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Differential resistance to proteinase K digestion of the yeast prion-like (Ure2p) protein synthesized in vitro in wheat germ extract and rabbit reticulocyte lysate cell-free translation systems

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Abstract The Ure2p yeast prion-like protein was translated in vitro in the presence of labeled [^{35}S]methionine in either rabbit reticulocyte lysate (RRL) or wheat germ extract (WGE) cell-free systems. When subjected to proteinase K digestion, the Ure2p protein synthesized in WGE was proteolysed much more slowly compared to that synthesized in RRL; this displays fragments of about 31–34 kDa, persisting over 8 min. Thus, the digestion rate and pattern of the protein synthesized in WGE, unlike that synthesized in RRL, revealed characteristic features of the [URE3] prion-like isoform of the Ure2p protein [Masison, D.C. and Wickner, R.B. (1995) Science 270, 93–95]. Chloramphenicol acetyltransferase, synthesized under the same conditions, differed fundamentally in its proteolytic sensitivity toward proteinase K (PK); in the RRL system it was more slowly digested than in WGE, proving specific PK inhibitors to be absent in both systems. Posttranslational addition of the WGE to the RRL-synthesized Ure2p does not protect Ure2p from efficient PK degradation either. The differences in Ure2p degradation may be ascribed to a specific structure or specific states of association of Ure2p synthesized in WGE; obviously, they yield a protein that mimics the behavior of the Ure2p in [URE3] yeast strains. The present data suggest that particular conditions of the Ure2p protein translation and/or certain cellular components (accessory proteins and extrinsic factors), as well as the nature of the translation process itself, could affect the intracellular folding pathway of Ure2p leading to the de novo formation of the prion [URE3] isoform.

Key words: Yeast prion; Ure2p; [URE3]; In vitro translation; Folding; Protease resistance; Prion origin

1. Introduction

Saccharomyces cerevisiae can use alternative nitrogen sources such as arginine, urea and allantoin, if preferred nitrogen sources like glutamine, asparagine, or ammonium ions are not available in the medium. The basis of this selectivity is known as nitrogen catabolite repression [1]. Utilization of alternative nitrogen sources requires both the relief of nitrogen repression and the induction of specific permeases and enzymes. The products of the GLN3 and URE2 genes are required for the appropriate transcription of numerous genes in alternative nitrogen assimilatory pathways [2–5]. GLN3 is known to activate their transcription when rich nitrogen sources are not available, and Ure2p appears to repress their transcription when alternative nitrogen sources are not needed [1–5]. However, the repression can be overcome in yeast [URE3] strains [6]. A cytoplasmically inherited element, [URE3], allows yeast to use ureidosuccinate in the presence of ammonium [1,6,7]. [URE3] is a non-Mendelian genetic element that mimics recessive mutations in the chromosomal URE2 gene. Recently it was suggested that [URE3] is an altered (prion-like) form of the chromosomally encoded Ure2p protein [8]. Several genetic and biochemical criteria were proposed to confirm this suggestion and [URE3] was shown to satisfy these criteria [7–9]. Evidence supporting the hypothesis that [URE3] represents a prion-like element in yeast came from the following observations: (1) [URE3] can be reversibly cured; (2) [URE3] propagation requires the natural URE2 gene; recessive chromosomal mutants exhibit the same phenotypes as those observed in the presence of the respective dominant non-Mendelian elements; (3) overproduction of Ure2p increases the frequency of cells acquiring [URE3]; (4) [URE3] is a dominant and invasive element [7–9].

Proteinase K resistance was among the first criteria to distinguish between the scrapie (altered PrP^{Sc}) form of the mammalian prion protein (PrP) and its cellular isoform (PrP^{c}) [10,11]; for [URE3] the same holds true [9]. Correspondingly, the cellular isoform Ure2p has been suggested to undergo a profound conformational change during its conversion into the cellular isoform [URE3] [7–9], in accordance with mammalian prion proteins [12,13].

