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Reaction of 3-Hydroxykynurenine with lens proteins and synthetic peptides

J. Andrew Aquilina

University of Wollongong

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REACTION OF 3-HYDROXYKYNURENINE WITH LENS PROTEINS

AND SYNTHETIC PEPTIDES

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Submitted in fulfilment of the requirements
for the Degree of Doctor of Philosophy

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Wollongong, AUSTRALIA

January 2000
Declaration

The work described in this thesis does not contain any material which has been accepted for the award of any other degree or diploma in this or any other University and to the best of my knowledge and belief contains no material previously published by any other person, except where due reference has been acknowledged.

J. Andrew Aquilina

January, 2000
In memory of Brett and Tim
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Sections of the work described in this thesis have been reported in the following publications:


Abstract

3-Hydroxykynurenine (3OHKyn), present in the human lens as a UV-filter, is a powerful antioxidant which has also been implicated as a carcinogen and neurotoxin. The neurotoxicity results from the oxidation of 3OHKyn due to the formation of H₂O₂ and possibly hydroxyl radicals. Oxidation of o-aminophenols, such as 3OHKyn, also results in the formation of highly reactive quinonimines. Thus one possible consequence of 3OHKyn oxidation may be the covalent modification of cellular macromolecules. Such a process could contribute to the neurotoxicity and may potentially be important in other tissues, such as the human lens, where 3-hydroxykynurenine functions as a UV filter.

In this study the roles of UV-light, pH, glutathione and oxygen were examined, with the objective of determining how these factors may possibly affect the binding of 3OHKyn to lens proteins (crystallins) under the conditions found within the lens itself. The presence of oxygen was found to be an important parameter for determining the extent to which 3OHKyn reacts with protein, and when it was totally excluded, little modification was observed. UV-light was not required for activation, but was found to augment the extent of modification and crosslinking, while an elevated pH, which is known to accelerate the rate of 3OHKyn oxidation, did not markedly increase the extent of reaction with the crystallins. 3OHKyn binding was accompanied by crystallin aggregation, pigmentation, and development of non-tryptophan fluorescence, all of which have been associated with cataract formation.

The inclusion of glutathione, a ubiquitous antioxidant, in reaction mixtures resulted in a delayed onset of crystallin modification. This effect was apparent at concentrations of glutathione greater than 1 mM. When glutathione levels fell below 1 mM, crystallins became modified by 3OHKyn. Since lens glutathione concentrations decrease with age, and are known to be lower in the lens nucleus than the cortex, this region appears particularly vulnerable to modification by this UV filter.

Using the model dipeptides glycyllysine (GK) and glycylglycine (GG), it was demonstrated that 3-hydroxykynurenine can bind to protein α-amino groups, and further, that under oxidative conditions, 3-hydroxykynurenine can function to cross-link polypeptide chains. The structures of a cross-linked moiety using the peptide GK and a covalently modified GG have been elucidated. The modified species were both coloured and fluorescent, and involved cyclisation of the N-terminal glycine to form a heteroaromatic ring at the C-2 and C-3 positions of 3OHKyn. GK was cross-linked through a benzoxazole moiety whereas GG preferably formed a benzimidazole linked compound. The compounds were named quinilinobenzoxamine (QBA) and 2-diglycylimidazolekynurenine.

The γ-crystallin family of lens proteins possess unblocked N-termini, the terminal residues of which are GK. In a reaction mixture of γ-crystallin and 3OHKyn a dimerised γ-
crystallin species was observed. LC-MS of a tryptic digest of this product failed to reveal a digest fragment corresponding to QBA. A synthetic peptide corresponding to the nine N-terminal residues of γ-crystallin was found to be cross-linked in the manner of QBA. Tryptic digestion of this product revealed a major product corresponding to a chymotryptic activity which was also present in the γ-crystallin / 3OHKyn dimer. As QBA could not be identified in the digests, it was proposed that trypsin may destabilise the benzoxazole moiety, resulting in a structurally altered product, the characteristics of which are not known.

Finally, the major reaction product of the tetrapeptide tuftsin (TKPR) and 3OHKyn was examined. This peptide which has threonine as the N-terminal residue, was able to form a dimer via the same mechanism as QBA. This result suggests that 3OHKyn can react with any peptide that has a free N-terminus, regardless of the identity of the amino acid (except proline). This finding has identified a host of potentially modified species in the reaction of 3OHKyn with the free N-terminal residues of proteins. In the lens where 3OHKyn is known to be continually synthesised, this may provide valuable biomarkers in disease states such as senile nuclear cataract.
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CHAPTER 1

GENERAL INTRODUCTION

1.1 Tryptophan Metabolism: The Kynurenine Pathway

Tryptophan is an essential amino acid which plays numerous biochemical roles apart from that of a protein building block. Tryptophan is the precursor to 5-hydroxytryptamine (serotonin), a neurotransmitter in the central nervous system, the coenzyme, acetyl-CoA as well as the cofactors NAD, NADP. Metabolism of tryptophan is carried out predominantly along the kynurenine pathway. In fact Fernstrom (1983) showed that this pathway is responsible for 90% of tryptophan metabolism in mammalian peripheral tissues. This oxidative pathway is summarised in figure 1.1. Initially, the indole ring is cleaved by the haem enzyme, tryptophan 2,3 dioxygenase (or indoleamine 2,3 dioxygenase outside the liver) to yield N'-formylkynurenine. N'-Formylkynurenine is hydrolysed by a non-specific formamidase to kynurenine in a step that occurs so readily, that the apparent product of tryptophan oxygenase is in fact kynurenine (Kyn).

Kynurenine is hydroxylated by a mitochondrial NADPH-dependent flavoprotein, kynurenine hydroxylase, specifically at the 3-position to yield 3-hydroxykynurenine (3OHKyn). Both Kyn and 3OHKyn are substrates for the pyridoxal phosphate (vitamin B6) dependent enzyme, kynureninase. This enzyme cleaves the sidechain of these molecules to give either anthranilic acid or hydroxyanthranilic acid. Another enzyme, kynurenine aminotransferase can also act upon Kyn and 3OHKyn, resulting in the formation of kynurenic acid or xanthurenic acid respectively, although these are relatively minor reactions.
Figure 1.1 The kynurenine pathway of tryptophan metabolism.
1.1.1 The Biochemistry of the Kynurenines

3-Hydroxykynurenine is predominantly metabolised by kynureninase to 3-hydroxyanthranilic acid (3OHA). The onward metabolism of 3OHA is shown in figure 1.2. A ferro-protein oxygenase, hydroxyanthranilate oxidase, catalyses the opening of the ring to yield 2-acroleyl-3-aminofumarate. This metabolite can undergo

\[\text{3-Hydroxyanthranilic acid} \xrightarrow{\text{oxidase}} \text{Acroleylaminofumarate} \xrightarrow{\text{Oxidase}} \text{Quinolinic acid} \]

\[\text{2 Acetyl CoA} \xrightarrow{\text{Dehydrogenase}} \text{NAD}^+\]

Figure 1.2: The catabolic pathway for 3-hydroxyanthranilic acid.
a non-enzymic cyclisation to quinolinic acid, a precursor for NAD synthesis.

3OHKyn, Kyn, kynurenic acid (KynA), xanthurenic acid (XA) and quinolinic acid (QA) are collectively referred to as kynurenines. The kynurenines are known to be neuroactive compounds, and have been implicated in the pathogenesis of several neurological disorders, particularly in neurodegenerative diseases. 3-Hydroxykynurenine, in particular, was shown to be cytotoxic to neuronal cells, postulated to be due to the formation of H$_2$O$_2$ as a result of 3OHKyn autoxidation.

It has been found that post-mortem brain levels of 3OHKyn were elevated 2-3 fold above controls in patients with Huntington’s disease. Subsequent studies revealed that the levels of 3OHKyn in the brain are also significantly elevated in other pathological conditions such as HIV associated dementia, hepatic encephalopathy and Parkinson's disease.

3OHKyn is also an excitotoxin which has been reported to be a convulsant, implicated in childhood epileptic seizures, convulsions observed in cerebral malaria and the seizure disorder associated with neonatal vitamin B-6 deficiency. This deficiency is also observed during pregnancy, increasing the susceptibility of humans to spontaneous seizures, in particular, those pregnancies complicated with preeclampsia / eclampsia (toxemia of pregnancy).

