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Scrambled Prion Domains Form Prions and Amyloid†

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The [URE3] prion of *Saccharomyces cerevisiae* is a self-propagating amyloid form of Ure2p. The amino-terminal prion domain of Ure2p is necessary and sufficient for prion formation and has a high glutamine (Q) and asparagine (N) content. Such Q/N-rich domains are found in two other yeast prion proteins, Sup35p and Rnq1p, although none of the many other yeast Q/N-rich domain proteins have yet been found to be prions. To examine the role of amino acid sequence composition in prion formation, we used Ure2p as a model system and generated five Ure2p variants in which the order of the amino acids in the prion domain was randomly shuffled while keeping the amino acid composition and C-terminal domain unchanged. Surprisingly, all five formed prions in vivo, with a range of frequencies and stabilities, and the prion domains of all five readily formed amyloid fibers in vitro. Although it is unclear whether other amyloid-forming proteins would be equally resistant to scrambling, this result demonstrates that [URE3] formation is driven primarily by amino acid composition, largely independent of primary sequence.

A wide range of diseases including Alzheimer's disease, Parkinson's disease, late-onset diabetes, and transmissible spongiform encephalopathies are associated with the formation of amyloid fibers. Amyloid fibers are highly organized protein aggregates characterized by filamentous morphology, high β-sheet content, protease resistance, and yellow-green birefringence upon staining with Congo red (23, 41). Although the proteins associated with amyloid diseases have diverse amino acid sequences, the amyloid fibers that they form seem to share a common structure.

Despite considerable study, much is still unknown about the relationship between amino acid sequence and propensity to form amyloid. In vitro, it seems that virtually any polypeptide can be made to form amyloid; however, in most cases, nonphysiological conditions such as low pH are required (13). Although many mutations increase the propensity of proteins to form amyloid by destabilizing the folded state of the protein and increasing the population of partially folded intermediates (19, 30), destabilization of the native state is not sufficient to explain the effects of all amyloidogenic mutations (26). The relation of amyloids to prions (infectious proteins) is also murky. Although prions are generally amyloid (with one known exception [38]), only PrP among the >20 human amyloids is infectious. Whether this reflects a qualitative feature of the amyloid facilitating seed formation or other factors remains unknown.

Yeast can serve as a useful model to study amyloid formation in vivo and its relation to prions. The nonchromosomal genetic elements [PSI] (10) and [URE3] (24) were discovered to be prion forms of Sup35p and Ure2p, respectively (50). Ure2p blocks the uptake of poor nitrogen sources in the presence of a good nitrogen source by binding to the transcription factor Gln3p (reviewed in reference 9). One of the genes activated by Gln3p is the allantoate permease gene, *DAL5*. Due to the structural similarity between ureidosuccinate (USA), an essential intermediate of uracil biosynthesis, and allantoate, Dal5p can also take up USA (47). Therefore, cells without active Ure2p, either because of deletion of the *URE2* gene or because of the presence of the [URE3] prion, can take up USA when grown on a good nitrogen source; [ure-o] cells (i.e., *URE2* cells lacking the [URE3] prion) cannot.

Numerous in vitro and in vivo studies show that amyloid formation by Ure2p is the basis for [URE3] (15, 29, 43, 44), although recent studies suggest that the largest aggregates are dead-end products (37). Ure2p fibrils have been directly visualized in [URE3]-containing cells (43). Ure2p-green fluorescent protein fusions are aggregated in [URE3] cells, but not in wild-type cells (15), and in extracts from [URE3] strains, Ure2p is partially resistant to proteases (29). Ure2p can form amyloid-like fibrils in vitro, and these fibrils show a pattern of protease resistance similar to that of extracts from [URE3] strains (44). The same region of Ure2p that is necessary and sufficient for prion formation in vivo is necessary and sufficient for fibril formation in vitro (27–29, 44).

Both Sup35 and Ure2p are composed of an N-terminal prion domain and a C-terminal functional domain (25, 29, 45). The Ure2p prion domain is composed of amino acids 1 to 89, whereas amino acids 90 to 354 are sufficient for nitrogen regulation activity (27). Amino acids 1 to 65 of the Ure2p prion domain have been shown to be sufficient for prion induction and propagation (28, 29) and comprise the protease-resistant core of Ure2p amyloid (3). The prion domains of both Ure2p and Sup35p have unusually high glutamine (Q) and asparagine (N) contents (44% for Sup35p and 46% for Ure2p). A similarly Q/N-rich domain is found in a third yeast prion protein, Rnq1p (12, 42). Mutagenesis studies of Sup35p and Ure2p have supported the important role of the Q/Ns in prion formation (11, 27). However, it is striking that, although Q/N-rich domains

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have been found in ~100 yeast proteins (31), relatively few of these have been shown to form prions. This suggests that, although Q/N content plays an important role in determining prion-forming ability, other sequence, composition, expression or localization features are also important.

We sought to determine whether there are specific sequence features of Ure2p that are required for prion formation or whether Ure2p is prone to forming prions solely because of its unusual amino acid content. To address this question, we constructed five mutagenized versions of Ure2p. In all cases, the nitrogen regulation domain was unchanged, and the order of the amino acids in the prion domain was randomized while keeping the amino acid content constant. We then tested the ability of each of these scrambled versions of Ure2p to form prions in vivo and to form amyloid fibers in vitro. Remarkably, four of the five were able to form stable prions in vivo, and the fifth formed unstable prions that could be maintained and transmitted under selective conditions. In vitro, the prion domains of all five readily formed amyloid fibers under native conditions. Although the primary sequence of the prion domain in the scrambled Ure2p variants had some impact on the frequency of formation and stability of [URE3] prions, these results demonstrate that the amino acid content of the prion domain is primarily responsible for [URE3] prion formation.

MATERIALS AND METHODS

Strains and media. A complete strain list is available elsewhere (see Table S1 in the supplemental material). Standard yeast media have been previously described (40). Galactose medium included 2% raffinose. USA medium was synthetic dextrose (SD) containing required supplements and 30 μg of sodium USA/ml in place of uracil. In all experiments, yeast were grown at 30°C.