It has been suggested that [URE3] arises spontaneously from the normal cellular isoform of the Ure2p protein, propagating through interactions of the N-terminal domain of the proteins [7–9]. The frequency of spontaneous occurrence of [URE3] is in the order of 10^{-5}; it can be enhanced up to 100-fold upon overexpression of the URE2 gene, or subjecting the yeast cells to stress (heat or cold shock, ethanol, etc.) [14]. On the other hand, [URE3] can be cured by growing the cells in non-mutagenic protein denaturants such as millimolar concentration of guanidinium chloride [7–9]. These observation lend additional support to the suggestion that [URE3] and Ure2p differ in their conformational state, i.e. protein-based inheritance in yeast can be determined by the conformational variants of the proteins involved. This possibility puts an intriguing question in the prion-folding problem, namely: how can one and the same gene expressed in a given cellular species give rise to different conformers in proportions depending on the environmental conditions? This question addresses a quite fundamental problem, and also bears on an important public health issue linked to prions in general, known to cause fatal neurological diseases in humans and animals [12,13]. In

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fact, two questions could be addressed in connection with the prion-folding problem: (1) is the initial conversion of the normal cellular isoform (PrP^c) of the prion protein into its scrapie isoform (PrP^{Sc}) stochastic and spontaneous, or could certain cellular factors and mechanisms affect this process? and (2) is the interaction of the PrP^{Sc} with PrP^c the only one factor which is sufficient and necessary for the propagation of the conversion?

The yeast prion-like element Ure2p may be considered a convenient object to elucidate the mechanism of the partitioning between the different conformers [7–9]. Here, we address this possibility by translating the Ure2p mRNA in various cell-free extracts. The results show that, depending on the factors present in the cell-free translation systems, the newly synthesized Ure2p protein differs in its structural properties. The Ure2p protein synthesized in vitro in wheat germ extract system (WGE) possesses major properties of the [URE3] prion isoform, displaying much higher resistance to proteinase K digestion, in contrast to the protein synthesized in rabbit reticulocyte lysate cell-free system (RRL). If these results apply in vivo, the occurrence and cytoplasmic inheritance of the [URE3] condition could result from the disbalance of the cellular translation apparatus, favoring the Ure2p prion-like isoform over the normal Ure2p conformer. Whether this is a co- or posttranslational process including other proteins is an open question.

2. Materials and methods

2.1. Plasmid construction

The pFL39URE2 and pBeGB/HFCURE2 plasmids bearing the genes for the natural Ure2p and modified protein (containing C-terminal hexahistidyl tail) were constructed by Christophe Cullin and Elisabeth Guillemet (to be published). The pUC18:IM3/CI-1 plasmid containing the chloramphenicol acetyltransferase (CATIII) gene was the gift of Prof. W.V. Shaw (University of Leicester). The *Eco*RI-*Xho*I (1421 bp) fragment bearing the URE2 gene of the pFL39URE2 plasmid was inserted into pBluescript II SK^+ vector and the *Hin*dIII-*Eco*RI (1158 bp) fragment of the pBeGB/HFCURE2 plasmid into pBluescript II KS^- vector, to obtain the genes under control of the T7 promoter. The *Bam*HI-*Hin*dIII (1034 bp) fragment of the pUC18:IM3/CI-1 plasmid was inserted into pBluescript II KS^- vector, under control of the T7 promoter.

2.2. In vitro transcription

The transcription reactions were carried out: (i) following the Promega Transcription in vitro systems Technical Manual or (ii) according to Gurevich et al. [15] in 100 μl (total volume) of 80 mM HEPES-KOH buffer, pH 7.5, containing 16 mM $MgCl_2$, 2 mM spermidine, 20 mM dithiothreitol (DTT), 3 mM ATP, 3 mM GTP, 3 mM UTP, 3 mM CTP, 2.5 μl (100 units) of RNasin (Promega), 5 μg of either *Eco*RI (pBSK$^+$/URE2), *Xho*I (pBKS$^-$/URE2his) or *Hin*dIII (pBluescript II KS^- CATIII) linearized DNA templates and 320 units of T7 RNA polymerase (Promega). The reactions were carried out at 37°C for 2.5 h and stopped by phenol/chloroform extraction. The transcripts were purified by LiCl precipitation and washed with 70% ethanol [16]. The purity and integrity of the RNA molecules were checked by 5% PAGE under denaturing conditions in the presence of 7 M urea. An aqueous solution (0.6 mg/ml) of the transcripts was used in translation experiments.

