sample buffer for PAGE. Unirradiated fibers, dissolved in the sample buffer, formed a single band on the gel at a position expected for the protein monomer (~36.5 kDa). UV-treated samples exhibited an extra up-shifted band with the mobility of a dimer. Additional weak bands corresponding to higher oligomeric species were also observed (Fig. 3).

**Mass Spectrometry**—Following CNBr digestion, five peptide fragments originated from Sup(1–61) (designated as F1–F5 sequentially) and cross-linked products could be obtained.

### TABLE 1

**Infectious fibers of mutant Sup(1–61)(+4M2A)-GFP pBpa substituted proteins are nucleated by prion particles of the VK and VL strain type**

Fibers are co-transformed with the YCplac111 (LEU2) plasmid into the 5V-H19 yeast background without the prion to check for infectivity. 5 VK/224: 5 VK colonies out of 224 Leu+ co-transformants. Protein: unseeded protein solution; Protein + VK: protein nucleated with VK seeds; Buffer + VK: VK seeds similarly diluted in buffer E.

| Protein   | D3pBpa | R28pBpa | Y32pBpa | Y52pBpa | Y55pBpa |
|-----------|--------|---------|---------|---------|---------|
| VK strain |        |         |         |         |         |
| Protein   | 0/224  | 0/224   | 0/224   | 0/224   | 0/224   |
| Buffer + VK | 5 VK/224 | 12 VK/224 | 11 VK/224 | 5 VK/224 | 11 VK/224 |
| Protein + VK | 29 VK/224 | 50 VK/224 | 53 VK/224 | 27 VK/224 | 46 VK/224 |
| VL strain  |        |         |         |         |         |
| Protein   | 0/224  | 0/224   | 0/224   | 0/224   | 0/224   |
| Buffer + VL | 2 VL/224 | 0/224   | 0/224   | 4 VL/224 | 1 VL/224 |
| Protein + VL | 16 VL/224 | 11 VL/224 | 13 VL/224 | 19 VL/224 | 10 VL/224 |
Digested peptides were analyzed by nano LC-nano electrospray ionization-MS/MS, which utilized CID to fragment peptides to yield sequences as well as sites of cross-linking (26). MS/MS peaks were assigned by MassMatrix (21), an automated search and match program, which scored the overall peak assignment and mass matches with a probability-based algorithm.

Clearly different mass spectra were obtained for inter- and intramolecularly linked peptides that were derived from fibers nucleated from equal molar mixture of 14N- and 15N-labeled protein. Only untruncated recombinant proteins are assembled into the fiber (judged with −UV lanes). UV-treated fibers exhibit additional up-shifted bands. Bands with apparent molecular masses of the protein monomer (36.5 kDa; labeled UV1) and dimer (73 kDa; labeled UV2) are excised, digested with CNBr, and analyzed by mass spectrometry. Band UV1 contains intramolecularly linked protein, which gives rise to a peptide mass spectrum of two peaks (corresponding to 14N/14N and 15N/15N species; B and E). Intermolecular linking exhibits the characteristic quadruplet pattern (corresponding to 14N/14N, 14N/15N, 15N/14N, and 15N/15N species); D and F are observed exclusively from band UV2. Satellite peaks are due to 13C isotope, which is ~1% natural abundance. Sequences of peptide fragments (F1, F2, F3, and F5) and cross-linked residues are indicated (in B and E). (M→Hsl) indicates homoserine lactone derived from methionine after reaction with CNBr.

FIGURE 2. Detection of intra- and intermolecular cross-linking. Cross-linked VL fibers assembled with equal molar 14N- and 15N-labeled Sup(1–61)(+4M2A)-GFP are analyzed. A–C, Asp3 substituted with the pBpa cross-linker; D–F, Tyr55 substituted with pBpa. UV-irradiated fibers (labeled + UV in A and D) together with unirradiated fibers (−UV) and unseeded protein controls (leftmost lanes) are resolved by SDS-polyacrylamide gel electrophoresis. Multiple bands of lower molecular mass are observed in unseeded protein controls; they likely result from translation initiation at different non-native methionine residues. Only untruncated recombinant proteins are assembled into the fiber (judged with −UV lanes). UV-treated fibers exhibit additional up-shifted bands. Bands with apparent molecular masses of the protein monomer (36.5 kDa; labeled UV1) and dimer (73 kDa; labeled UV2) are excised, digested with CNBr, and analyzed by mass spectrometry. Band UV1 contains intramolecularly linked protein, which gives rise to a peptide mass spectrum of two peaks (corresponding to 14N/14N and 15N/15N species; B and E). Intermolecular linking exhibits the characteristic quadruplet pattern (corresponding to 14N/14N, 14N/15N, 15N/14N, and 15N/15N species); D and F are observed exclusively from band UV2. Satellite peaks are due to 13C isotope, which is ~1% natural abundance. Sequences of peptide fragments (F1, F2, F3, and F5) and cross-linked residues are indicated (in B and E). (M→Hsl) indicates homoserine lactone derived from methionine after reaction with CNBr.