Outside of the central nervous system, Kuznezova (1969) showed that 3OHKyn causes chromatid breakages in human somatic cells. Thus, along with 3OHA, 3OHKyn was deemed to be mutagenic. Metabolites of tryptophan, including 3OHKyn and kynurenine, have been found in the urine of patients with leukemic diseases and bladder cancer. These compounds were found to induce tumours in experimental animals and the subcutaneous injection of 3OHA and 3OHKyn into mice was shown to induce leukaemia.
1.1.2 3OHKyn: Oxidative Stress Generator or Antioxidant?

Eastman and Guilarte (1989)\textsuperscript{10} observed that the \textit{in vitro} toxicity of 3OHKyn was attenuated by antioxidant treatments, and abolished when catalase was present in neuronal cell culture medium, suggesting that H$_2$O$_2$ plays a critical role in 3OHKyn toxicity. Desferrioxamine also reduced the toxicity of 3OHKyn\textsuperscript{11} indicating that metal-mediated hydroxyl radical formation was also involved in the damaging effects of 3OHKyn.

Ironically, 3OHKyn has also been found to be an effective scavenger of peroxyl radicals, inhibiting the radical-mediated oxidation of phosphatidylcholine and $\beta$-phycoerythrin, leading to the proposal that it may act as a local antioxidant in mammalian inflammatory diseases\textsuperscript{29}. In insects, Goshima et al, (1986)\textsuperscript{30} first reported that 3OHKyn is a scavenger of active oxygen; indeed it is a major radical scavenger in the respiration of the blowfly, \textit{Aldrichina grahami}, protecting against tert-butyl-hydroperoxide injury to body fat cells\textsuperscript{31}. Thus, the presence of 3OHKyn in biological systems appears to represent a delicate equilibrium between promoting oxidative stress through the formation of H$_2$O$_2$ or acting as an antioxidant. The presence of other endogenous antioxidants may be critical in maintaining this equilibrium and thus reducing the cytotoxicity of 3OHKyn.
1.2 The Human Lens

The transparent crystalline lens of the eye is located immediately behind the iris (Fig. 1.3) and its primary function is to transmit and focus light upon the retina. Proteins constitute some 30% of the lens by weight, the remainder being mostly water.

Figure 1.3: Schematic cross-section of the eye showing the location of the lens (left), and a superior cross-section of the lens showing the concentric layers of fibre cells.

These proteins, or crystallins are found within the cytoplasm of the avascular fibre cells, (Fig. 1.3), which themselves constitute the bulk structure of the lens. It is the exceptional clarity at very high protein concentrations (>300 mg/ml) which provides the lens with the requisite refractive power to focus light upon the retina. Fibre cells are formed from the single anterior layer of epithelial cells that divide and elongate at the lens equator. 

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In common with lenses from other species, the human lens grows by building up layer after layer of fibre cells around the original core, which is present at birth \textsuperscript{33}. The lens contents are contained within a clear membrane-like structure called the capsule. As a result, the lens naturally tends towards a rounder or more globular configuration, a shape it must assume for the eye to focus at a near distance \textsuperscript{34}.

Since no cells are lost as the younger cells envelop the older ones, the lens contains cells that were present even prior to the birth of the organism \textsuperscript{32}. It is this absence of cell turnover as the lens grows, and the absence of cellular organelles, which makes it susceptible to cumulative oxidative and non-oxidative insults, particularly within the older lens nucleus which becomes increasingly remote from the lens nutrient and antioxidant supply as the lens grows. Depending on age, the nuclear protein concentration can be several times higher than that of the cortex \textsuperscript{35}, and it has been proposed that a barrier to the transport of small molecules exists in the region surrounding the nucleus \textsuperscript{36}.

1.2.1 Crystallins: Structural Proteins of the Lens

The high refractive index of the human lens can be attributed to the sheer abundance and short-range packing of three families of structural proteins called crystallins, namely the $\alpha$-, $\beta$- and $\gamma$-crystallins \textsuperscript{37,38}. Other crystallin families do exist, for example the $\delta$-crystallins which replace $\gamma$-crystallins in birds and reptiles \textsuperscript{39}, and $\varepsilon$-crystallins, a major component of the duck lens \textsuperscript{40}. Although initially thought to be lens specific, it is now clear that most of the crystallins occur at low levels in tissues outside the lens \textsuperscript{41}.

Crystallins of the human and bovine lens account for approximately 90\% of the total lens protein \textsuperscript{42}, and consist of variably aggregated subunits which can be readily separated into $\alpha$-, $\beta$- and $\gamma$-crystallins using size exclusion chromatography. The order
Chapter 1  Introduction

of elution is a highly aggregated high-molecular-weight component, followed consecutively by the α-, β- and γ-crystallins, reflecting their decreasing states of aggregation (Fig. 2.1).

1.2.2 Age Related Changes in Crystallin Protein

The exceptionally long lifetimes of the crystallins makes them susceptible to the accumulation of a variety of post-translational modifications that are thought to disrupt their tertiary structure. These post-translational modifications have been thought to include the following:

Phosphorylation of serine residues, particularly of the αA- and αB-crystallins, which results in four major subunits arising from the two gene products; deamidation of glutamine and asparagine residues; non-enzymatic glycation of the ε-amino group of lysines to form fructoselysine, which has been shown to increase from infancy to age five, after which the level remains constant; formation of Nε-(carboxymethyl)lysine (CML) by the autoxidation products of ascorbic acid; truncation of the C-terminus or N-terminus of many crystallin subunits; oxidation of tyrosine residues to ortho-tyrosine and dityrosine.

The significance of these and other proposed modifications to the crystallins is unknown, however it does seem reasonable that the charge networks responsible for maintaining short-range order and packing may be disrupted, resulting in a protein aggregation and a decrease in lens transparency.

With ageing, the lens has also been observed to become progressively more yellow and fluorescent. These changes result in increased absorption of visible light, particularly by proteins within the lens nucleus, leading to reduced colour perception by older individuals. Wood and Truscott (1993) observed that levels of the tryptophan-derived UV-filter compounds, 3OHKyn and 3HKG decreased with the onset of
adulthood. It has recently been proposed that the loss of 3HKG is due to the photochemical attachment of this molecule to lens proteins\textsuperscript{55}, resulting in the aforementioned increase in lens fluorescence.

1.2.3 3-Hydroxykynurenine in the Primate Lens

3OHKyn plays a variety of diverse roles in the biological kingdom. It is a precursor for the strongly coloured compounds (ommochromes) in insect eyes and butterfly wing scales,\textsuperscript{56, 57} plays a physiological role in regulating the molting of crustaceans\textsuperscript{58} and may regulate the balance between excitatory and inhibitory processes in the insect central nervous system\textsuperscript{59}.

The human lens contains a group of tryptophan derived, fluorescent compounds, which act to absorb ultraviolet light in the 300-400 nm region of the spectrum\textsuperscript{60}. At present, five such compounds have been identified. The major component is the glucoside of 3OHKyn, 3-hydroxykynurenine glucoside (3HKG)\textsuperscript{61}. The identification of 3HKG in the lens represents a unique pathway of tryptophan metabolism since 3OHKyn is not converted to 3OHA, but instead is conjugated with glucose. Smaller amounts of Kyn and 3OHKyn have been detected in human lens extracts\textsuperscript{54, 62}. Another UV-filter compound was identified by Truscott et al (1994)\textsuperscript{63} as AHBG (4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid O-glucoside), which is derived from tryptophan and constitutes the second most abundant UV-filter in the human lens. Recently, a glutathionyl adduct of deaminated 3HKG was isolated and shown to be a novel fluorophore in aged human lenses\textsuperscript{64}.

Of the compounds described above, only 3OHKyn has a free aromatic hydroxyl group, and as such is an \(\sigma\)-aminophenol. \(\sigma\)-Aminophenols are known to readily undergo complex oxidative processes, Dillon (1983)\textsuperscript{65} observed that 3OHKyn in solution at pH 7.4 tended to decompose on storage, even at 4 °C in the dark. Under
severe oxidative stress induced via the hydrogen peroxide - horseradish peroxidase system, 3OHKyn was observed to form hydroxyxanthomattin and xanthomattin, the products of multi-electron oxidations\textsuperscript{66}. When 3OHKyn was autoxidised at neutral pH, more than 50% of 3OHKyn was converted into long wavelength-absorbing material after 10 hours\textsuperscript{67}. This material was not characterised, but appeared related in structure to xanthomattin when observed by TLC.