2.3. Cell-free protein synthesis

Cell-free translation of the Ure2p and CATIII mRNAs was performed using either the RRL or WGE in vitro translation systems in the presence of [^{35}S]methionine (15 mCi/ml, Amersham) as described in the above Promega Technical Manual. The final concentration of Ure2p mRNAs in both systems was 132 μg/ml and that of CATIII mRNA 128 μg/ml. The translation was carried out at 34°C for 1 h in the case of RRL and at 25°C for 1.5 h in the case of the WGE system to enrich the same final Ure2p protein concentration in equal volumes of the translation systems. Chloramphenicol acetyltransferase translation was performed for 40 min in the case of RRL and 80 min in the case of the WGE system. The translation reactions were stopped by the addition of an equal volume of 20 mM Tris-HCl buffer, pH 7.6, 200 mM KCH_3COO, 20 mM $Mg(CH_3COO)_2$, 2 mM DTT and 0.2 mM EDTA cold at 0°C (buffer A).

2.4. Proteinase K susceptibility of the in vitro synthesized Ure2p and CATIII

Proteinase K (0.2–0.5 μg, Stratagene) was added to 25 μl fractions of the stopped translation mixtures and the incubation was performed at 37°C. Periodically 3 μl aliquots were removed, mixed with 50 mM Tris-HCl, electrophoresis sample buffer pH 6.8, containing 4% SDS, 2% mercaptoethanol (v/v), 12% glycerol (w/v), 0.01% Serva Blue G and immediately incubated at 80°C for 10 min. The samples were further analyzed by electrophoresis according to [17] in a 10% T, 3% C or 16.5% T, 6% C gel, overlaid with a 4% T, 3% C stacking gel. 'Rainbow [^{14}C]methylated colored proteins' (MW = 2350–46000 Da, Amersham) were used as molecular weight markers. Gels were fixed, dried in vacuo and subjected to autoradiography using the Molecular Dynamics PhosphorImager.

2.5. Miscellaneous

Molecular cloning was performed following general procedures described in [16].

Radioactivity was monitored using a Packard 2200CA Tri-Carb liquid scintillation analyzer. Trichloroacetic acid precipitable radioactivity was determined in sample aliquots spotted onto GF/C (Whatman) glass filters after NaOH hydrolysis. Protein determination was done using the bicinchoninic acid assay [18].

3. Results and discussion

Synthesis of Ure2p made use of either the RRL or WGE cell-free translation systems. In both cases equal amounts of mRNAs were subjected to translation, optimizing the reaction so that equal amounts of the proteins (~0.4 ng) were synthesized in a total final volume of 50 μl of both systems. The translation reactions were stopped by the addition of an equal volume of cold on ice buffer (A) and subjected to digestion with proteinase K (PK) as described in Sections 2.3 and 2.4.

The translation of the Ure2p in either the RRL or the WGE cell-free system resulted in a major polypeptide of about 42 kDa (Fig. 1), in accordance with its known mobility on SDS-PAGE [9] and close to the molecular weight of the Ure2p protein calculated from its amino acid sequence (40 kDa). An additional 30 kDa fraction may be ascribed to an 'abortive' polypeptide (apparently the product of premature termination and release of the incomplete nascent Ure2p chains). Interestingly, a polypeptide of the same size was also detected in vivo during the expression of Ure2p in yeast cells by immunoblotting with a polyclonal antibody to Ure2p [9]. The major polypeptide synthesized in RRL was digested by PK in less than 1 min, yielding major fragments of 18–19 kDa as well as minor polypeptides of 6–7 and 4 kDa respectively (Fig. 1, lane 1′ RRL). The major species are digested slowly (life-time 5 min); the 6–7 kDa fragment is very short-lived, whereas the 4 kDa fragment resists proteolysis for at least 25 min. For the polypeptide synthesized in WGE, the proteolysis pattern closely resembled that obtained in RRL; however, the kinetics of proteolysis differed significantly (Fig. 1, lane 1′–25′ WGE). Most of the 42 kDa polypeptide synthesized in WGE was digested within 1 min into at least three fragments, two of 34 and 32 kDa, and one overlapping with the 30–31 kDa 'abortive' polypeptide. As in the case of the RRL system, a characteristic fragment of about 18–19 kDa