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The pBpa substitution at amino acid residue 52 did not yield any specific cross-linking. However, again, nonspecific intramolecular cross-linking with Tyr45 was observed in every sample and control.

To further support observed specific cross-linking in VK and VL fibers, two more pBpa substitutions, R28pBpa and Y55pBpa, were prepared. Their ability to transmit VK and VL characteristics was confirmed by infection of yeast expressing the wild type Sup35 (Table 1). Although D3pBpa was cross-linked to Arg28 in the VL fiber, no specific cross-linking between R28pBpa and Asp3 was detected. The R28pBpa residue instead had nonspecific intramolecular cross-linking to the Gln30 residue. The Y55pBpa mutant had specific intra- and intermolecular cross-linking to Tyr32 in the VL fiber and also intermolecular cross-linking to Tyr32 in the VK fiber (Fig. 5). Again, additional ubiquitous intramolecular cross-linking was detected between Y55pBpa and Tyr45 in all samples. All results were reproduced with independent preparations.

**Constraining the Chain Folds**—The cross-links identified above were used to constrain strain-specific chain folding patterns of Sup(1–61)(+4M2A) is shown. Green letters indicate residues of the linker between Sup(1–61) and GFP. CNBr fragments are demarcated by red lines. B, CID tandem mass spectrum of a triply charged ion of cross-linked 15N/14N peptides from band UV1 of VL fibers (shortened hereinafter as VL, uv1 (14N/14N)) indicates specific intramolecular linking between D3pBpa and Arg28. Standard nomenclature is used to annotate the CID products with an apostrophe (’) indicating loss of water and an asterisk (*) indicating loss of NH3. C, MS/MS spectrum of a cross-linked peptide ion (VL, uv2 (15N/14N)) indicates specific intermolecular linking between D3pBpa and Arg45 in VL fibers. D, nonspecific intramolecular cross-linking is detected between D3pBpa and Gln10 in all samples. A representative MS/MS spectrum is shown (Protein (15N/14N)).
a parallel in-register Sup35 arrangement mentioned above, the combined results indicated close proximity of residues 32 and 55 in the VK fiber as well. The absence of Y55pBpa-Tyr32 intra-molecular cross-linking in the VK fiber probably reflected local asymmetry—for efficient cross-linking to happen, the Y55pBpa residue and a reacting methylene group would have to be in a

FIGURE 4. Intramolecular cross-linking between pBpa32 and Tyr55. A, CID tandem mass spectrum of a cross-linked peptide ion (VL, uv1 (15N/15N); charge = 3+, m/z 1094.17) indicates the intramolecular cross-linking for the VL fiber. B, MS/MS spectrum of a cross-linked peptide ion (VK, uv1 (15N/15N); charge = 3+, m/z 1107.13) indicates the intramolecular cross-linking for VK. The nomenclature is the same as in Fig. 3.

FIGURE 5. Tandem mass analysis of Y55pBpa cross-linking. A, cross-linking observed by tandem mass spectrometry in different samples of the Y55pBpa protein (labeled as in Fig. 3). B, MS/MS spectrum of a cross-linked peptide ion (VL, uv1 (15N/15N)) indicates specific intramolecular linking between Y55pBpa and Tyr32 for the VL fiber. C, MS/MS spectrum of a cross-linked peptide ion (VL, uv2 (15N/14N)) indicates specific intermolecular linking between Y55pBpa and Tyr32 for VL. D, MS/MS spectrum of a cross-linked peptide ion (VK, uv2 (15N/14N)) indicates specific intermolecular linking between Y55pBpa and Tyr32 for VK.
favorable stereogeometrical configuration, which occurred in the VL fiber but apparently did not in the VK structure.

We further tested whether Sup(1–61)-GFP, with residues 32 and 55 cross-linked, could be incorporated into [PSI] fibers in vitro (27). Sup(1–61)(Y32C,Y55C)-GFP was treated with oxidized DTT to obtain disulfide-linked monomers, which was then nucleated with prion particles purified from yeast harboring the VK or the VL strain. After overnight incubation at room temperature, numerous fibers were observed in seeded samples of both strain types; unseeded controls remained clear (Fig. 6).