1.3 Human Cataract

A cataract is a lens opacity that interferes significantly with vision. As a result the light from an object produces a degraded image, or if severe, no image at all. It is often thought that there are a number of risk factors associated with the development of cataract\textsuperscript{68}. Some forms of cataract have been considered to be environmentally, chemically or lifestyle induced. For example, it has long been known that diabetics are a high risk group due to the elevated levels of blood glucose associated with the disease\textsuperscript{69}. Higher plasma levels of bilirubin and creatinine, and lower levels of total protein phosphate and cholesterol have also been observed in cataract patients compared with controls\textsuperscript{70}. When normal and age-related cataract patients were administered an oral dose of tryptophan, it was found that a major subgroup of the cataract patients exhibited a dysfunction in the metabolism of tryptophan\textsuperscript{71}.

Corticosteroids, if taken for a long time are proposed to upset numerous biochemical pathways of lens metabolism\textsuperscript{39}. Steroids possessing a reactive carbonyl group have been shown to form adducts with lens proteins\textsuperscript{72} in a manner similar to the non-enzymic glycosylation involved in diabetic cataract\textsuperscript{73}, which involves attack on the protein amino groups by the reactive carbonyl group. Harding\textsuperscript{74} has suggested that severe episodes of diarrhoea and heatstroke, with consequent dehydration and uraemia, might generate levels of cyanate sufficient to inflict cataract-promoting injury upon the
lens. When lenses of renal failure patients were searched for evidence of carbamylation at lysyl or cysteinyl residues of the α-crystallins however, carbamylation was not detected. The only modifications observed were glutathione adducts to Cys 131 and Cys 142.

Other risk factors such as smoking and high alcohol intake, hypertension and psychiatric illness treated with tranquillisers and even poverty have been implicated in cataractogenesis. However, in the general scheme of cataract epidemiology, the proportion of cases arising specifically from these conditions is not large, and is and difficult to attribute to individual factors.

1.3.1 Senile Nuclear Cataract

The form of cataract that is associated with ageing is called senile cataract. There are two main types: cortical and nuclear. In cortical cataract the regions of opacity are localised in the outer regions of the lens, and appear to be associated with an ionic imbalance. A nuclear cataract is the result of changes to proteins in the centre of the lens, resulting in a progressive increase in the lens protein fraction which is insoluble in 8 M urea or 6 M guanidine hydrochloride. In time the nucleus may become deep yellow, brown or nearly black, the degree of colour and severity classified as types I-IV as first proposed by Pirie.

The changes to nuclear proteins observed in a cataractous lens differ from those observed in a normal ageing lens. Apart from an increase in the levels of urea-insoluble protein, there is an increase in the amount of oxidised cysteine and methionine residues and progressive loss of protein sulphydryl groups as well as an increase in the level of crystallins cross-linked by disulphide and other covalent bonds.
It is also possible that protein modifications could be the result of the reaction of lens crystallins with hydrogen peroxide derived from superoxide \(^{84}\), as peroxide is known to oxidise cysteine and methionine efficiently \(^{85}\). Fu et al. (1998) \(^{86}\) demonstrated that as nuclear cataracts develop an associated increase in hydroxylated amino acid oxidation is observed. The oxidised amino acids observed were DOPA, \(\alpha\)- and \(m\)-tyrosine, 3-hydroxyvaline, 5-hydroxyleucine as well as dityrosine. The modifications were thought to be consistent with metal-mediated hydroxyl radical formation. In support of these findings, it has recently been observed that a type IV cataractous lens generates a more intense hydroxyl radical signal than a type II lens in the presence of 1 mM \(\text{H}_2\text{O}_2\), as observed by electron paramagnetic resonance spectroscopy \(^{87}\). Furthermore, type IV cataractous lenses were found to have higher Fe levels than type II lenses, supporting a role for transition metal-mediated hydroxyl radical formation in the aetiology of cataract.

Glutathione (GSH), the principal antioxidant in the lens, plays an important role in the prevention of oxidative damage to lens tissue \(^{88}\). With age, GSH levels in the lens decrease \(^{89}\), from about 6 mM to 3 mM in the cortex, and 5 mM to less than 1 mM in the nucleus \(^{90}\), leading to an environment where oxidants are less likely to be scavenged. A common feature of nuclear cataract is the low concentration of GSH in the centre of the lens \(^{83}\). Sweeney and Truscott (1998) \(^{36}\), observed that a barrier to GSH diffusion exists in lenses from adults over the age of thirty, which is not present in younger lenses. It was proposed that development of this barrier, which corresponded to the area of the sclerotic zone of cataractous lenses, may precondition the nucleus to cataract by allowing oxidative modification to occur.

Cataract is responsible for blindness in over half of blind people worldwide \(^{91}\). There is no efficacious treatment available to either slow the progress of or prevent the onset of cataract; the only solution is surgery, of which there are two basic techniques. In developed countries, the intracapsular lens contents are removed by phaco-
emulsification and an artificial lens is implanted inside the capsule. The annual cost of this treatment in the USA alone was estimated to be more than $15 billion in the year 1991 \(^9\). In developing countries, the number of operations required necessitates a simpler procedure, in which the whole lens is completely removed and optical correction achieved with aphakic spectacles rather than an artificial lens - still a major improvement on blindness.

The difficulties involved in the development of an effective treatment for cataract, whether preventative or curative, arise from the mechanism of cataract formation being poorly understood. Age-related cataract is just that, an affliction borne through longevity of the individual, and as such, is intrinsically related to the protein changes described above (Sec. 1.2.1). One of the earliest descriptions of nuclear cataract described it thus, "The sclerosis of the lens-fibres is the analogue of the hardening of the cuticle, only that while in the cuticle the older cells lie on the external surface, in the lens they are constantly driven inwards towards the centre ... the older they grow the more they give up their water and become ... compacted and amber coloured. Since the centrally situated fibres are for the most part the oldest, the sclerosis begins at the centre of the lens..."\(^{93}\). This sclerosis implies insolubilisation and aggregation of the crystallins, an event very much a part of cataract formation.

### 1.3.2 Ultraviolet Radiation and Cataract

One of the most controversial proposed causative factors of age-related cataract concerns the effects of UV-B radiation on the lens. Since early this century, scientists have suggested an association between sunlight and cataract \(^{94}\). Not surprisingly this area has become increasingly topical in light of ozone layer depletion and the subsequent increase in UV-B energy which reaches the earth's surface. UV-rays of wavelengths below 290 nm (UV-C) are completely absorbed by the cornea, and only
two percent of UV-B radiation (290-315 nm) reaches the lens (Fig. 1.4). Thus, even though ninety eight percent of the UV-radiation which reaches the lens is UV-A, it is the higher energy UV-B radiation which has created more interest with respect to UV-induced cataract via direct damage to the lens structural proteins.

**Figure 1.4:** Schematic of percentage of UV-energy absorbed by the cornea and lens. Note that only two percent of UV-B radiation is absorbed by the lens compared with up to fifty-two percent of UV-A.

In the past 20 years a number of epidemiological studies have attempted to document the association, yet quite polarised views still exist in the scientific community. An examination of six nuclear cataract studies in which individual exposure levels were assessed found no association between solar UV-B and nuclear cataract, however there was an association with cortical cataract 94.

One problem encountered in linking UV-radiation with cataractogenesis is that many of the changes associated with ageing of the crystallins probably mask the
initiating events by years, thus attributing such events to UV radiation is fraught with speculation. Furthermore, one of the first changes observed during exposure of lenses to sunlight in the laboratory was the destruction of tryptophan, however, this damage was not found in brunescent cataractous lenses, in fact a brown nuclear cataract could not be produced artificially in the laboratory using UV or sunlight exposure. Similarly, Barrow, et al (1987) found that caged guinea pigs developed no lens opacification when exposed to UV-radiation in the range of 305 to 410 nm for a period of 9 months. Although in lens tissue, particularly cortical proteins, minor changes have been observed upon \textit{in vivo} UV-exposure, it appears that nuclear proteins experience no damage.