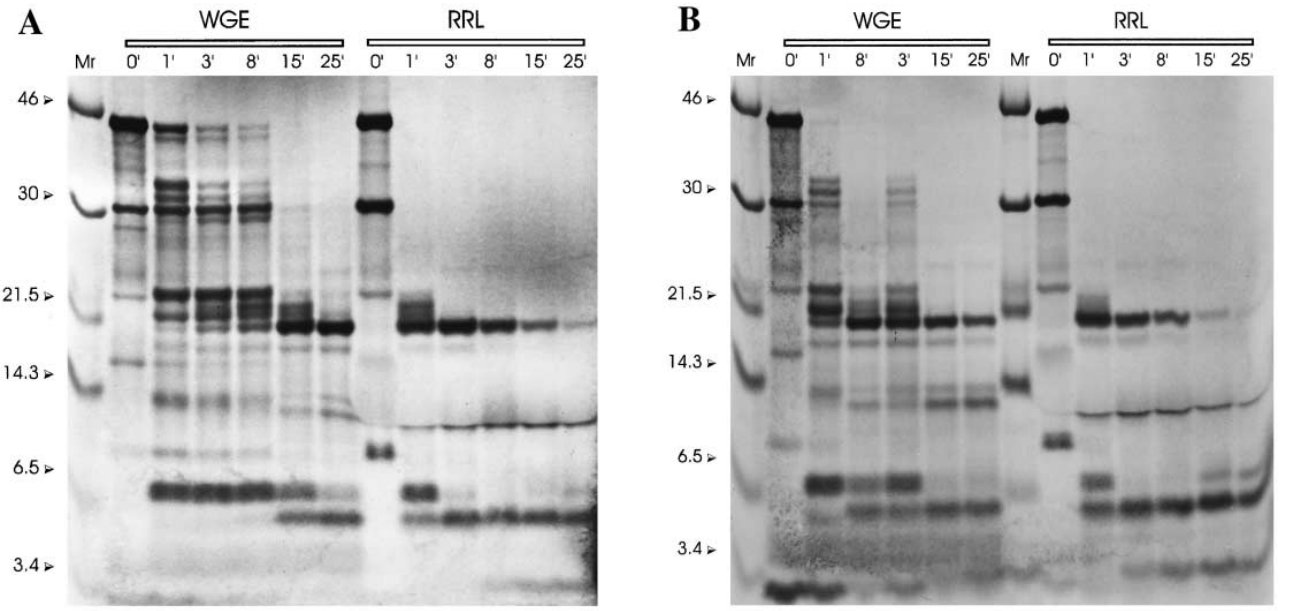

Fig. 1. Autoradiogram of SDS gel electrophoresis of the Ure2p mRNA cell-free translation products and PK digestion. Numbers at the top of the slots indicate the times of digestion by PK (time 0′: no protease added). WGE and RRL refer to the cell-free systems used to translate Ure2p mRNA. Arrows indicate the positions of molecular weight standards. Molecular mass is given in kDa. Mr refer to the slots with molecular weight standards. A: 0.25 μg of PK was added to a 25 μl aliquot of the translation mixture as indicated in Section 2. B: 0.5 μg of PK was added to a 25 μl aliquot of the translation mixture as indicated in Section 2.