***URE2* prion domain randomization.** Codons for amino acids 2 to 89 of Ure2p were listed in an Excel spreadsheet. For each randomization, each codon was assigned a random number. The codons were then sorted based on their random number. Since the scrambled sequence was to be constructed by PCR with four overlapping oligonucleotides (see below), the nucleotide sequences of the overlapping regions were modified to allow for specific priming. Conservative changes were made in the oligonucleotide sequence while taking care to avoid codons that are poorly represented in the yeast genome.

Building scrambled *URE2* sequences. Plasmid VTG20 is a *CEN LEU2* plasmid expressing *URE2* from its native promoter (15). To insert a BsmI restriction site at the 3′ end of the *URE2* prion domain, inverse PCR (33) was performed on VTG20 with the oligonucleotides EDR14 and EDR16 (see Table S2 in the supplemental material). These primers both contain an XbaI site to allow circularization of the PCR product. The PCR product was digested with XbaI and ligated closed, generating plasmid pER26.

For each *URE2* variant, four 70- to 100-nucleotide (nt) overlapping oligonucleotides were combined in a single PCR to generate the full-length scrambled *URE2* prion domain flanked by BamHI and BsmI restriction sites (see Table S2 in the supplemental material). The PCR products for *URE2-21*, *-23*, and *-25* were digested with BamHI and BsmI, inserted into BamHI/BsmI-cut pER26, and transformed into *Escherichia coli*. Plasmids expressing *URE2-21*, *-23*, and *-25* were named pER30, -32, and -34, respectively. *URE2-22* and *URE2-24* were not well tolerated in *E. coli* and were therefore cloned by recombination in *Saccharomyces cerevisiae*. The previously described PCRs were reamplified (by using primers EDR47 and -48 for *URE2-22* and EDR51 and -56 for *URE2-24*) and cotransformed with XbaI/EagI-digested pER26 into 4131 (*MAT*α *ura2 leu2 his3 ade2 ure2 kar1-1*). Correct insertion was confirmed by PCR, and the PCR products were sequenced. The yeast strains carrying successful clones of *URE2-22* and *-24* were named YER75 and YER77, respectively. The plasmids carried in YER75 and -77 were named pER31 and -33. pER31 was isolated from YER75 and transformed into *E. coli*. *E. coli* cells carrying pER31 grew very slowly but were viable, allowing for plasmid purification. pER33 was apparently not tolerated by *E. coli*.

Integration of scrambled *URE2* sequences into the *URE2* locus. To allow for easy selection during integration into the yeast genome, the *HIS3* gene was inserted downstream of the scrambled *URE2*s in pER30-34 and VTG20. The *HIS3* gene was PCR amplified from pH405 (a pRS423 derivative provided generously by Herman K. Edskes, National Institutes of Health) with primers EDR85 and EDR86. These primers inserted a SalI site at the 5′ end and at the 3′ end added a XhoI site and 45 bp of a region located 3′ of *URE2* in *Saccharomyces*. The PCR product was digested with XhoI and SalI and inserted into XhoI-digested pER30, -31, -32, -34 and VTG20. Successful clones were PCR amplified with EDR106 and EDR107, and the resulting PCR product was transformed into YHE886 (*MAT***a** *ura2 leu2 his3 trp1 ure2*::*G418*, Σ1278b background). Transformants were selected on SC−His and then replica plated to test for loss of G418 resistance and loss of ability to utilize USA in the place of uracil. Transformants were analyzed by PCR and DNA sequencing. Successfully integrated clones of VTG20 and pER30-32, -34 were named YER109, -111, -113, -115, and -119, respectively. Because pER33 (expressing *URE2-24*) was not tolerated by *E. coli*, the construct to integrate was built without subcloning by using PCR. *URE2-24* was PCR amplified with EDR106 and EDR116, and *HIS3* was amplified with EDR117 and EDR86. These two PCR products overlap and were therefore combined in a PCR to generate a fragment containing both genes. EDR106 and EDR107 were added to amplify the full-length product. The resulting PCR product was transformed into YHE886. A successfully integrated clone was named YER117. Integrated strains were then crossed with YHE825 (*MAT*α *ura2 leu2 trp1 his3 mks1*::*G418*, Σ1278b background) to generate strains for spontaneous [URE3] generation assays and with 4131 to generate cytoduction recipients and dominance testers. The strains expressing *URE2-20*, *-21*, *-22*, *-23*, *-24*, and *-25* used for spontaneous [URE3] generation assays were YER159, -139, -143, -147, -151, and -155, respectively (*MAT***a** *ura2 leu2 his3 trp1 URE2-x*::*HIS3*, Σ1278b background). The strains used for dominance testing were YER214, -184, -188, -191, -211, and -195, respectively (*MAT*α *ura2 leu2 his3 ade2 URE2-x*::*HIS3*). The cytoduction recipients expressing *URE2-21 -22*, *-23*, *-24*, and *-25* were YER197, -249, -133, -209, and 206, respectively (*MAT*α *ura2 leu2 his3 ade2 kar1-1 URE2-x*::*HIS3*).

Western blot analysis. Yeast strains YER159, -139, -143, -147, -151, and -155 were grown in SD+Leu+Ura liquid medium to an absorbance at 600 nm of 0.4. Cells were harvested and lysed, and Western blot analysis was performed as previously described (29). Rabbit antiserum Ure2-3C (3), specific for the C-terminal domain of Ure2p, was used as the primary antibody.