Epidemiological studies have attempted to address the high prevalence of cataract in developing nations due to sunlight. A comprehensive review of these data led Harding to conclude that sunlight was not responsible for a significant portion of the cataracts beyond those observed in Western countries. Studies relating equatorial proximity and cataract and altitude and cataract were found to reveal no significant correlations, in fact a decrease in the prevalence of cataract higher in the mountains was observed, even though the levels of ultraviolet light in sunlight are greater at altitude.

\subsection{1.4 Ultraviolet Radiation and Photosensitisers}

It has been proposed that the kynurenine pathway of tryptophan metabolism generates two photosensitisers: kynurenic acid and $N$-formylkynurenine which can generate reactive oxyradical species. $3\text{OH}Kyn$ and $3\text{HKG}$ on the other hand have been shown to possess little photochemical activity, rather, in the human lens, the function of these compounds may be to protect the lens and retina from UV-
induced photodamage by absorbing most radiation between 295 and 400 nm. The lack of evidence supporting direct UV-induced nuclear cataract suggests that if photooxidation is an important contributor to cataract formation, it may arise from a photosensitized reaction. Photochemical loss of the glucoside of 3OHKyn (3HKG) has been shown to occur when it is exposed to UV-A for long periods of time, resulting in a concomitant yellowing of the lens. More recently, Dillon et al. (1999) concluded that 3HKG attaches to human lens proteins following exposure to UV-light, however no data on the structure or binding site of the bound moiety were provided.

It has recently been shown that in the ageing lens, one mechanism of modification by 3HKG involves deamination of the aliphatic 3HKG side-chain, to yield an unsaturated ketone which is susceptible to non-oxidative nucleophilic attack by cysteine, histidine and lysine residues. Garner et al. (1999) found that Kyn could modify lens crystallins under non-oxidative conditions, even in the absence of UV-light. The mechanism of formation was proposed to be the same as that described by Hood et al. for 3HKG.

These findings show that UV-radiation is not necessary for either Kyn or 3HKG to bind to proteins. The mechanism proposed by Dillon (1999) is an oxidative one which relies on the initial radiation-catalysed cleavage of the o-glucoside from 3HKG, for which no mechanism was provided. The resultant phenoxy radical can then be oxidised to a quinonimine, in the same way that the autoxidation of 3OHKyn is thought to occur (Fig 1.5).
Figure 1.5: Proposed autoxidative mechanism of 3OHKyn, and formation of the products xanthommatin and DHQCA, (from Vazquez et al. 106).
1.5 Oxidation Products of 3-Hydroxykynurenine and Cataract

The readiness of 3OHKyn to form a quinonimine in the presence of oxygen leaves it susceptible to nucleophilic attack on the ring via a Michael addition. This is known to be the case in the formation of xanthommatin, a 3OHKyn dimer which is readily degraded to a quinone product, DHQCA, (Fig. 1.5) under slightly basic conditions, a process accelerated by hydrogen peroxide \(^{106}\).

3OHKyn can react with lens proteins to give a tanned product resembling cataractous material \(^{67}\), and can react with free glycine in vitro to form fluorescent products \(^{107}\), the structures of which have not been elucidated. The fact that 3OHKyn is readily autoxidised at neutral pH suggests that its reactions with proteins and peptides would not be expected to be similar to that of Kyn and 3HKG, which do not have a free phenolic group, and thus are not so susceptible to autoxidation. Deamination of the aliphatic side chain is a slow non-oxidative process \(^{108}\) which is a relatively minor reaction on the time scale of normal *in vitro* oxidative conditions, in comparison to the conversion of 3OHKyn to a reactive quinonimine. This quinonimine product may be susceptible to nucleophilic attack by protein amino groups.

The availability of oxygen in the lens, and the presence of antioxidants such as glutathione and ascorbate may dictate whether this initial autoxidation of 3OHKyn occurs. In the majority of experimentally induced cataracts, and in the centre of nuclear cataract lenses, a large decrease in lenticular GSH is observed \(^{32, 83}\).
Stutchbury and Truscott (1993) found that both the autoxidation of 3OHKyn and its ability to tan proteins was inhibited by the inclusion of GSH in \textit{in vitro} reactions and it was not until most of the GSH had oxidised that 3OHKyn became reactive.

3OHKyn is continuously biosynthesised in the lens with concentrations in the region of 5 \textmu M \textsuperscript{54}. Oxygen tension in the nucleus is thought to be considerably lower than other tissues, however the availability of even catalytic amounts of molecular oxygen would be sufficient to propagate a mechanism such as that proposed in figure 1.5. It is feasible then, that over time, the presence of micromolar concentrations of 3OHKyn in an increasingly oxidative environment such as the aged lens nucleus, may lead to covalent modification of the crystallins by 3OHKyn autoxidation products. The work in this thesis is primarily concerned with the interaction between proteins (and peptides) and 3OHKyn in oxidative environments and their relevance towards the aetiology of human nuclear cataract.

\textbf{1.6 Some Known Reactions of Low Molecular Weight Molecules With Structural Proteins}

Many organisms produce catechols which, together with specific oxidative enzymes, can react with proteins to form melanoproteins in animals, lignopolysaccharides in plants and sclerotins in insects\textsuperscript{109}. Catechols are precursors of quinones, a great variety of which occur in biological systems. Catechols are oxidised by specific enzymes called catecholoxidases, to quinones, which are highly reactive
compounds. \( \text{o-Quinones are susceptible to Michael-type addition of a nucleophile, such as protein amino groups as shown in figure 1.6.} \)

![Figure 1.6: Michael addition of a nucleophile to o-quinone](image)

An early observation of this process in nature was the hardening of cockroach egg capsules, a result of the oxidative reaction between 3,4 dihydroxybenzoic acid and enzymes, which are secreted from separate glands. The actual hardening process involves oxidation of the o-dihydroxy acid to the o-quinone, followed by condensation with the protein amino groups. Numerous 4-substituted catechols have been implicated in the cross-linking of protein during the hardening of the insect cuticle (sclerotisation), and it was proposed that nucleophilic addition occurs at the C-4 sidechain.

Lipofuscin, the pigmented autofluorescent granules found in human and animal tissues, is thought to be composed of free-radical-damaged protein and fat that concentrates beneath the skin, in the muscles, liver and other vital organs. It is particularly troublesome in brain tissue where accumulation is associated with neuronal cell death. The small carbonyl containing compounds malondialdehyde, ascorbic acid and reducing sugars have each been implicated as mediators in the formation of the coloured lipofuscin complexes. Lipofuscin, then, may be a product of carbonyl-protein interaction and cross-linking, not dissimilar to the mechanism of sclerotisation described above.
Some \( \sigma \)-quinonimine compounds have also been found to be involved in protein tanning. 3-Hydroxyanthranilic acid (3OHA) for example (Fig. 1.2), is responsible for the tanning of moth cocoon protein and the formation of coloured pigments in some Australian marsupials. The oxidised form of 3OHA is an \( \sigma \)-quinonimine as is the oxidised form of 3OHKyn. These molecules, like \( \sigma \)-quinones, are susceptible to nucleophilic Michael addition by protein amino groups at the C-5 position (below).

\[
\text{NuH}
\]

Recent studies have indicated that kynureninase activity is lacking in human lenses and therefore 3OHA is absent. In this thesis the reactivity of 3OHKyn with small peptides and lens proteins was examined to provide a basis for understanding the mechanism of reaction with proteins, and to discover if characteristic markers of reaction of 3OHKyn could be detected.
1.7 Aims of the Project

Four physicochemical parameters applicable to the lens, which are thought to influence the reactivity of 3OHKyn towards lens proteins, were studied initially. The roles of UV-light, pH, glutathione and oxygen were examined and assessed for their relative contributions towards covalent modification of the crystallins.

Since 3OHKyn has been found to be involved in modification of proteins in vitro, knowledge of the nature of such adducts would represent valuable biomarkers for the process of cataractogenesis. In the present study, 3OHKyn was reacted, under oxidative conditions, with the dipeptides, glycylllysine and glycylglycine in an effort to isolate and characterise any major products of these reaction. These peptides were chosen as they both have a free N-terminus, (as do the γ-crystallins), and they may provide clues as to whether the ε-amino group of GK can play a significant role in the formation of a product.