also appeared, but significantly later in time of digestion. Remarkably, the digestion pattern of the proteins produced in WGE after 15 min of digestion closely resembled that after only 1 min of digestion of the proteins produced in RRL. In addition, it is important to note that the total endogenous protein concentration in RRL exceeded that in WGE threefold (98 mg/ml in RRL, 31 mg/ml in WGE), meaning that the relative ratio of PK to the total endogenous as well as to the newly synthesized protein content is 3 times higher in WGE. If the rate of proteolysis is assumed to be first-order with respect to the protease concentration, this would mean that the proteolysis kinetics is about 40 times faster for Ure2p synthesized in RRL compared to the protein produced in WGE. Similar digestion features were also observed for the Ure2p protein containing a His-tag at its C-terminal end (Fig. 2). Ure2p His-tagged protein has also been shown to give rise to the [URE3] yeast phenotype (C. Cullin, unpublished data). It needs to be emphasized that differences in PK digestion are relative. Increasing the concentration of PK two-fold (0.5 μg PK per 0.1 ng of the newly synthesized Ure2p) resulted in a more efficient degradation of the protein translated in WGE (Fig. 1B). However, even under this condition the difference in PK digestion was significant: the 31–34 kDa fragments persisted for more than 3 min in the case of the protein synthesized in WGE; the major 18–19 kDa fragment remained unaffected for over 25 min. In this time range, the degradation of the protein synthesized in RRL is practically complete (Fig. 1B). It should be underlined that Ure2p synthesized in both cell-free systems was stable until PK was added and no difference in peptide patterns was observed upon incubation of the translation mixtures at 37°C for 25 min (not shown). It should also be underlined that the indicated differences of Ure2p degradation are not due to specific PK inhibitors present in the WGE system; rather they are attributable to structural differences or to variations in the state of association of Ure2p. Control experiments to support this made use of the in vitro synthesis of chloramphenicol acetyltransferase in both cell-free systems. PK digestion here resulted in a lower efficiency for the RRL system compared to WGE (Fig. 3). This finding may be attributed to the fact that the overall concentration of endogenous proteins in RRL is 3 times higher that in WGE. Posttranslational addition of WGE (up to 80% of its

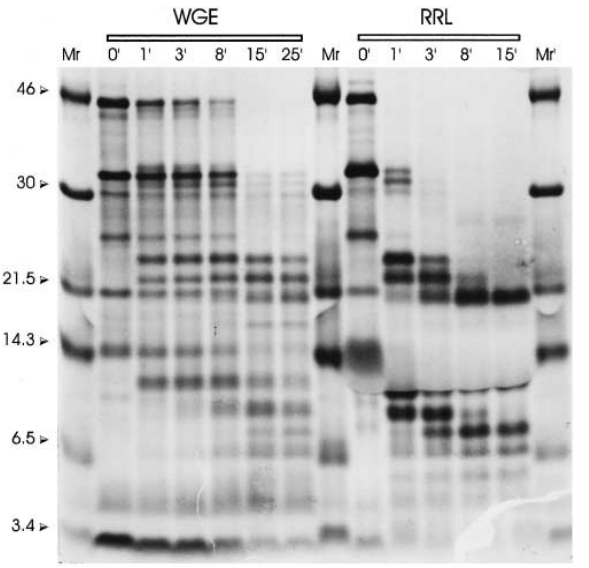

Fig. 2. Autoradiogram of SDS gel electrophoresis of the Ure2p His-tailed mRNA cell-free translation products and PK digestion. Numbers at the top of the slots indicate the times of digestion by PK (time 0′: no protease added). 0.2 μg of PK was added to a 25 μl aliquot of the translation mixture as indicated in Section 2. WGE and RRL refer to the cell-free systems used to translate Ure2p mRNA. Arrows indicate the positions of molecular weight standards. Molecular mass is given in kDa. Mr refer to the slots with molecular weight standards.