Plasmid for galactose-inducible expression of *URE2* variants. The prion domains from plasmids pER30-34 were amplified by using primers EDR57 and EDR60-64. The resulting PCR products were cotransformed with BamHI/XhoI-digested pKT20 (a *TRP1*, 2μm plasmid containing the *GAL1* promoter, provided by Herman K. Edkes; originally constructed by Kimberly L. Taylor) into YHE888 (*MAT*α *ura2 leu2 trp1 ure2*::*G418*, Σ1278b background). Plasmids were isolated from yeast, transformed into *E. coli*, and purified. The resulting plasmids expressing the prion domains of *URE2-21*, *-22*, *-23*, *-24*, and *-25* were named pER42 to pER46, respectively. The BamHI-XhoI cassettes from pER30, -32, -34, and -42 to -46 were cloned into BamHI/XhoI-digested pH317 (16) under the control of the *GAL1* promoter. The resulting *LEU2*, 2μm plasmids expressing full-length *URE2-21*, *-23*, and *-25* were named pER95 to pER97, respectively. Plasmids expressing the prion domains of *URE2-21*, *-22*, *-23*, *-24*, and *-25* were named pER98 to pER102, respectively. Again, cloning of full-length *URE2-22* and *-24* was done directly into yeast by PCR amplifying from pER31 and pER33 with EDR57 and EDR82 and then cotransforming the resulting product with BamHI/XhoI-digested pH317 into YER113 and YER117, respectively. The resulting plasmids were named pER112 and pER113.

***E. coli* expression vectors.** His_6-tagged wild-type *URE2* was generated by PCR amplifying VTG20 with primers EDR115 and EDR118. These primers add a BamHI site and the nucleotides coding for a His_6 tag to the N terminus of *URE2*. In addition, primer EDR115 contains a BglII site, allowing the BglII/XhoI-digested PCR product to be inserted into BglII/XhoI-digested pUB6, a derivative of pET-17b (Novagen) in which the unique BglII site had been removed and a new BglII site installed just downstream of the ribosome-binding site. The resulting plasmid expressing full-length, His_6-tagged *URE2* was named pER94. To generate His_6-tagged versions of *URE2-21*, *-22*, and *-25*, the BamHI-XhoI cassettes from pER30, -31, and -34 were inserted into BamHI/XhoI-digested pER94, producing pER103, pER104, and pER106. Similarly, to generate expression vectors for each of the scrambled prion domains, the BamHI-XhoI cassettes from pER42-46 were cloned into BamHI/XhoI-digested pER94, producing pER107 to pER111. Attempts to clone full-length *URE2-23* and *-24* into pER94 by the same method were not successful.

Prion generation. Strains carrying an integrated copy of each *URE2* variant were transformed with a derivative of pH 317 in which the same full-length *URE2* variant or prion domain were under control of the *GAL1* promoter or with vector alone (pH317). Strains were grown for 3 days in galactose-raffinose dropout medium lacking leucine. Serial 10-fold dilutions were then plated on

SD+USA+Trp to select for [URE3] cells. Frequencies of USA^+ colony formation were determined as the average of at least three independent experiments.

Stability, curability, and dominance tests. USA^+ colonies were resuspended in water in a 96-well microtiter plate and spotted onto YPAD plates and mating plates (YPAD plates spread with a lawn of *ade2*, *TRP1* cells of the opposite mating type expressing the same *URE2* variant). After 48 h, the YPAD plates were replica plated to SD+USA+Leu+Trp to test for maintenance of the USA^+ phenotype. The mating plates were grown for 24 h and replica plated to SD+Ura+Leu to select for diploids. These plates were grown for 48 h and then replica plated to SD+USA+Leu to test diploids for [URE3].

To test curability, USA^+ cells were streaked for single colonies on YPAD or on YPAD plus 5 mM guanidine HCl. Single colonies were then tested for loss of [URE3] by streaking onto SD+USA+Leu+Trp.

Cytoduction. Cytoductions were performed by using donors and recipients expressing the same *URE2* variant. Donor (ρ^+) and recipient (ρ^0) cells were mixed in water and mated on YPAD for 7 h at 30°C. Recipient cells were *kar1*, which dramatically reduces the efficiency of karyogamy during mating (8). Cells were then streaked onto medium selecting for the recipient. Cytoductants are identified as ρ^+ cells with the recipient nuclear genotype (36). Cytoductions for strains expressing *URE2-22* were performed in the same manner, except that matings were incubated for only 6 h at 30°C and were then streaked directly onto ammonia medium with USA in the place of uracil and with limiting adenine (10 μg/ml).

Protein expression, purification, filament assembly, and visualization. Full-length Ure2p variants were expressed and purified as previously described (3). Proteins were dialyzed into 20 mM Tris–200 mM NaCl (pH 8.0). Fibers were formed during overnight rotation at 4°C. Prion domains were expressed in *E. coli* as previously described (3), and the *E. coli* cells were resuspended in 100 mM NaH_2PO_4–10 mM Tris–6 M GuHCl (pH 8.0). Cells were lysed by high pressure, and insoluble material was removed by centrifugation for 15 min at 17,000 × *g*. The supernatant was bound to preequilibrated Ni-nitrilotriacetic acid Superflow resin (Qiagen). Resin was washed with 100 mM NaH_2PO_4, 10 mM Tris, and 6 M GuHCl (pH 8.0). All subsequent washes were done with 100 mM NaH_2PO_4, 10 mM Tris, and 8 M urea. Resin was sequentially washed at pH 8.0, 6.3, and 5.9 and eluted at pH 4.5. Purified prion domains were dialyzed overnight into water at 4°C. All but Ure2-25p$^{1\text{-}90}$ precipitated during dialysis. Ure2-25p$^{1\text{-}90}$ showed only a small amount of precipitate after dialysis. It was further rotated for 12 h at 4°C, during which time significant precipitate formed. Filaments were stained with uranyl acetate and examined by negative staining electron microscopy as previously described (2). Congo red staining of aggregates was performed by incubating them for 1 h at room temperature in 0.1 volume 0.5% Congo red (Sigma) and washing them twice with 1 volume of water. Small amounts of the stained precipitate were placed on glass slides and viewed under a polarization microscope in bright field and with crossed polarizers.