Total γ-crystallin isolated from bovine lenses and a synthetic nonapeptide representing the N-terminus of all bovine γ-crystallins, were reacted with 3OHKyn to ascertain if similar products would result to those found in the dipeptide experiments. Finally, a tetrapeptide with threonine as the N-terminal residue was reacted with 3OHKyn to determine if the identity of this residue affected the mechanism of formation of reaction products, and to elucidate a general mechanism of reaction between 3OHKyn and proteins.
CHAPTER 2

Reaction of 3-Hydroxykynurenine with Bovine Crystallins

2.1 Introduction

Changes that occur in the human lens with ageing may contribute to the processes responsible for age-related cataract. Of particular note is the slow transformation of constituent crystallin proteins to high molecular weight (HMW) aggregates, and an increase in the insoluble protein fraction, which, at high concentration, can cause significant light scattering.

The human lens contains fluorescent, low-molecular weight compounds, which have the capacity to absorb long-wavelength UV-light and reduce chromatic aberration. One of these, 3OHKyn has been shown to react with lens proteins resulting in a tanned product resembling cataractous material. Other investigators have shown that 3OHKyn can react with both calf lens protein and free glycine, in vitro, to form products with distinct blue fluorescence characteristics.

It has been demonstrated that under continual exposure to 300-400 nm (UV) light, absorption of this light by 3HKG, the major UV filter, can lead to the photochemical loss of 3HKG and concomitant yellowing of the lens. Dillon proposed that this is due to the photochemically-induced attachment of 3HKG to lens proteins. Other groups have proposed that xanthommatin, a dimerised compound of 3OHKyn, and oxidised xanthurenic acid (OXA) may be potentially cataractogenic molecules. Each of these compounds is formed from 3OHKyn: 3HKG by glucosyl transferase, xanthommatin by oxidative dimerisation and OXA via kynurenine aminotransferase. The question arises then whether 3OHKyn is ultimately
responsible for all of the changes observed with these derivative species, and if so, is there a distinct and reactive intermediate involved?

In accord with its known ability to be readily autoxidised, 3OHKyn has been described as possessing antioxidant activity and to be up-regulated in regions of high superoxide concentration \(^{29}\). Secondly, in the respiration of the blowfly, *Aldrichina grahami*, 3OHKyn effectively protected the fat body cells from peroxidation through radical scavenging \(^{31}\). Hence, with reference to lens chemistry, if there is a region of the lens where the primary antioxidant, glutathione (GSH) has become depleted, for example in the centre of nuclear cataract lenses \(^{83}\), 3OHKyn may act as the antioxidant of choice. In the young human lens 3OHKyn and 3HKG are major absorbing species in the range 295-400 nm \(^{121}\) hence it is feasible that 3OHKyn, as well as 3HKG, can become photochemically attached to lens proteins under conditions of long term photooxidative stress. The resultant yellowing of lens proteins leads to an increase in the number of photons absorbed by the lens \(^{118}\), and UV-light may then become a potential causative cofactor in cataract.

This chapter describes four environmental parameters present in, or encountered by the lens, and their role in the protein binding properties of 3OHKyn. Specifically, the roles of UV-light, pH, glutathione - an endogenous lens antioxidant - and oxygen were examined, with the objective of determining how these factors may possibly affect the binding of 3OHKyn to crystallins under the conditions found within the lens itself.
Chapter 2 Reaction of 3-Hydroxykynurenine with Bovine Crystallins

2.2 Materials and Methods

3OHKyn, GSH and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma (St. Louis, MO, U.S.A.). 3OHKyn was tritiated by exchange with TCI at the University of New South Wales (Kensington, N.S.W.), by Dr Mervyn Long and purified by HPLC prior to use. All other chemicals were of analytical quality.

2.2.1 Crystallin Preparation

Bovine lenses (2-4 weeks old), obtained from a local abattoir were homogenised in 50 mM Tris buffer containing 5 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride and 0.04% sodium azide (1.5 ml / lens), centrifuged at 4°C for 15 min at 10 000g, and the supernatant decanted for dialysis and lyophilisation. Separation of the resultant total calf lens protein (CLP) into the major classes of crystallins, namely α, β, and γ was achieved using size exclusion chromatography as follows: 300 mg of lyophilised protein was solubilised in 3 ml of elution buffer (0.05M Tris.HCl, 0.02% sodium azide, pH 6.8), and centrifuged at 4°C for 15 min at 10 000g. The supernatant was loaded onto a 1200 x 25 mm column, packed with Pharmacia S-300 gel, and eluted at a constant flow rate of 20 ml hr⁻¹, similar to the method of Slingsby and Bateman (1990). Fractions from peaks corresponding to the α-, β-, and γ-crystallin families were pooled and concentrated with a Diaflo ultrafiltration apparatus (Amicon) using a YM10, 10000 Da cutoff membrane. The protein was dialysed and freeze-dried as for CLP (above).
2.2.2 **Incubations**

All incubations were carried out at ambient temperature at concentrations of 5 mgml\(^{-1}\) calf lens protein and 2.2 mM 3OHKyn in freshly prepared 100 mM phosphate or bicarbonate buffers (pH 7.0 and 8.4 respectively) in a total volume of 2 ml in screw cap glass vials.

Lyophilised, tritiated 3OHKyn (3O\(^3\)HKyn) was received in eppendorf tubes and was of unknown activity. To determine the radioactivity of these samples, 1000 \(\mu\)l of 20 mM sodium acetate/acetic acid buffer pH 4.50 \(^{123}\) was added to an eppendorf from which a 20 \(\mu\)l aliquot was taken, injected onto a 250 x 4.6 mm Spherisorb S5ODS2 GoldPak column (Activon Scientific Products) and eluted with the same buffer. The eluant was run into an Canberra-Packard Radiomatic 140TR Flow Scintillation Analyser where it mixed with a 2.2:1 ratio of Packard Ultima-Flo M LSC-cocktail for counting in a six second residence time window. The area under the resultant DPM elution curve was integrated to determine the activity of the sample. 3O\(^3\)HKyn was added in 50 \(\mu\)l aliquots (= 8 x 10\(^5\) dpm) to the prepared incubation mixtures, and the solutions were sterile filtered through 0.22 \(\mu\)m Amicon syringe membranes; 50 \(\mu\)l of chloroform was added as an antibacterial agent.

The initial incubations were either left exposed to sunlight on the laboratory bench, or wrapped in foil and stored in the dark at room temperature. 100 \(\mu\)l aliquots were removed in triplicate at 24 hr intervals and immediately frozen. For counting, aliquots were thawed, precipitated and centrifuged twice with cold 10% trichloroacetic acid, washed once with H\(_2\)O and resolubilised in 0.5 ml of 9 M urea. 10 ml of aqueous counting scintillant (Amersham) was added to each sample and counting was performed on a Beckman LS 6000TA counter for 5 min per sample vial.
2.2.3 **SDS-PAGE, UV and Fluorescence Spectrometry**

Incubations for crosslinking and tanning experiments were prepared as above. Anaerobic samples were obtained by purging and blanketing the solutions with argon gas prior to capping. UV samples were constantly exposed to an ultraviolet tube (NIS GL-30, 30W germicidal) at a distance of 40 cm. The radiant intensity of UV was not determined. Control solutions prepared were identical except for the exclusion of 3OHKyn. SDS-PAGE was performed on 15% homogeneous gels, and gels scanned with a Biorad densitometer. UV-visible spectra were acquired on a Shimadzu UV-265 spectrophotometer, and fluorescence spectra on a Hitachi F4500 fluorimeter. Protein concentrations were determined using a Biorad dye binding assay.

2.2.4 **Digestion and HPLC of 3OHKyn-Modified Crystallins**

Solutions from the 96 hr incubations were lyophilised, washed twice with 1.5 ml of cold 80% ethanol, and re-dried. The resultant protein pellets were resolubilised in 0.2 ml of 8 M urea, 1 mM DTT, 1 ml of NH₄CO₃ (100 mM, pH 8.0) and pronase (Sigma, type XIV) added to give a final protein:enzyme ratio of 60:1. Digestion was undertaken for 24 hr at 37°C, after which samples were immediately lyophilised.