We further determined that 90% of the free cysteines were oxidized in the protein solution used for nucleation, and ~46 and 65% of the total protein was incorporated into the fiber after seeding with VK and VL particles, respectively. It was thus concluded that disulfide-linked monomers could indeed join a growing fiber and continue.

Molecular ions revealed VL-specific intra- and intermolecular cross-linking between D3pBpa and Arg28, but no Asp3-R28pBpa cross-linking was detected. Similarly, this could be due to asymmetric stereogeometry in the two pBpa substitution mutants; in addition, if everything else is equal, the side chain of an interacting arginine residue is clearly a bit longer than that of an aspartate. Mutating the Asp3 residue to arginine (i.e. Arg3 and Arg28) in the wild type Sup35 destabilized the VL strain in yeast, causing misnucleation to generate VK (10 [VL]/10); however, mutating residue 28 to a smaller aspartate (Asp3 and Asp28) in the wild type Sup35 did not cause VL to change (10 [VL]/10). Both mutants supported VK propagation (10 [VK]/10; 10 [VK]/10). The involvement of Sup35 N-terminal residues in the structure of VL, but less so in VK, was also consistent with our previous genetic finding that deleting residues 5–10 from the wild type Sup35 protein in vivo cured the VL strain but still allowed the cellular propagation of VK (7). Based on the analysis above, constrained chain folds of the VK and VL fiber are illustrated (Fig. 7).

**Discussion**

In this study, cross-linking was obtained from multiple Sup(1–61)(+4M2A)-GFP derivatives, each of which had a single pBpa substitution at a different amino acid position. Different local structural perturbations might occur to result in cross-linking that did not correspond to amino acid contacts in the parental Sup(1–61)(+4M2A)-GFP fiber and cause inconsistencies when linkages were taken together as constrains. The likelihood of such artifact was very low when cross-linking between a pair of amino acid residues was consistently detected, no matter which residue was replaced with pBpa, as in the case of residues 32 and 55. Otherwise, additional supporting evidence was sought, as in the case of residues 3 and 28 in the VL fiber.

The similarity between fibers of Sup(1–61)(+4M2A)-GFP and of wild type Sup35 requires discussion. In the present work, all mutant proteins were nucleated by prion particles purified from yeast, and the resulting fibers were able to faithfully induce the same [PSI] strains in yeast expressing the wild type Sup35. This indicated that structural elements responsible for interacting with soluble Sup35 and converting it into strain-specific conformations were faithfully retained in all mutant fibers. Furthermore, no new element that caused mis-seeding was created, either by exposing a protected element or by de novo generation. The strain-specific folding topology of Sup(1–61)(+4M2A)-GFP constrained by the cross-linkages thus should not conflict with that of the wild type Sup35 N-terminal domain.

The ability to distinguish strain-specific infectivity was arguably the most important aspect in the design of the present work. It is known that single amino acid changes in an amyloidogenic protein could cause the aggregates to adopt a completely different structure, and pBpa substitutions are presumably not exceptional. For example, the W8 strain of [PSI] was observed to transmute to VK when propagated by Sup35 with a single proline substitution at amino acid positions 5, 6, 7, or 43 (5).

With pBpa substitution at more amino acid positions, additional proximity information could be obtained to further constrain chain topologies of different [PSI] strains. We noticed that UV-activated pBpa cross-linking was inefficient such that less than 10% of irradiated polypeptides were actually cross-linked. Furthermore, with only a few CNBr-digested fragments from the proteins, we were operating far below the capacity of modern mass spectrometers and the analytical power of their software. It might be possible to introduce pBpa residues at several amino acid positions in a single protein and efficiently characterize the whole array of sparsely cross-linked products. This development would further mitigate the concern that each single pBpa mutant changes the fiber structure in a different way.

**FIGURE 6. Nucleation of disulfide-linked Sup(1–61)(Y32C,Y55C)-GFP.** Disulfide-linked protein was nucleated by yeast prion particles of the VL and VK strain type (A and C, respectively) to form fibers (B and D, respectively). Unseeded protein controls remain clear under the microscope (not shown).
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The experiments described here can be applied to other prions, such as Prp\textsuperscript{Sc} of mammals (28, 29), as well as other pathological amyloids for which fine strain typing methods can be developed (30–32). Tertiary contacts obtained by cross-linking experiments can be combined with additional secondary structural information attained by NMR (8, 33) or hydrogen exchange mass spectrometry (34), to better define chain folding patterns. The knowledge would be useful in providing mechanistic insights in prion strain mutation and in validating future higher resolution fiber structures.

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