Protease treatment. A 0.1 volume of 1 mg of proteinase K/ml was added to filament preparations, followed by incubation for 16 h at 37°C. Samples were centrifuged for 30 min at 38,000 × *g*, washed twice with 20 mM Tris–200 mM NaCl (pH 8.0), resuspended in the same buffer, and visualized by electron microscopy.

RESULTS

Randomization of Ure2p. To examine whether Ure2p forms prions solely as a result of the amino acid content of the prion domain or whether there are specific sequence features within the prion domain that drive prion formation, we generated five variants of the Ure2p prion domain in which the order of amino acids 2 to 89 was randomly shuffled without changing the amino acid content. These scrambled versions of *URE2* were designated *URE2-21*, *-22*, *-23*, *-24*, and *-25* (Fig. 1). The nucleotide sequences coding for the prion domains were constructed by PCR and inserted in place of those coding for amino acids 2 to 89 in the wild-type protein. Each of these scrambled versions of *URE2* was then integrated into the *URE2* locus under control of the *URE2* promoter. As a control, a wild-type copy of *URE2* was reintegrated in the same manner and designated *URE2-20*. Expression levels of each scrambled variant were checked by Western blot with an antibody specific to the C terminus of Ure2p; expression levels were similar for all variants (data not shown). If there are specific sequence features of the prion domain that are required to form [URE3], it is unlikely that they would have been maintained when the prion domain sequence was shuffled; therefore, it is unlikely that any of the scrambled proteins would be able to form prions. In contrast, if the ability to form prions is solely dependent on amino acid content, changing the order of the amino acids should have little impact on prion formation.

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wt Ure2:    MMNNNGNQVSNLSNALRQVNIGNRNSNTTTD
            QSNINFEFSTGVNNNNNNNSSSNNNNVQNNN
            SGRNGSQNNDNENNIKNTLEQHRQQQQ...

URE2-21:    MVDGNQMNNNKSRRNSSQRGNSNQRVNNQNE
            NNFNGLAQSSNNNNSITTTFTNNNQINSQLN
            GINNNVNQTDQNVQNHGNSNENNSENL...

URE2-22:    MQSHQAESNSSQNGDQNGTNNLQNNRSNGIN
            NFGNNNRNQNNLESQRVNNTINNNKLNQFNG
            NNEVNNVQNQSSDNTNNNMSIVTTRNS...

URE2-23:    MNIRNQNQSTAVLNVNQQSNNGTSNSVNNLN
            FNNSGMQNHGRNFNQSTRNNNTNEKGGNNIL
            NSNDERINNQQNQENNNTVDNSQNNSS...

URE2-24:    MMQRNGQQEGTNNNHSNINTQRNVFNNSANN
            NRNNNEGLNNNNSNFNNLVSNNQQVNVSSNS
            NINNQDNNKSILSGTSNDTTENRGQQQ...

URE2-25:    MNTNNSQGSFVDENQNRSIVKSRTVNMSQNN
            NTGNNNNAQLNNILNNTDSGHVSNNENRLGR
            QNNEFNQNSSQTNNGNNQQQSNNNNNI...
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FIG. 1. Amino acids sequences of scrambled prion domains. Amino acids 1 to 89 are shown for wild-type Ure2p and for each of the scrambled Ure2p variants. For each of the Ure2p variants, amino acids 90 to 354 are the same as in wild-type Ure2p.

Spontaneous [URE3] generation. The inability to utilize USA can be used as an assay of *URE2* activity. [ure-o] cells become USA^+ (able to take up USA) at a very low frequency (~$1/10^6$ cells; Table 1). Introduction of a plasmid overexpressing Ure2p from a *GAL1* promoter increases the frequency of prion formation due to the increase in protein available to spontaneously form prion seeds (Table 1) (50). We found that for all five of the Ure2p variants, cells expressing the proteins at wild-type levels formed USA^+ colonies at low frequency (Table 1). In all cases, overexpression of the Ure2p variants resulted in an increase in the number of USA^+ colonies, although the increase for *URE2-23* was not statistically significant.

For strains expressing wild-type *URE2*, overexpression of the prion domain is sufficient to induce formation of USA^+ colonies (Table 1) (29). We found that overexpression of the prion domains from each of the scrambled versions of Ure2p increased the frequency of USA^+ colony formation in strains carrying a chromosomal copy of the same *URE2* variant (Table 1). In fact, in all cases the prion domains induced to a greater degree than the full-length protein, although for *URE2-24* the difference was not statistically significant.

USA^+ stability, dominance, and curing by guanidine HCl. USA^+ colonies from each of the five scrambled prions were tested for the stability of the USA^+ phenotype. First, a low-

TABLE 1. Formation, stability, and dominance of USA^+ colonies in strains with integrated *URE2* variants

Integrated *URE2*	Plasmid[a]	Mean no. of USA^+ colonies/10^6 cells[b] ± SEM	USA^+ stability[c]	USA^+ dominance[d]
URE2-20	Vector, pH317	1.3 ± 0.2	19/44	14/19
	F.L., pER62	30 ± 6	12/22	11/12
	P.D., pER63	225 ± 65	8/22	5/8
URE2-21	Vector, pH317	67 ± 27	2/44	2/2
	F.L., pER95	220 ± 110	16/22	14/16
	P.D., pER98	1970 ± 640	20/22	18/20
URE2-22	Vector	8.2 ± 1.5	0/44	0/0
	F.L., pER112	21 ± 3	2/44	0/2
	P.D., pER99	360 ± 68	0/44	0/0
URE2-23	Vector	1.3 ± 0.8	1/44	0/1
	F.L., pER96	4.0 ± 2.0	1/22	1/1
	P.D., pER100	7.7 ± 0.3	2/22	2/2
URE2-24	Vector	2.7 ± 1.0	16/36	12/16
	F.L., pER113	60 ± 26	17/44	17/17
	P.D., pER101	68 ± 27	37/44	34/37
URE2-25	Vector	2.7 ± 0.9	12/26	8/12
	F.L., pER97	11 ± 2	2/22	2/2
	P.D., pER102	70s ± 40	2/22	2/2