Digested protein was dissolved at 5 mg ml⁻¹ for HPLC separation on a Brownlee C18 OD300 column (250 x 4.6 mm, 7 μm), using a 0 to 48% acetonitrile gradient over 30 min at a flow rate of 0.8 ml min⁻¹. Detection was at 229 nm using a Varian 2050 UV detector, and 2 min fractions were collected using a Gilson 201 fraction collector. TFA was included in all buffers at 0.05% v/v, and scintillation counting was as described above.
2.2.5 **Anaerobic Incubation**

Nitrogen was bubbled through freshly prepared 100 mM N-ethyl morpholine, pH 8.4, and phosphate buffer, pH 7.0 for 45 min. Two sample vials containing pre-weighed calf lens protein (CLP) and 3OHKyn were placed under nitrogen streams, to each of which deoxygenated buffer was added. These vials were placed quickly into an Atmosbag™ (Aldrich) under positive nitrogen pressure and half of each solution was then sterile filtered (0.22 μm) and transferred into individual incubation vials, after which the Glovebag was sealed. The remaining solutions were taken for aerobic incubation. The experiment was carried out on the laboratory bench with minimal light exposure, and samples were removed and processed as described above. In other aerobic incubations, GSH was included at a concentration of 5 mM. The concentration of GSH was determined by reaction with DTNB.

2.2.6 **The Effect of 3OHKyn Concentration on Radiolabelling**

In order to explore the effect that lower levels of 3OHKyn would have on labelling, incubations using 200 μM and 5 μM concentrations of 3OHKyn were prepared. Furthermore, identical samples, but with the addition of 5 mM GSH, were included in the experiment. All other parameters were as for those described in 2.1.5.

To accurately prepare 5 μM and 200 μM 3OHKyn, a standard curve of 3OHKyn concentration versus HPLC elution peak area for absorbance at 365 nm was prepared using ICI DP800 software. The resultant curve was used to calculate the molarity of a sample of 3O³HKyn, an aliquot of which had been chromatographed on the Flow Scintillation Analyser as described to determine the relationship between activity and $A_{365}$. A 2.00 mM stock of cold 3OHKyn was used to obtain accurate final concentrations.
2.2.7  $\alpha$, $\beta$, and $\gamma$-Crystallin Incubations

The purified crystallin families, as well as CLP, were incubated as described in section 2.1.2 for the dark stored samples, except that pH 8.4 was not incorporated. The reason for this experiment was to ascertain if any differences existed between the relative binding capacities of the individual crystallin families at biological pH, and to what extent this contributed to the total label bound to the CLP. Aliquots of labelled 3OHKyn were added from a stock solution to ensure equivalent dosing of samples, and the incubations were carried out in the dark with exposure to atmospheric oxygen.

2.3  Results

This study utilised both the total water-soluble protein of bovine lenses (CLP), as well as the separated and purified major classes, namely the $\alpha$-, $\beta$-, and $\gamma$-crystallins. As described in section 2.1.1, CLP was obtained by exhaustive dialysis of the supernatant of the centrifuged lens homogenate, which, after lyophilisation, resulted in a white crystalline powder devoid of salt and small molecules, consisting almost exclusively of crystallins.

Subsequent separation of the CLP into the crystallin classes was achieved using size exclusion chromatography (SEC). Figure 2.1 illustrates a typical separation of the $\alpha$-, $\beta$-, and $\gamma$-crystallin families obtained by this method. SEC is not denaturing, thus it does not deaggregate the proteins to their constituent subunits. This property permits the separation of the crystallin families based on their aggregate rather than monomeric subunit size.
Figure 2.1: Low pressure size-exclusion chromatographic separation of total calf lens protein (Pharmacia S-300 gel media, 0.05M Tris.HCl buffer, pH 6.8, flowrate 20 ml hr^{-1})

In figure 2.1, α-crystallin, apart from the high-molecular-weight peak, represents the most highly aggregated species, with an average molecular weight of ~800 kDa. β_H-Crystallin, an octomer of heterogeneous subunits, constitutes the next peak, followed by a mixture of trimers and dimers (β_L-crystallin), and finally the monomeric γ-crystallin species.

An SDS-PAGE separation of the three major crystallin classes described is depicted in figure 2.2 Lane 1 contains as molecular weight markers, ovalbumin (45 kDa), upper and trypsinogen (24 kDa), lower. Lanes 2 - 5 show α-, β-, γ- and total lens crystallin respectively. α-Crystallin appears as 2 bands, αA (lower) and αB, which are the two major post-translational products of the α-crystallin family. The β-crystallins (lane 3) consist of some 8 individual subunits, four basic (βB_3), and four
acidic ($\beta A_y$). Ranging in molecular weight from 28055 Da ($\beta B_1$) to 22140 Da ($\beta A_2$), these subunits are labelled accordingly in figure 2.2. The $\gamma$-crystallins are the only monomeric species found in vivo. The family consists of 5 highly homologous subunits, $\gamma A$ - $\gamma E$, which are not resolved by SDS-PAGE, and thus electrophorese as a broad band of molecular weights 20750 - 21000 Da (lane 4) \(^{42,126-129}\). Lane 6 was loaded with the calf lens extract prior to SEC separation of the aggregates, as such it contains all of the aforementioned components.

Figure 2.2: Twelve percent homogeneous SDS-PAGE gel of total and class separated bovine crystallins. Lanes: 1 - Stds. 45 kDa (upper), 24 kDa (lower) 2 - $\alpha$-crystallin 3 - $\beta H$-crystallin 4 - $\beta L$-crystallin 5 - $\gamma$-crystallin 6 - total CLP
2.3.1 Incubations of CLP with 30HKyn Under Aerobic Conditions: the Influence of Sunlight

Our initial investigation focussed on the effects of sunlight on the incorporation of 3OHKyn into CLP in the presence of oxygen, utilising 3O³HKyn. Aliquots were removed from the incubation mixtures at 0, 24, 48 and 96 hr, the protein precipitated, washed and counted. A period of 96 hours was chosen as it was found by HPLC analysis that all of a 2.2 mM solution of 3OHKyn in the presence of 5 mg ml⁻¹ CLP was oxidised after such time. Figure 2.3 shows the incorporation of label at a pH value of 7.0 in the presence and absence of sunlight.

![Graph showing the incorporation of label at pH 7.0](image)

**Figure 2.3:** Effect of sunlight on the incorporation of radiolabelled 3OHKyn into CLP. Incubations were at ambient temperature and either exposed to sun-light, or kept in the dark. 100 mM phosphate buffer, pH 7.0 with —— and without —— sunlight exposure respectively.
The intensity of sunlight at midday under these conditions was measured at approximately 1.5 mW/m². It is clear that sunlight exerted a significant effect in the samples taken after 24hr. This finding shows that sunlight at the levels to which the lens is exposed can markedly increase the degree to which crystallins are modified by 3OHKyn.

Incubations of CLP with 3OHKyn were performed at pH 8.4 since o-aminophenols (eg. 3OHKyn) are known to oxidise at a faster rate at higher pH. It was of interest, therefore, to discover if reaction with proteins was accelerated when the rate of 3OHKyn oxidation was increased. Bicarbonate buffer was used initially due to the observation that 3OHKyn undergoes an intramolecular condensation to 3OHKyn-yellow when incubated under these conditions. The initial rate of incorporation of label was found to be increased under these conditions, however, the pH of the bicarbonate buffer was found to have increased to 9.5 after 50 hr, due to the production of CO₂. Thus, later experiments were repeated at pH 8.4 using N-ethyl morpholine instead of bicarbonate buffer to avoid any possible complications arising from an increase in basicity over time.

UV-visible spectra of light-exposed protein/3OHKyn mixtures were taken after 0 hr and 96 hr incubation (Fig 2.4) At 0 hr, pH was shown to have a marked effect on the λ_max and molar absorptivity of 3OHKyn. This effect presumably arises from the deprotonation of the aromatic amino, or hydroxyl groups at pH 8.4, which was also apparent by eye. After 96 hr, there was little difference between the pH 7 and pH 8.4 solutions. The 3OHKyn peak at ~370 nm had decreased in intensity, and longer wavelength-absorbing species were evident. Similar changes have been observed by Stutchbury and Truscott (1993) for oxidised 3OHKyn in the absence of protein.
Figure 2.4: UV-visible spectra of ten fold diluted aliquots from incubations described in figure 2.3 at, (a) 0 hr incubation, (b) 96 hr incubation, in (I) phosphate buffer pH 7.0, (II) bicarbonate buffer pH 8.4.

