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Chapter 6

Conclusions and Future Directions

The aim of this thesis was to examine the role of 3OHKyn in the human lens and in particular its interaction with proteins. Initially model studies were undertaken to mimic the role of 3OHKyn in the normal lens. 3OHKyn amino acid adducts were synthesised and characterised. Like 3OHKyn, the 3OHKyn amino acid adducts were found to be unstable at pH 7.2. The fragment ions identified for each adduct will be important in future studies, since they can be used as markers in protein digests. In addition a novel oxidation product of 3OHKyn-*t*-Boc-His was also identified.

Hydrolysis has previously been used to show Kyn and 3OHKynG bound covalently to lens proteins. However, acid hydrolysis was not an appropriate method for determining the levels of 3OHKyn bound to proteins. A new and simple method was developed to determine the levels of protein-bound kynurenines. 3OHKynG was found to be present at the highest amounts followed by Kyn with 3OHKyn being attached in the lowest amount. The ratio was approximately 145:4:1 (3OHKynG:Kyn:3OHKyn). This is in agreement with the finding that 3OHKynG is the most abundant free UV filter in the lens followed by Kyn and 3OHKyn. The findings from this study also showed that there was no relationship between the levels of bound and free UV filters, suggesting that the binding of UV filters to proteins reflects not so much the concentration of available UV filter, but more probably the degree of lens barrier formation. This study also showed that all of the UV filters are bound to normal human lens nuclear proteins after middle age. The levels in the nucleus were found to be much higher than in the cortex. In addition the levels of bound UV filters in normal lens proteins was much higher than in cataract lens proteins. Bound 3OHKyn could not be detected in cataract lens proteins, indicating that once bound to normal lens proteins, 3OHKyn may possibly oxidise and play a role in the progression of cataract.

Since 3OHKyn is indeed bound to human lens nuclear proteins, another key objective was to characterise the properties of such 3OHKyn-modified proteins. The level of bound 3OHKyn in the nuclear proteins is extremely low, and 3OHKyn is reactive. Therefore, it was unlikely that a peptide could be readily isolated and analysed from

human protein. A model study with CLP modified by 3OHKyn at pH 7.2 failed to identify 3OHKyn modified peptides, since CLP contains several crystallin subgroups, and many modifications may result from a reaction involving 3OHKyn. A peptide modified at Cys was however identified when purified α-crystallin was incubated with 3OHKyn. In this same study, major Met and minor Trp oxidation were also observed. Oxidation of Met is a characteristic feature of cataract lens proteins. The findings from this study suggest that 3OHKyn may contribute to the formation of cataract, since oxidation of 3OHKyn results in the formation of H_2O_2, and H_2O_2 readily induces protein oxidation. However, there are other sources of H_2O_2 in the lens, and 3OHKyn may not be the sole agent responsible for the protein oxidation that occurs in cataract.

Crosslinking is another feature of ARN cataract. The theory that 3OHKyn may crosslink lens proteins was examined in preliminary experiments. Model studies with the 3OHKyn amino acid adducts were first undertaken. Incubation of 3OHKyn-*t*-Boc-His yielded a compound with a molecular ion of *m/z* 550, which eluted as a doublet on the HPLC, and had a wavelength maximum centred at 408 nm. This product was produced in the absence and presence of excess amino acids, and its molecular weight suggests that crosslinking may have occurred, but unfortunately, structural characterisation of this compound was not achieved. Model studies with CLP modified with 3OHKyn at pH 7.2 and 9.5 also failed to show any evidence of crosslinking when the protein was allowed to incubate over an extended period of time. 3-D Fluorescence profiles showed a shift in fluorescence over the incubation period, however the SDS-PAGE data did not show crosslink formation. Acid hydrolysis of the modified proteins following the incubation showed numerous fractions eluting on the HPLC, but none could be conclusively identified by mass spectrometry, as crosslinked compounds. The study did however show that during the incubation, bound 3OHKyn cleaved and formed xanthurenic acid via oxidation. This demonstrates that in cataract lenses when 3OHKyn is attached to lens proteins at pH 7, it may ultimately result in the formation of xanthurenic acid.

3OHKyn was found to be facilely transferred between amino acid residues during the incubation. For example, the protein originally modified at pH 7.2, was modified

predominantly at Cys. However following incubation over 15 days, the recovery of 3OHKyn-Cys diminished, and His modification was observed after 6 days. This demonstrates the instinsic instability of 3OHKyn adducts in proteins, and also suggests how the UV filter may react *in vivo*.

In future studies it would be ideal if the product, *m/z* 550, resulting from 3OHKyn-*t*-Boc-His incubations could be structurally characterised, since 3OHKyn-*t*-Boc-His is the most stable of the three 3OHKyn amino acid adducts. It is possible that this stable product may be present in aged normal or cataract lens proteins, but this was not examined.

Acid hydrolysis of human cataract lens proteins showed that 6 novel peaks eluted in the HPLC chromatogram of the hydrolysate. These peaks were not observed in the hydrolysate of CLP or normal aged lens protein. Isolation and examination of each peak by UV-visible spectroscopy and mass spectrometry showed that several of the 5 peaks, P1-P5, may be UV filter derived compounds based on the UV-visible data. However, the mass spectral data alone was insufficient to identify characteristic UV filter ions except for P4. P4 contained a Kyn fragment ion and extensive mass spectral analysis showed that the unknown Kyn derivative in P4, appears to have a molecular ion of *m/z* 670. Unfortunately at this stage NMR characterisation was unsuccessful due to a lack of sufficient material.

In summary, in the normal human lens, 3OHKyn binds to lens proteins after middle age, and in this sense behaves like the other two UV filters. The reason for the development of ARN cataract is not known, but, since it is now known that 3OHKyn is bound to proteins, 3OHKyn may have a role in cataract formation, and this requires further investigation. The model studies outlined in Chapter 4 provided the means to investigate the problem. Initial investigation suggest it may be fruitful to isolate, and to structurally characterise fractions from acid hydrolysed cataract lenses, since this may reveal the identity of the major species that modifies lens proteins in cataract.