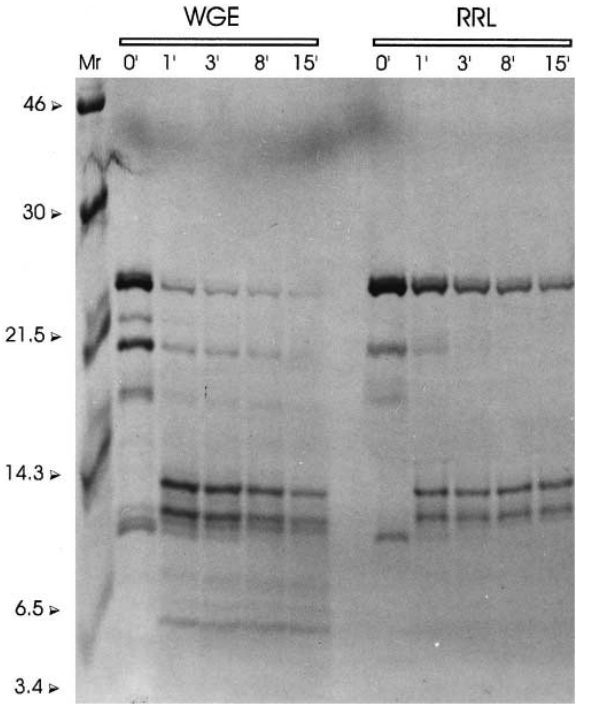

Fig. 3. Autoradiogram of SDS gel electrophoresis of the CATIII mRNA cell-free translation products and PK digestion. Numbers at the top of the slots indicate the times of digestion by PK (time 0′: no protease added). 0.25 μg of PK was added to a 25 μl aliquot of the translation mixture as indicated in Section 2. WGE and RRL refer to the cell-free systems used to translate CATIII mRNA. Arrows indicate the positions of molecular weight standards. Molecular mass is given in kDa. Mr refer to the slots with molecular weight standards.

final concentration in the standard WGE translation mixture, Fig. 4) does not protect Ure2p, synthesized in RRL from efficient digestion by PK, proving once again that specific PK inhibitors are absent in WGE. Neither the full-length nor the characteristic 31–34 kDa fragments could be observed in this case in contrast to the digestion of the protein synthesized in WGE (compare Figs. 1A and 4). Thus, one can conclude that the observed difference in Ure2p degradation could be attributed to the different folding states of the protein synthesized in WGE and RRL. The slight difference in Ure2p degradation in the RRL+WGE condition in comparison to the pure RRL mixture could be evidently ascribed to the higher overall protein concentration in the RRL+WGE mixture.

It should be emphasized that similar differences regarding the time scale and the presence of 30–32 kDa fragments were previously demonstrated in PK-dependent degradation of Ure2p expressed in either [URE3] or wild type yeast cells [9]. Thus, we conclude that the isoform of the Ure2p protein with the characteristic PK resistance of the [URE3] prion-like element can be produced in the WGE system unlike in the RRL. It should also be stressed that features of degradation of the major 18–19 kDa Ure2p proteolytic fragment closely resemble those of the N-terminal Ure2p prion inducing domain fragment [9]. This fragment separately expressed in wild type and prion containing yeast cells displays differential resistance to PK digestion [9]. We argue that the 18–19 kDa fragment observed in our experiments originates from the N-terminal part of the Ure2p protein. This fragment displays similar mobility on SDS-PAGE as revealed after PK treatment of the C-terminal His-tagged and wild type Ure2p protein run on one and the same gel (not shown).

The results presented clearly indicate that [URE3] can arise nonstochastically and that the cellular environment could affect the Ure2p intracellular folding pathway.

As has been previously reported [19–21], the specific properties of cell-free translation systems can affect protein folding in vivo. In the case of the precursor of liver mitochondrial aspartate aminotransferase (pmAspAT), the RRL system seems to contain some relevant factors that are effective in protein folding, but absent in the WGE system [20,21]. Fully folded pmAspAT is known to be largely resistant to trypsin hydrolysis. The enzyme synthesized in vitro in WGE, unlike that in RRL, does not become trypsin resistant. Obviously, it is partially misfolded in contrast to the enzyme obtained by the in vitro synthesis in RRL [20,21]. The differences in Hsp70 association between the two forms of pmAspAT support the assumption that early in translation one and the same polypeptide chain may exhibit different structural features in the two cell-free systems. The stability of the Hsp70 complex with the WGE enzyme confirms its non-native conformation [20,21]. The same kind of partitioning between the native and a misfolded state may be assumed for Ure2p. However, in both cases it is unclear what the misfolded proteins are. In the given context an alternative explanation could be that the WGE system contains all the necessary factors, but that they