[a] All strains carry a *LEU2* plasmid containing the *GAL1* promoter (vector, pH317) or a version of pH317 modified to express either a full-length version of the Ure2p variant (F.L.) or the prion domain of the Ure2p variant (P.D.).
[b] That is, the number of USA^+ cells per 10^6 cells after growth in galactose-raffinose dropout medium. Standard errors are indicated.
[c] That is, the fraction of USA^+ colonies that remained USA^+ after 48 h of growth on YPAD.
[d] That is, the fraction of stable USA^+ colonies whose USA^+ phenotype was dominant when mated with cells carrying the same *URE2* variant and unable to utilize USA.

stringency stability test was performed to identify potential stable USA^+ colonies. Colonies were suspended in water, spotted onto YPAD, grown for 48 h, and replica plated to test for the ability to utilize USA. This is a relatively low stringency test for stability, since only a small fraction of the cells need to be USA^+ for a given patch to appear USA^+. For all of the scrambled versions of *URE2*, some of the colonies were found to have maintained the USA^+ phenotype; however, the fraction that remained USA^+ varied dramatically among the *URE2* variants, with *URE2-21* and *URE2-24* most resembling wild-type *URE2* (Table 1).

When [URE3] cells are mated with [ure-o] cells, the diploids are [URE3], whereas *ure2* mutants are generally recessive. We therefore tested whether the colonies identified as stable in our low-stringency screen were dominant by mating them with strains expressing the same scrambled *URE2*. For strains expressing each of the *URE2* variants except *URE2-22*, the USA^+ phenotype was found to be dominant for the majority of cases (Table 1), suggesting that in most, if not all cases, the USA^+ phenotype results from prion formation.

Both [URE3] and [PSI] can be cured by growth on low concentrations of guanidine HCl (46; M. Aigle, unpublished data [cited in reference 50]). Guanidine acts by inhibiting the chaperone Hsp104p (17, 20, 21), which is required for the generation of prion seeds (32, 34, 49). To test whether the USA^+ phenotype in the dominant USA^+ colonies from strains expressing *URE2-21*, *-23*, *-24*, and *-25* was curable, we streaked cells from these colonies for single colonies on YPAD containing 5 mM guanidine HCl. Single colonies were then tested for their ability to utilize USA. In all cases the USA^+ phenotype was curable by guanidine treatment in 100% of the colonies (Fig. 2). When reselected on USA containing medium, cured clones became USA^+ at a similar frequency to the original cells (data not shown). As a control in the curing experiments and to further assay the stability of the USA^+ colonies, all strains were also streaked for single colonies onto YPAD, and the single colonies were then tested for their ability to utilize USA. For each *URE2* variant, a spectrum of stabilities was observed among the strains tested. For each, at least two USA^+ isolates (isolates A and B in Table 2) were identified with >90% stability; these were used for subsequent cytoduction experiments.

Transmission by cytoduction. Cytoplasmic mixing experiments (cytoductions) are used to test the infectivity of yeast prions. Cytoduction uses *kar1* mutants, which have a nuclear fusion defect during mating (8). When a donor strain carrying [URE3] is mated with a *kar1* [ure-o] recipient strain of the opposite mating type, the cells form mating pairs, but in most cases the nuclei do not fuse. During cell division, the nuclei separate; the resulting cells have cytoplasm that is a mixture of donor and recipient cytoplasm, while maintaining their original nuclei. Since the [URE3] prion is maintained in the cytoplasm, both donor and recipient cells are [URE3] after cytoduction. When the USA^+ cells from strains expressing *URE2-21*, *-23*, *-24*, and *-25* were used as cytoduction donors and cells expressing the same variant of *URE2* were used as recipients, in all cases the USA^+ phenotype was transmissible (Table 2).

The fact that formation of USA^+ colonies by cells expressing the scrambled versions of Ure2p is induced by overexpression and that the USA^+ phenotype is reversibly curable, dominant, and transmitted by cytoduction confirms that *URE2-21*, *-23*, *-24*, and *-25* are forming stable prions.

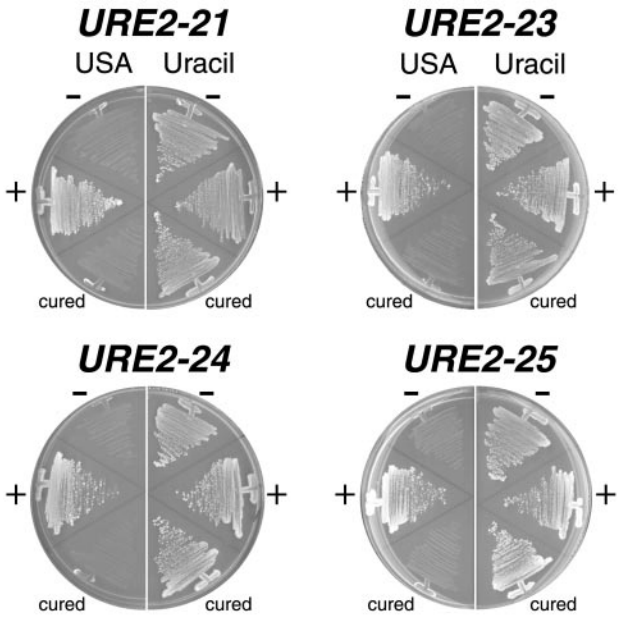

FIG. 2. Stability and curability of the USA^+ phenotype. Strains expressing *URE2-21*, *-22*, *-23*, and *-25* form stable, curable USA^+ colonies. USA^+ colonies from strains expressing the *URE2* variants were streaked for single colonies onto YPAD and YPAD with 5 mM guanidine HCl. Single colonies were taken from the YPAD plate (+) and from YPAD plus guanidine (cured) and streaked on ammonia medium with uracil (right) or with USA (left) to test for the ability to utilize USA. The [ure-o] parent was included as a negative control (−).