2.3.2 Effects of pH, Air and UV-light on SDS PAGE Patterns

In a second series of incubations, a low-pressure UV-light source was used to deliver a constant amount of radiation (50 mW/m²), and parallel samples were left in the dark. UV intensity reaching the lens has been estimated as 1 J/hr/cm², which is equivalent to 2.8 W/m². It is clear then that we are utilising very modest intensities of UV-light, that are much less than estimated outdoor lenticular exposure. In addition, the use of glass containers in this study approximates the transmission properties of the cornea, i.e., little light below 300 nm is transmitted to the sample.
SDS-PAGE gels of the 96 hr incubation samples with and without 3OHKyn, under eight different conditions, are shown in Figure 2.5. The upper gel (A) represents control samples of CLP exposed to the same conditions as the samples below (B), except for the omission of 3OHKyn from the mixtures. It is clear that, in the absence of 3OHKyn, under these conditions of pH, oxygen and UV-light exposure, the distribution of crystallins by gel electrophoresis is unaffected; i.e., no crosslinking or major modification was noted.

When 3OHKyn was included in the mixtures, (Fig. 2.5B), distinct changes were observed in the distribution and aggregation of the crystallins compared with controls (Fig. 2.5A). Lanes 1 and 2, where incubations were performed at pH 7.0 under aerobic conditions, in the presence and absence of UV-light respectively, distinct changes to the distribution of crystallins were observed. The β-crystallin bands, (23-30 kD) are less distinct in both lanes from the 3OHKyn-exposed samples, and substantially weaker in the UV-exposed lane 1. Furthermore, the α- and γ-crystallins (20 kD) appear to have formed a more heterogeneous population, once again particularly in lane 1, as judged by the loss of sharpness of the bands. Lanes 3 and 4, representing the anaerobic analogues of lanes 1 and 2, contain all bands shown in the controls above, albeit with less sharpness. There appears to be a slightly greater degree of modification by 3OHKyn in the UV samples exposed to air (lane 1(B) cf. lane 3(B)). The appearance of lanes 1(A) and 3(A) suggest that UV-light alone, in the presence or absence of oxygen, plays only a minor role in gross polypeptide modification, with slightly less definition observed around 26 kD.

Lanes 5 and 6 (Fig. 2.5B), which show the effects of exposure to 3OHKyn, under aerobic conditions at high pH, indicate that a substantial portion of the crystallins have been incorporated into covalently crosslinked, high-molecular-weight species, as evidenced by a loss of crystallin bands and the failure of some material to enter the gel. Lane 5, with UV-light exposure, has lost all β-crystallin bands, apparently to aggregation, as well as some α-, and γ- crystallins, while only a small percentage of
Figure 2.5: 15% homogeneous SDS-PAGE gel of CLP incubated for 96 hr in the absence, (A) and presence, (B) of 3OHKyn. Lanes 1-4 represent pH 7.0 incubations, and lanes 5-8 pH 8.4 incubations. Odd numbered lanes were exposed to UV-light as described. Lanes 1, 2, 5, and 6 were exposed to oxygen, while lanes 3, 4, 7, and 8 were blanketed with argon to exclude contact with atmospheric oxygen.
Chapter 2 Reaction of 3-Hydroxykynurenine with Bovine Crystallins

the β-crystallins remain in lane 6. Under analogous anaerobic conditions (lanes 7 and 8) the extent of protein modification appears less. Blurred bands are evident, however, and the UV-exposed sample in lane 7 has a less distinct βB1 band than lane 8.

The appearance of lanes 1, 2, 5, and 6, which were exposed to oxygen, indicates that 3OHKyn under oxidative conditions, has the ability to bind to and crosslink protein, and that under more basic conditions, the extent of this modification is enhanced.

In the absence of 3OHKyn, UV-light appeared not to alter the SDS PAGE patterns but, in the presence of 3OHKyn, UV-light exposure resulted in an increase in the heterogeneity of the crystallin distribution through a more marked smearing of bands compared to parallel samples, which were kept in the dark.

To assess the degree of protein tanning (colouration) by 3OHKyn, an index of A370/A595 was calculated, where the A370 value is indicative of the incorporation of 3OHKyn into the protein and A595 is λ_max for the Biorad protein dye binding assay. Figure 2.6 presents the index of tanning for the samples represented by lanes 1 to 8 in the SDS-PAGE gel of figure 2.5(B).

It is clear from this graph that a synergistic effect exists between UV exposure and oxygen which resulted in an increase in the proportion of tanned material in the samples that were exposed to light and oxygen. In both buffer systems, aerobic samples attained a greater degree of tanning than the corresponding anaerobic equivalents. Furthermore, UV-light contributed to a higher level of tanning than the non-exposed samples within each treatment. pH also had an influence on the degree of tanning, as the bicarbonate system had a higher overall index, in concordance with the generally increased incorporation of radiolabel at the higher pH. Protein concentration of samples was followed throughout the incubation, and no significant decrease was observed (results not shown), indicating that precipitation was not a variable in the index of tanning figures presented.
Figure 2.6: Index of tanning of the eight samples described in figure 2.5(B) after 96 hr incubation. The values were obtained by taking the ratio of absorbance at 370 nm to the total protein concentration to give an indication of the degree to which the protein has been modified. Lane number refers to corresponding samples described in figure 2.5(B).

2.3.3 UV and Fluorescence Spectra of Tanned CLP

UV-visible absorbance and fluorescence spectra were taken of the precipitated and washed 0, 48, and 96 hr samples from the two incubations shown in lanes 1 and 5 of the SDS-PAGE in figure 2.5(B). UV-visible spectra of urea-solubilised precipitates showed a progressive increase in absorbance in the range ~350-450 nm with time of incubation in samples reacted with 3OHKyn (figure 2.7). A similar spectral shift is also observed in cataractous proteins.
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Figure 2.7: UV-visible spectra of ethanol precipitated and washed CLP from the UV-light and oxygen exposed pH 7.0 (i), and pH 8.4 (ii) incubations, correlating to lanes 1 and 5 in figure 2.5(B). Precipitates were resolubilised in 8 M urea and spectra were acquired on the 0, 48 and 96 hour reaction samples in the wavelength range of 300 - 500 nm.

Non-tryptophan fluorescence can give an indication of chromophores attached to a protein \(^{134}\). In the normal human lens, blue fluorescence is found, which increases with age, and typically has an excitation of ~340 nm, and an emission maxima of ~420 nm. It is clear from the spectra in figure 2.8, that from a baseline value at 0 hr,
fluorophores were increasingly bound to the CLP, and that over time the emission became both more intense and encompassed increasingly longer wavelengths. A similar result was noted for samples exposed at pH 8.4.

**Figure 2.8:** Fluorescence spectra of ethanol precipitated and washed CLP from the UV-light and oxygen-exposed incubations at (i), pH 7.0 and (ii), pH 8.4 in the presence of 3OHKyn, corresponding to lanes 1 and 5 in figure 2.5(B). Precipitates were resolubilised in 8 M urea and spectra were acquired on the samples which had been exposed to 3OHKyn for 0, 48 and 96 hr of reaction. Excitation was at 340 nm, and emission recorded in the range 350-600 nm.
2.3.4  *Digestion of Labelled and Modified Protein*

In order to confirm that 3OHKyn was indeed bound covalently to the protein, CLP from the phosphate incubation at pH 7.0 that had been exposed to both UV and oxygen for 96 hr was precipitated and digested with pronase to assess the distribution of modified proteolytic fragments. Digests were separated by HPLC and monitored at 229 nm and 340 nm. The collected fractions were counted to give the absorption and radiochemical profiles represented in figure 2.9. From figure 2.9, it is apparent that the distribution of labelled digest products reflects both the 229 nm and 340 nm profiles, indicating that 3OHKyn had indeed covalently bound to CLP, and that it was distributed across numerous proteolytic fragments. This finding suggests that the colour associated with modification of proteins by 3OHKyn is due to covalent attachment of a coloured, and therefore probably oxidised, derivative of 3OHKyn. In addition, as evidenced by the area under the radiochemical profiles shown in figure 2.10, it was clear that covalent attachment of label was greater in oxygen and UV-light-exposed samples (A), when compared with profiles generated from samples which were not exposed to oxygen and UV-light (B).
Figure 2.9: Absorption, at 229 nm and 340 nm, and radiochemical profiles of a RP-HPLC separation of pronase digest fragments of CLP. The sample corresponds to that of lane 1 in figure 2.5(B), i.e., after incubation with 3O\textsuperscript{3}HKyn at pH 7.0 in the presence of UV-light and oxygen for 96 hours.
Figure 2.10: Radiochemical profiles of pronase digests of the precipitated and washed CLP corresponding to incubation samples described for lane 5 (A), and lane 8 (B) from figure 2.5(B).
2.3.5 Anaerobic versus Aerobic Incubation in the Absence of UV-light

In the preceding experiments, attempts were made to exclude air by performing reactions under a blanket of argon, however it was considered possible that some oxygen may have been able to enter via the caps of the vials. In order to avoid this problem, an Atmosbag™ was employed to obtain an anaerobic environment and all samples were kept in the dark (see Methods). To avoid the complications arising from rising pH in the bicarbonate buffer with time, N-ethyl morpholine was used at pH 8.4 to compare the effects of pH on oxidation under aerobic and anaerobic conditions. Samples were taken as described, and the resultant incorporation of label was calculated. The pH of each solution remained within 0.05 units throughout the incubation period.