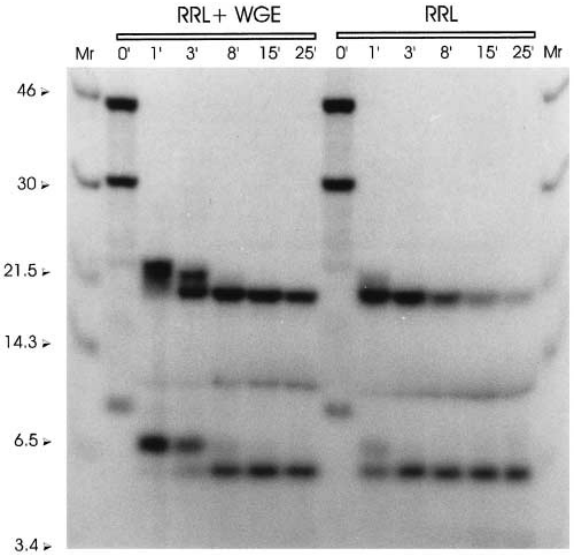

Fig. 4. Autoradiogram of SDS gel electrophoresis of the Ure2p mRNA cell-free translation products and PK digestion. Numbers at the top of the slots indicate the times of digestion by PK (time 0′: no protease added). RRL+WGE and RRL refer to the conditions used to analyse Ure2p PK digestion. After 1 h of incubation, the RRL translation reaction was stopped by the addition of cold in ice buffer A (see Section 2) and the reaction mixtures were subdivided into two equal portions. The WGE extract (80% of its final concentration in the standard WGE translation mixture) was added to one of the portions (RRL+WGE) and an equal volume of the half-concentrated buffer A to the other (RRL). The ratio of PK to the Ure2p synthesized in RRL was 0.25 μg PK per 0.1 ng of the Ure2p, as described in Fig. 1a. Arrows indicate the positions of molecular weight standards. Molecular mass is given in kDa. Mr refer to the slots with molecular weight standards.

are not adapted to assist the production of the native Ure2p state. Hamster PrP expressed in vitro in either the WGE or the RRL cell-free system supplemented with microsomal membranes was also shown to represent different folding states [22]. The characteristic transmembrane form of the PrP was observed in the WGE system in contrast to the native completely translocated (secretory) form observed in RRL. Likewise it was concluded that hamster PrP synthesized in vitro could display two system-dependent topologies.

A fundamental question in the pathogenesis of the prion diseases is the mechanism of conversion of the cellular isoform of prion protein (PrP^c) into the infectious scrapie isoform (PrP^{Sc}). It was suggested that conversion of PrP^c into PrP^{Sc} occurs by a conformational mechanism due to a post-translational interaction of PrP^{Sc} with PrP^c [12,13].

Similar mechanisms were postulated to explain the origin of the yeast prion-like proteins [URE3] and [PSI] [7,14,23].

However, several lines of evidence call the postulated mechanisms into question for at least two reasons: (i) conversion of mammalian PrP^c into PrP^{Sc} (observed in vitro) requires significant pretreatment of PrP^c (PrP^{Sc}) with 1–6 M GdnHCl [23,24], conditions one can hardly achieve in vivo; (ii) at least in the case of the yeast [PSI] prion-like element it was shown that several chaperones and in particular Hsp104 and Ssa1 provoke efficient elimination of [PSI], being overexpressed, whereas an intermediate amount of Hsp104 is required for the propagation of [PSI] [14].

In an attempt to elucidate the mechanism by which [URE3] can arise de novo, we have studied the resistance to proteinase K digestion of the Ure2p yeast prion-like protein after its synthesis in vitro using either a mammalian system, rabbit reticulocyte lysate, or a plant system, wheat germ extract.

The results of the present study indicate that Ure2p protein when newly synthesized in WGE (unlike the RRL protein) reveals characteristics of the [URE3] prion-like isoform. This means that particular conditions of Ure2p translation can lead to either the Ure2p or the [URE3] variant. This observation strongly suggests that in vivo *S. cerevisiae* can produce [URE3] de novo. Evidence showing that overproduction of Ure2p increases the frequency of cells acquiring [URE3] [7–9] suggests that the [URE3] isoform could result from the disbalance of the protein biosynthetic apparatus. Similar mechanisms could explain the initial formation of the PrP^{Sc} isoform in the case of mammalian prion diseases. Different cytosolic factors are assumed to contribute to determine the final proper protein conformation in vivo, ranging from those controlling ribosome traffic to chaperones, stress proteins and foldases. The particular balance of these components is expected to be specific for a given organism. Very recently the structure of the full-length recombinant murine prion protein, mPrP (23-231) has been characterized by UV-CD and NMR [25,26]. Their data showed that N-terminal (23-120) part of the protein is represented (in aqueous solution) by a flexible extended coil, which could enable structural transitions of PrP^C to PrP^{Sc} in vivo [25,26] and apparently be an 'attractive' target for chaperones. Similarly one can expect the same and for the N-terminal part of the prion-like elements of yeasts.

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