***Ure2-22p* forms unstable prions.** For strains expressing *URE2-22*, 130 of the 132 USA^+ colonies tested were unstable, and neither of the two stable ones was dominant (Table 1). Interestingly, the unstable USA^+ colonies were able to maintain the ability to utilize USA as long as they were grown under constant selective pressure; however, when grown on nonselective medium (YPAD), most of the cells lost the ability to utilize USA after 48 h (Fig. 3A). These results were reminiscent of those obtained for Sup35-Δ22/69, a mutant of Sup35p (4). Sup35-Δ22/69 forms prions that are defective in aggregate shearing. Strains expressing Sup35-Δ22/69 can switch to an inactive state ($[PSI]^{\Delta 22/69}$) that, like the USA^+ phenotype in cells expressing *URE2-22*, can only be maintained under selective pressure. $[PSI]^{\Delta 22/69}$ prions can be transmitted by cytoduction when the cytoductions are performed under selective conditions. We therefore tested whether the USA^+ phenotype could be transmitted by cytoduction from cells expressing *URE2-22*. USA^+ colonies from strains expressing *URE2-22* were grown for 48 h either on YPAD or USA medium. These cells were used as cytoduction donors, with recipient cells also expressing *URE2-22*. The recipient cells carry an ade2 mutation, causing them to accumulate red pigment when grown on limiting adenine and making it easy to distinguish between cytoductants, which are red when grown on limiting adenine, and diploids, which are white. Cytoductions were streaked directly onto medium containing USA and limiting adenine. When cells maintained on USA were used as donors, both USA^+ cytoductants (red) and diploids (white) were observed; however, when donor cells were pregrown on YPAD, no USA^+ cytoductants or diploids were observed (Fig. 3B). Select USA colonies were picked and spotted onto YPAD, and their genotypes were confirmed by replica plating (data not shown). The ability of USA^+ colonies to transmit the USA^+ phenotype by cytoduction clearly shows that the USA^+ phenotype is a result of an unstable prion.

TABLE 2. USA^+ phenotype is transmitted by cytoduction

URE2 variant	No. of USA^+ clones/total no. tested[a]	
	Isolate A	Isolate B
URE2-21	19/20	20/20
URE2-23	20/20	14/20
URE2-24	20/20	20/20
URE2-25	18/20	20/20

[a] Spontaneous USA^+ clones from strains expressing the *URE2* variants were used as cytoduction donors. Recipient cells expressed the same scrambled *URE2*. The numbers indicate the fractions of cytoductants that were USA^+.

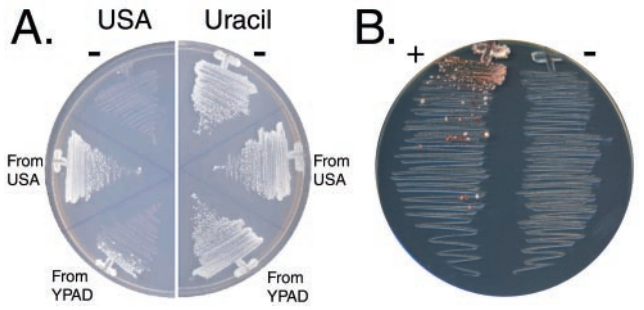

FIG. 3. *URE2-22* strains form unstable prions. (A) USA^+ colonies from strains expressing *URE2-22* retain the ability to utilize USA when maintained under selective pressure but lose the ability when maintained under nonselective conditions. USA^+ cells were pregrown on either YPAD or ammonia medium with USA in the place of uracil and then tested for the ability to grow on ammonia medium containing uracil (right) or USA (left). The [ure-o] parent was included as a negative control (−). (B) The USA^+ phenotype in *URE2-22* cells is transmissible by cytoduction when cells are maintained under selective pressure. Cytoductions with donors that were maintained either on ammonia medium containing USA (+) or on YPAD (−) were streaked directly onto ammonia medium containing limiting adenine and with USA in the place of uracil. For cells maintained under selective pressure, both USA^+ diploids (white) and cytoductants (red) are observed.

Amyloid fiber formation in vitro. Synthetic $Ure2p^{1\text{-}65}$ forms amyloid fibers in vitro rapidly (within 1 h [44]). These fibers are protease resistant and when stained with Congo red produce yellow-green birefringence under polarized light. We purified His_6-tagged versions of the prion domains of all five scrambled Ure2p variants to determine whether they would also form amyloid fibers. The prion domains were expressed in *E. coli* and purified under denaturing conditions. All but $Ure2\text{-}25p^{1\text{-}90}$ precipitated during overnight dialysis into water at 4°C. $Ure2\text{-}25p^{1\text{-}90}$ formed a small amount of visible precipitate during dialysis that was enhanced by an additional 12 h of rotation at 4°C. When examined by negative staining electron microscopy, the precipitates were all found to consist of filaments (Fig. 4). The diameters of the protofilaments were ca. 4 to 6 nm for all constructs. These protofilaments were assembled into polymorphic mixtures of higher-order fibrillar structures, with the

