ESRF	**Experiment title:** Self-assembly mechanisms of prion Ure2p fibril formation studied by sulfur K-edge XANES spectroscopy	**Experiment number**: SC-1354
Beamline: ID26	**Date of experiment**: from: 03 oct 2003 to: 07 oct 2003	**Date of report**: Nov 2004
Shifts: 12	**Local contact(s)**: L. Alvarez	*Received at ESRF:*

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Report:

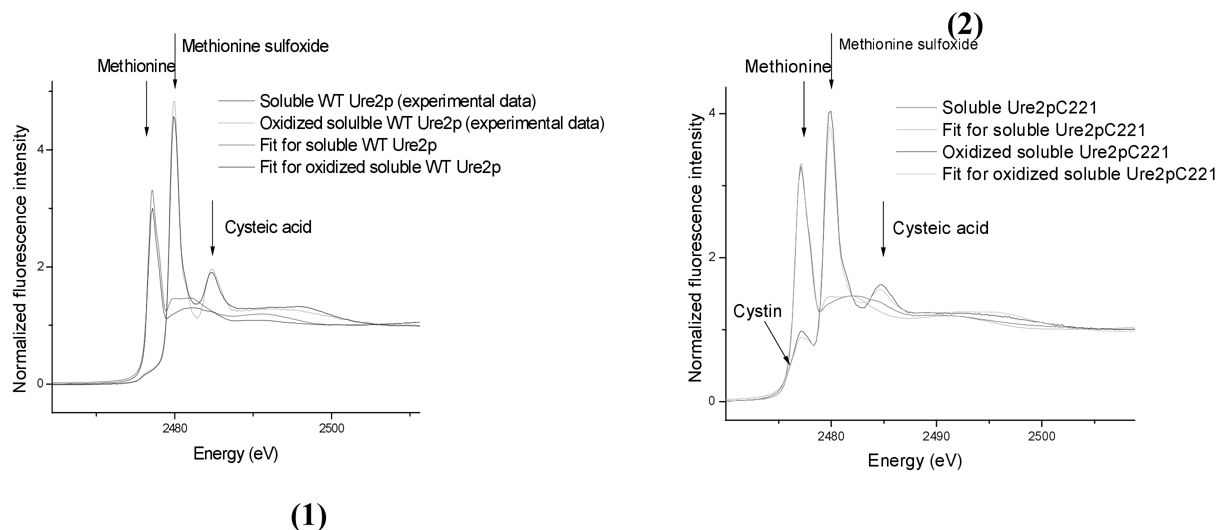
Ure2p is a protein involved in the signal transduction pathway that regulates nitrogen catabolism in yeast cells. Self-propagating altered forms of Ure2p are responsible of the phenotype [URE3]. In wild type cells, Ure2p is dispersed through out the cytoplasm while it forms large globular or filiform aggregates in cells carrying the [URE3] phenotype. Ure2p constitutes an excellent model system to study the prion-forming mechanisms, in particular because it does not yeast cells death and is harmless to mammals, in particular humans.

The molecules contain two domains, a poorly structured prion-forming domain (residues 1-65) and a compactly folded functional domain (residues 65-354). In solution the Ure2p molecules are mostly assembled into dimers. In contrast with many amyloid forming proteins, Ure2p assembles *in vitro* into fibrils under physiologically relevant conditions.

We aimed to determine the packing of Ure2p molecules within the fibrils. To access whether monomeric Ure2p needs to reassociate into a dimer prior to assembly, a Ure2p variant in which Ser221 (at the interface between the two monomers within the dimer) was mutated to Cys was generated. In the crystal structure Ser221 from one polypeptide chain faces the same residue in the partner chain. Under oxidative conditions a disulfide bond is established between the two monomers. Under reducing conditions Ure2pC221 assembles into fibrils, in contrast to the amorphous aggregates which are formed under oxidative conditions. This result suggests that the dissociation of the dimeric form of the protein to its monomeric constituents is necessary for fibril formation. Alternatively, the reduction of the flexibility within the dimer following disulfide bond formation could generate an assembly incompetent molecule. We have dissected the assembly process of Ure2p and tried to determine wether Ure2p is in its native dimeric form or in a non-native monomeric or dimeric form by the use of XANES spectroscopy at the sulfur K-edge, a technique which is sensitive to the oxidation state of sulfur atoms.

