Rebecca Murphy - Summer Vacation Studentship Report

Project Aims

Ubiquitin C-terminal Hydrolases (UCHs) are de-ubiquitinating enzymes involved in the cleavage of ubiquitin molecules from the oligopeptides products of cellular proteolysis. There are currently four known UCH isozymes, UCH L1-4, all of which display a complex 5_2-knotted topology in their tertiary structure. UCH-L1, the focus of this project, is expressed exclusively and abundantly in neuronal tissue and comprises between 1-5% of the total soluble protein in the brain [1].

As a component of Lewy bodies [2], the insoluble proteinaceous bodies found in the brains of Parkinson's disease (PD) patients, UCH-L1 is significantly implicated in the pathology of PD. A single amino acid substitution, Ile93Met (I93M), has been linked with PD. Several recent publications have provided extensive characterisation of the UCH-L1^{I93M} variant, demonstrating that it displays several aberrant molecular properties consistent with a causative association with PD, including increased insolubility and increased interactions with other cellular proteins [3]. Furthermore, although the association of the UCH L1^{I93M} mutation with familial PD has been the subject of significant study, the molecular basis of sporadic PD, which accounts for over 90% of cases, is less well understood.

Oxidative stress is implicated in the pathogenesis of PD and UCH-L1 has been shown to be a major target of oxidative modification [4]. One of the species most responsible for oxidative damage to proteins is 4-hydroxy-trans-2-nonenal (HNE). HNE reacts with the sulphydryl groups of proteins and is believed to target the cysteine residues C90, C132 and C152 in UCH L1. It has recently been demonstrated that HNE-modified WT UCH L1 displays similar aberrant properties to UCH-L1^{I93M}: reduced ubiquitin binding, increased insolubility and increased interactions with other cellular proteins, indicating that oxidative damage to UCH-L1 may contribute to sporadic PD [3]. The aim of the project was to undertake a preliminary investigation of the molecular properties of the heterogeneous population of carbonylated UCH-L1 and the preparation of UCH-L1 variants lacking various combinations of cysteine residues. These can be used in further studies to analyse the alteration in the molecular properties of UCH-L1 on modification by HNE at specific cysteine residues.

Experimental Procedures

Human wild type (WT) UCH-L1 was expressed in *E. coli* XL1 cells as a fusion protein with GST and purified by affinity chromatography and gel filtration. The solution of purified protein was run on an SDS-PAGE gel to verify purity. A QuikChange Site-Directed Mutagenesis kit was used to produce UCH-L1 variants in which various cysteine residues had been replaced by serine residues (C220S, C132S, C220S+C132S and C220S+C132S+C152S). The DNA was purified using a MiniPrep kit and the mutations verified by sequencing; there was insufficient time to express these variants.

WT UCH-L1 in sodium phosphate buffer was incubated at 37 °C for two hours with various concentrations of HNE. Samples of each reaction mixture were then subjected to analysis by mass spectrometry to ascertain the extent of modification and the number of sites modified by HNE.

The far-UV CD spectra of the unmodified and modified wild-type protein were taken, to assess the effect of HNE modification on the secondary structure of UCH L1. As HNE was supplied in ethanol, it was necessary to control for the effect of ethanol on the structure of UCH L1 by analysing a sample of UCH L1 incubated with ethanol only.

Finally, a refolding reversibility assay was performed on both the modified and the unmodified WT UCH L1. The far-UV CD and fluorescence emission spectra of WT and HNE modified UCH L1 that had been unfolded in 6 M guanidinium chloride and then refolded were compared to the spectra of modified and unmodified native UCH L1 and to the unfolded protein spectra. Unfortunately, the results of this experiment were inconclusive, so a repetition will be required to verify refolding reversibility.

Results and Discussion

Analysis of HNE-modified UCH-L1 by mass spectrometry revealed that a maximum of three amino acid residues are modified per UCH-L1 molecule, indicating that there are three HNE-accessible sites on the UCH-L1 protein; these sites are likely to be the three solvent-exposed cysteine residues C90, C132 and C152. Furthermore, the decline in peak intensity in the mass spectra with increasing concentrations of HNE suggests that HNE-modification may have a destabilising effect on the native state of UCH L1.

Further evidence for the destabilising effect of HNE-modification on UCH-L1 was provided by preliminary analysis of the secondary structure of UCH-L1 molecules in a heterogeneous population of HNE-modified UCH-L1 by far-UV CD. The far-UV CD spectra showed a decrease in the α-helical content of HNE-modified UCH L1 relative to the unmodified protein, consistent with a destabilisation of the native state of the protein.

Although these results provide preliminary evidence that carbonylation of UCH-L1 by HNE causes alterations in the secondary structure of the protein, it is important to recognise that all experiments were performed on a heterogeneous population of modified protein molecules. To characterize further the effects of HNE modification on UCH-L1 molecules, it will be necessary to prepare UCH-L1 variants that have just a single residue accessible for modification. Modification of these variants with HNE should enable the precise effects of carbonylation at specific cysteine residues to be elucidated.

Furthermore, to ensure a significant population of modified protein molecules, the modification experiments described above used HNE concentrations far in excess of those found in the neuronal cells in which UCH L1 is expressed. Using lower concentrations of HNE and longer incubation times would provide information about HNE modification of UCH-L1 that is considerably more physiologically relevant than that described above.

References

1. Wilkinson, K.D., Lee, K.M., Deshpande, S., Duerkensen-Hughes, P., Boss, J.M., Pohl, J. (1989) The neuron-specific protein PGP 9.5 is a ubiquitin carboxy-terminal hydrolase, *Science 246*, 670-673.
2. Lowe, J., McDermott, H., Landon, M., Mayer, J.D., Wilkinson, K.D. (1990) Ubiquitin carboxy-terminal hydrolase (PGP 9.5) is selectively present in ubiquitinated inclusion bodies characteristic of human neurodegenerative diseases, *J. Pathol. 161*, 153-160.
3. Kabuta, T., Setsuie, R., Mitsui, T., Kinugawa, A., Sakurai, M., Aoki, S., Uchida, K., Wada, K. (2008) Aberrant molecular properties shared by familial Parkison's disease-associated mutant UCH-L1 and carbonyl-modified UCH-L1, *Hum. Mol. Gen. 17*, 1482-1496.
4. Choi, J., Levey, A.I., Weintraub, S.T., Rees, H.D., Gearing, M., Chin, L.S., Li, L. (2003) Oxidative modifications and down-regulation of ubiquitin carboxyl-terminal hydrolase L1 associated with idiopathic Parkinson's and Alzheimer's diseases, *J. Biol. Chem. 279*, 13256-13264.

Importance of the Studentship

As my first experience of working in a laboratory, the studentship experience was extremely important in increasing my understanding of how research is carried out and in cementing my desire to pursue a career in academic research. The preliminary studies that I carried out during my project also demonstrated that HNE modification of UCH-L1 is a tractable research area, so have been of importance to the Jackson group as more detailed analysis of the effect of HNE modification on UCH-L1, particularly at specific cysteine residues, will be a significant area of further research.