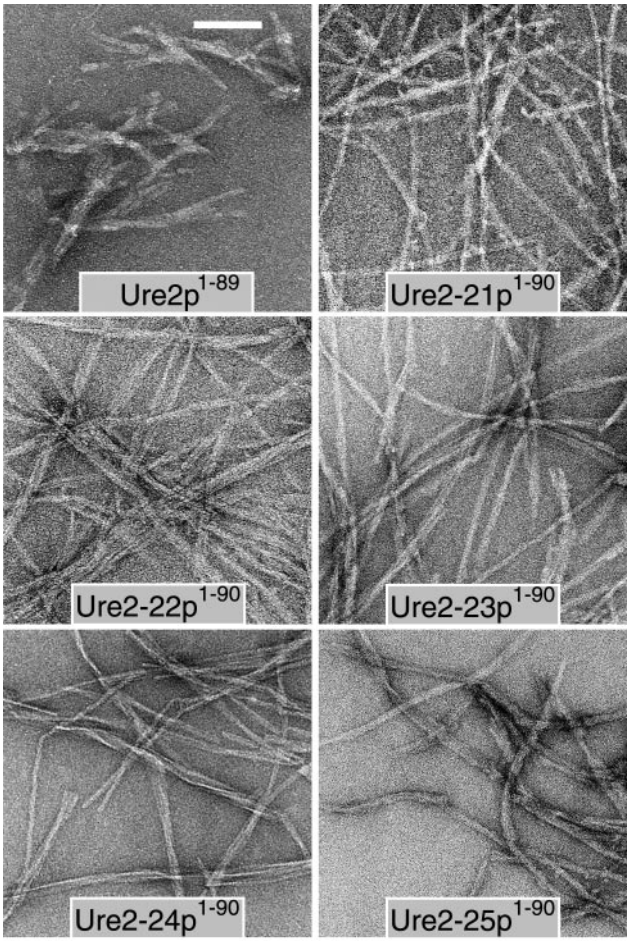

FIG. 4. Scrambled Ure2p prion domains form filaments. Prion domains were purified under denaturing conditions and dialyzed into water overnight at 4°C. Significant precipitate was visible for all constructs except Ure2-25p$^{1-90}$. Precipitate was observed for Ure2-25p$^{1-90}$ after further 12 h rotation at 4°C. Precipitates were stained with uranyl acetate and visualized by negative-staining electron microscopy. Bar, 100 nm.

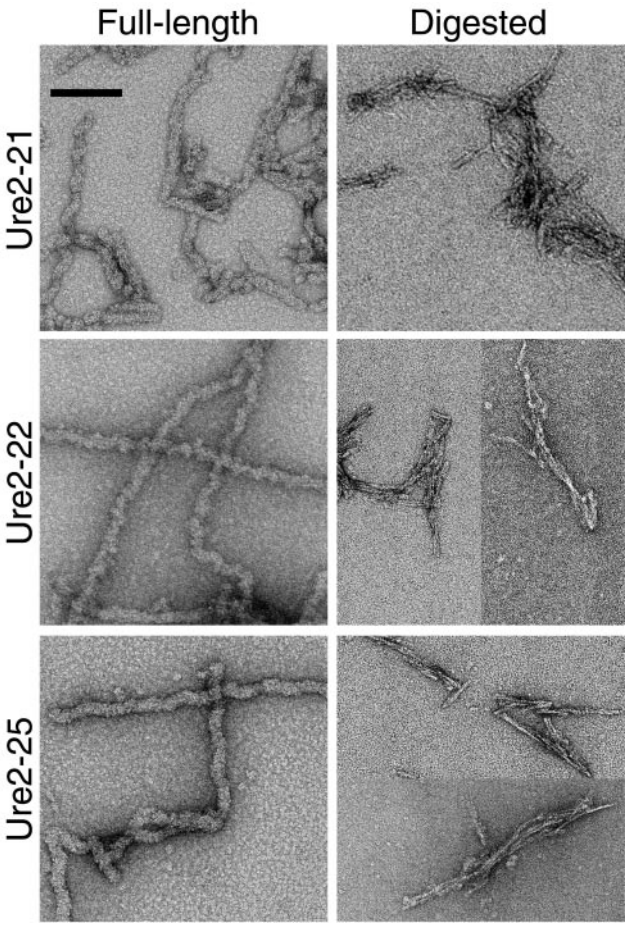

FIG. 5. Full-length scrambled Ure2p variants form fibers with protease resistant cores. Full-length Ure2-21p, -22p, and -25p were purified under native conditions. Significant precipitate was observed after overnight rotation at 4°C. Precipitates were digested with 0.1 mg of proteinase K/ml at 37°C for 16 h, and the insoluble fraction was collected, washed, and stained with uranyl acetate. Electron micrographs compare undigested (left) and digested (right) filaments. Bar, 100 nm.

array of higher order structures varying among the samples. This kind of polymorphism is typical for amyloid-like structures (1, 18). These filaments, when stained with Congo red, showed the yellow-green birefringence that is characteristic of amyloid (data not shown).

Full-length Ure2p can form self-seeding filamentous aggregates in vitro (44). When digested with proteinase K, the fiber diameter is narrowed, with a resulting fiber diameter comparable to that of fibers formed by the prion domain alone (3, 44). Mass spectrometry analysis of digested filaments has demonstrated that they are composed entirely of prion domain fragments (3). We constructed His_6-tagged versions of full-length URE2-21, -22, and -25; expressed them in *E. coli*; and purified the proteins under native conditions. All three formed visible aggregates during overnight incubation (rotation at 4°C in pH 8 buffer). When examined by negative-staining electron microscopy, the aggregates were all found to consist of filaments (Fig. 5). The appearance and diameters (20 to 25 nm) of these filaments are very similar to filaments of Ure2p. After extensive digestion with proteinase K (100 μg/ml for 16 h at 37°C), sedimentable filaments remained. These filaments had a tendency to stick together, making visualization more difficult. However, it was clear that they were much thinner (~4 nm) than those formed by the full-length proteins (Fig. 5) and were consistent in diameter with proteinase K-treated wild-type Ure2p filaments (3, 44).

DISCUSSION

The Ure2p prion domain is necessary and sufficient for prion formation in vivo (28, 29) and for amyloid fiber formation in vitro (3, 44). We generated five variants of Ure2p in which we randomly shuffled the order of the amino acids in the prion domain. In all cases the randomized prion domains formed amyloid fibers in vitro under native conditions and supported prion formation in vivo, albeit with various frequencies and stabilities. Thus, although the primary sequence of the Ure2p prion domain has some impact on the frequency of formation

and stability of [URE3] prions, the ability of the Ure2p prion domain to support prion formation is primarily a result of its amino acid content.