Ure2p is a 40,2 kD protein that assembles into dimers. Each monomer contains 9 methionine residues and no cystein. By contrast, the variant Ure2pC221 in which the two C221 of each monomer face themselves with a Cα-Cα 4.1Å distance under reducing conditions, contains one Cys residue per monomer. Under oxidative conditions a disulfide bond is established. Sulfur K-edge XANES spectroscopy was used to detect the presence or absence of disulfide bonds in Ure2pC221 fibers, under oxidative conditions. The XANES spectra of sulfur compounds depends on sulfur oxidation state and sulfur environment. In particular, the XANES spectrum of cystin, the oxidized form of cystein with disulfide bonds, presents a characteristic double peak at

an energy of 2 476 eV. All the other forms of oxidized cystein or methionine exhibit a single resonance, at higher energy. We have taken advantage from these characteritics to analyze sulfur oxidation state of solutions of wild type and C221 variant soluble proteins and fibrils, under reducing and oxidative conditions.



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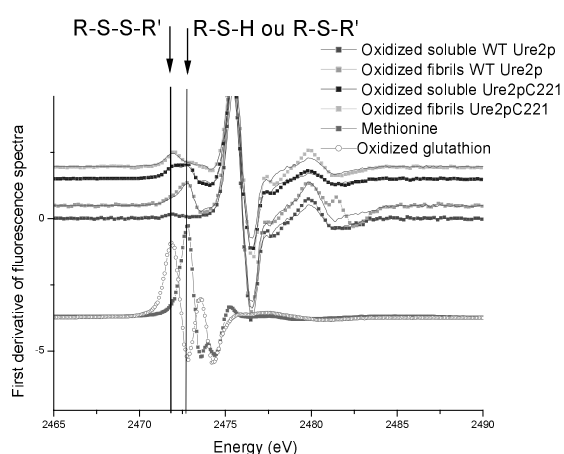

(3)

Figure (1) : Experimental and adjusted XANES spectra for soluble wild-type protein (no Cys) under reducing and oxidzing conditions. The arrows indicate the characteristic peak positions for the XANES spectra of standard solutions.

Figure (2) : Experimental and adjusted XANES spectra for soluble Ure2p C221 variant protein (1 Cys per monomer) under reducing and oxidzing conditions.

Figure (3) : First derivative of XANES spectra for oxidized WT and C221 variant soluble proteins and fibrils. The symbols correspond to experimental data, the lines correspond to the best adjustement by linear combinations of standard spectra. The characteristic disulfide bond peak (R-S-S-R') is equally present in soluble and fibrillar Ure2pC221 spectra.

The experiment was carried out at ID26 beamline. The samples were freeze-dried before analysis. Several reference solutions were also analyzed for energy calibration and oxidation state identification: each experimental spectrum was adjusted using a linear combination of reference spectra obtained in the same experimental conditions.

We checked that disulfide bond (S-S) background signal level in wild type proteins solution was low (Figure 1). After adjustment the contribution of S-S signal to the full spectra of wild type proteins was estimated to be 3%. We checked that we could observe the formation of a disulfide bond in soluble Ure2pC221 under oxidative conditions (Figure 2). In this case, the S-S contribution to the signal was measured to be 9%. According to the ratio Cys/Met of 1/9 in each monomer, this results confirms that about one disulfide bond is established between the two C221 of each dimer. Finally, we have compared the signals obtained for all the oxidized forms of the proteins (Figure 3). We clearly showed that oxidized Ure2pC221 fibrils exhibit a disulfide signal, representing 11% of the total sulfur signal. Again, this proportion coincides with a disulfide bond established between the two C221 of each dimer. This means that even in the fibril form, the two C221 of each dimer remain close enough to establish a disulfide bond under oxidizing conditions. In other words, we show for the first time, that Ure2p fibril formation mechanism would involve dimer units or units close to the native form rather than a mechanism based on the stacking of monomers with a prior dimer dissociation process.

In order to confirm our findings we have recently repeated the experiment on ID21 as part of a inhouse program. Instead of freeze-dried protein solution, fully hydrated cryo-fixed solutions were analyzed. Similar conclusions were drawn. These results will be the object of a publication, in preparation.