Although a variety of lines of evidence have indicated that high Q/N content is an important factor in prion formation by Ure2p and Sup35 (11, 27), only Rnq1p, of the ~100 additional yeast proteins with Q/N-rich domains (31, 42), has been shown to form prions. The Q/N-rich domain of one other, New1p, can support prion formation when attached to the Sup35 C terminus but has not been shown to form prions in the context of full-length New1p (39). The apparent inability of other Q/N-rich domain protein to form prions is likely in part due to amino acid content features other than Q/N-content playing a role in prion formation. The high serine content (12%) or relative lack of charged residues of the Ure2p prion domain may contribute to prion formation. In addition, cellular expression and localization may influence prion formation. Proteins expressed at very low levels or not expressed during parts of the cell cycle would likely not be able to maintain stable prions. Intra- or intermolecular interactions may stabilize potential prion-forming domains, preventing prion formation. Finally, as seems to be the case with New1p, features of the protein outside of the Q/N-rich domain may influence prion formation.

Ure2p has two features that may make it more prone to forming amyloid than other proteins with comparable amino acid content. First, although partial unfolding of the native state is required for amyloid formation by many proteins (22), the Ure2p nitrogen-regulation domain folds independently of the relatively unstructured prion domain (5, 35, 48) and remains folded in amyloid fibers (2, 6); therefore, unfolding of Ure2p is probably not required for prion formation. Furthermore, since the order of the amino acids in the prion domains of the scrambled Ure2p variants is random, these domains are unlikely to contain significant stably fold structures.

Second, [URE3] amyloid fibers have a core composed of the prion domains with the C-terminal domains arrayed around the outside (2, 3). For other proteins that contain potentially amyloidogenic domains with similar amino acid content to the Ure2p prion domain, the remaining part of the protein might be sterically unsuited for incorporation into amyloid fibers. Similarly, the C-terminal domain of Ure2p may be particularly well suited to support the maintenance of [URE3]. In vitro, the presence of the C-terminal domain of Ure2p slows down amyloid fiber formation (44); perhaps in vivo the C-terminal domain acts similarly, since the largest aggregates may be dead-end products (37), the presence of the C terminus may slow down amyloid formation sufficiently to allow the propagons to be maintained by preventing the accumulation of larger, dead-end aggregates.

Although all five scrambled Ure2p variants are able to form prions, the frequency of prion formation and fraction of spontaneous USA^+ colonies that are able to maintain the USA^+ phenotype without selection vary significantly among the variants. Not surprisingly, attempts to use alignments of the scrambled prion domain amino acid sequences to correlate prion-forming activity with amino acid sequence were not particularly informative. The high asparagine content resulted in a high number of identities (14–28) for all pairwise alignments; however, there was no apparent correlation between the alignment scores and the prion-forming behavior of the proteins in vivo. Similarly, the alignments did not reveal any obvious primary sequence features that were conserved among the sequences that most readily formed stable prions. It is striking that *URE2-22*, which only formed unstable prions, and *URE2-23*, which forms USA^+ colonies at the lowest frequency and only rarely forms stable prion, are the only two variants that contain no runs of asparagines longer than three amino acids. This might suggest that runs of asparagines and not just asparagine content could be important in determining prion stability. However, if glutamines and asparagines are assumed to be functionally interchangeable for amyloid formation, then it becomes more difficult to rationalize the various stabilities of the prions formed by each of the variants, since all of the variants have Q/N-runs of at least four amino acids in length.

Although the *URE2-22* strain formed USA^+ colonies spontaneously at a rate comparable to the other scrambled *URE2* variants, and Ure2-22p readily forms amyloid fibers in vitro, the prions formed by *URE2-22* could only be maintained under selective pressure. This could be because the fibers are not sufficiently stable to be maintained without selective pressure or because they are too stable, and therefore are not distributed efficiently during mitosis and rapidly lost without selective pressure. This second explanation has been observed for the Sup35-Δ22/69 mutant of the prion protein Sup35 that forms prions that are defective in aggregate shearing (4). Regardless of the explanation, the anomalous behavior of *URE2-22* highlights the limitations of in vitro experiments in predicting amyloid formation and propagation in cells.

Would the amyloid-forming ability of other amyloidogenic proteins be similarly resistant to scrambling? On the surface, this idea would seem to clash with the numerous examples of proteins in which single point mutations significantly impact amyloid-forming ability. For the yeast prion protein Sup35, there are multiple examples of point mutations and deletions in Sup35p that block [PSI] formation (11, 14, 45). However, each of these mutations eliminates a glutamine or asparagine and/or inserts a charged residue. Since both Q/N content and absence of charges are likely important features driving [URE3] and [PSI] prion formation, the effects of these mutations may simply result from critical changes in the amino acid composition in the regions that drive [PSI] formation. For other amyloidogenic proteins, as previously noted, the effects of many single point mutations can be rationalized based on their destabilizing effect on the native state (22). Similarly, point mutations could disrupt inter- or intramolecular interactions involving the potential amyloid-forming domains, thereby making them more accessible and more prone to forming amyloid.

Some unique feature of Ure2p may make its amyloid-forming ability insensitive to amino acid order; for example, if [URE3] formation is driven entirely by Q/N-runs, then the scrambled variants would also tend to form prions, since any sequence with 46% Q/Ns would be likely to have Q/N runs. If this is the case, it would be interesting in its own right because it would suggest that most of the numerous other proteins with regions of amino acid content similar to the prion domain of Ure2p should possess the intrinsic ability to form amyloid under physiological conditions and that it is only the context of these sequences within their respective proteins or the cellular expression, localization, and interactions that prevents them

from forming amyloid. Amyloid formation by other proteins may be driven by more complex sequence motifs and therefore be blocked by scrambling. However, we find it intriguing to note that for human muscle acylphosphatase, the effects of individual mutations on intrinsic amyloid-forming ability in vitro under denaturing conditions can be largely explained based on their effects on hydrophobicity, charge, and propensity to convert from α-helix to β-sheet (7). Of these, hydrophobicity and charge, and to some extent even β-sheet propensity, are independent of the order of amino acids, suggesting that this and perhaps other amyloid-forming systems may likewise show a degree of primary sequence independence.

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