Figure 2.11 shows that under strictly anaerobic conditions, little incorporation of label occurred at pH 7.0 over the incubation period. At pH 8.4 an increased amount of labelling was observed. In contrast to this, the samples exposed to air exhibited a relatively rapid and linear accumulation of label over the same period, attaining molar ratios of 3OHKyn to CLP of 0.65 at pH 7.0, and 0.73 at pH 8.4. The only slightly greater incorporation at pH 8.4, implies that under increasingly basic conditions, where 3OHKyn oxidises more rapidly, it is not appreciably more reactive toward protein. This increase in labelling with pH was not as pronounced as in the bicarbonate buffer system, where a more rapid incorporation occurred in conjunction with an increase in basicity up to about pH 9.5.
Figure 2.11: Effect of pH and oxygen on the incorporation of radiolabelled 3OHKyn into CLP. Incubations were at ambient temperature and either incubated in a nitrogen filled Glovebag™, or exposed to atmospheric oxygen. Light was excluded from the solutions except when time point samples were taken. Incorporation ratios were calculated by division of the counts in washed and precipitated aliquots by the total counts in an untreated 100 μl aliquot of 100 mM phosphate buffer, pH 7.0 with —•— and without —■— oxygen exposure respectively. 100 mM N-ethyl morpholine buffer, pH 8.4, with —○— and without —●— oxygen exposure respectively.
2.3.6 Effect of Glutathione on the Incorporation of Label into CLP

When GSH was included in the aerobic incubation systems, no incorporation of 3OHKyn was observed during the first 24 hr period at pH 7.0 (fig. 2.12A), and only a very small amount at pH 8.4 (fig 2.12B). From 24 hr onwards, however, an increase in the rate of incorporation was observed at both pH values, in parallel with a decrease in GSH concentration. Measurement of GSH levels over the period of the experiment indicated, as expected, a more rapid oxidation of GSH at pH 8.4. At pH 8.4, the GSH level fell to 0.5 mM after 24 hr and to 0.0 mM at 48 hr (fig 2.12B).

![Figure 2.12](image)

**Figure 2.12:** Assessment of the contribution of pH to GSH oxidation and 3OHKyn incorporation. (A) 100 mM phosphate buffer, pH 7.0, (B), 100 mM N-ethyl morpholine buffer, pH 8.4. Incubations were in the dark at room temperature. Open symbols represent GSH concentration and filled symbols the incorporation of radiolabelled 3OHKyn.

In order to estimate more precisely the concentration of GSH at which reactivity of 3OHKyn commenced, a reaction was carried wherein aliquots were removed for
analysis more frequently across the window of perceived onset of binding. The incubation was carried at pH 7.0 only, and a higher specific activity of $3O^3$Hkyn was included to avoid background ambiguities. The results are presented in figure 2.13. It can be seen from the results that no modification was observed above a 1 mM concentration of GSH. However, when the level of GSH fell below 1 mM the protein became modified, as evidenced by the rise in counts (right axis).

Figure 2.13: Effect of GSH on the incorporation of radiolabelled $3OHKyn$ into CLP by a sample exposed to oxygen exposed sample in 100 mM phosphate buffer, pH 7.0. Incubation was in the dark, and 5 mM GSH was included in the initial solution. Symbols represent concentration of GSH —— and incorporation of radiolabel, —— respectively.
2.3.7 3OHKyn Concentration, GSH and Radiolabelling

It was of interest to investigate whether labelling occurs at the much lower 3OHKyn concentrations of 5 μM and 200 μM compared to the previously used 2.2 mM solutions, and whether glutathione affords greater protection against oxidative modification. Initially, a standard curve of HPLC peak areas versus 3OHKyn concentration was prepared over the range 50 μM to 2 mM. This enabled a relationship to be drawn between concentration and peak area in μV² (Fig. 2.14).

Figure 2.14: Standard curve of peak area obtained by HPLC versus concentration of 3OHKyn detected at 365 nm. The peak areas were obtained by injection of 20 ul aliquots and integration of the chromatograms using Waters DP800 software.
An HPLC-LSC run of 20 µl of a 500 µl stock 3O^3HKyn solution yielded a 365 nm peak area of 8.07 x 10^6, µV^2 and a corresponding activity of 4.80 x 10^5 dpm (data not shown).

From the standard curve

\[ 1 \text{ µV}^2 = 5.92 \times 10^5 + 1.38 \times 10^4 \text{ µM} \]

from the HPLC-LSC

\[ 1 \text{ µV}^2 = 5.95 \times 10^2 \text{ dpm} \]

therefore

\[
\begin{align*}
\text{dpm} & = \frac{(5.92 \times 10^5 + 1.38 \times 10^4 \text{ µM})}{5.95 \times 10^2} \\
\text{µM} & = \frac{(\text{dpm} - 9.95 \times 10^6)}{2.32 \times 10^3}
\end{align*}
\]

For the stock solution containing 500 µl, this equates to 1.15 x 10^7 dpm; substituting

\[
3\text{OHKyn} = 6.68 \text{ µM} = 3.34 \times 10^{-9} \text{ moles}
\]

To achieve a 5 µM concentration of 3OHKyn in 2 ml of solution 100 x 10^10 moles were required. Thus the stock was divided into four portions such that each sample contained 8.35 x 10^10 moles of 3O^3HKyn. For the 5 µM samples, a further 91.65 x 10^10 moles of 3OHKyn were added from a 200 µM stock solution (45.8 µl). The contribution of the 3O^3HKyn to the 200 µM samples was insignificant, thus the phosphate buffered 200 µM stock was used as the incubation buffer.

Incubations were carried out in the dark, identical to those used in section 2.2.6, except for the concentrations of 3OHKyn. Figure 2.15 represents the extent of radiolabelling over a 144 hour period for the four combinations of 3OHKyn concentration and presence of GSH.

From figure 2.15, it is clear that the concentration of 3OHKyn has an effect on the extent of radiolabelling. When a concentration of 2.2 mM 3OHKyn was used (Fig 2.11), a molar-binding ratio of 0.65 was reached after 96 hours. An 11-fold dilution of 3OHKyn to 200 µM under the same incubation conditions resulted in a molar binding
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ratio of 0.057 after the same period, indicated by the dotted-line intersection in the main figure 2.15, which suggests a direct, linear relationship between 3OHKyn concentration and label incorporation.

Figure 2.15: Effect of 3OHKyn concentration and GSH on the incorporation of radiolabelled 3OHKyn into CLP. Incubations were at ambient temperature exposed to atmospheric oxygen in 100 mM phosphate buffer, pH 7.0. Light was excluded from the solutions except when time point samples were taken. Incorporation ratios were calculated by division of the total counts in washed and precipitated aliquots by the total counts in an untreated 100 μl aliquot. CLP concentration was 5 mgml⁻¹. 5 μM 3OHKyn with ■—■— and without □—□— 5 mM GSH. 200 μM 3OHKyn with •—•— and without —○— 5 mM GSH respectively. The inset graph depicts the rescaled 5 μM solutions.
The results of further dilution to 5 μM 3OHKyn are depicted in the inset of figure 2.15. In this case, after 96 hours in the absence of GSH, a 5 μM concentration of 3OHKyn yielded a molar binding ratio of approximately $6.5 \times 10^{-4}$. This is less than that suggested by an extrapolation of the linear relationship exhibited between 2.2 mM and 200 μM, where the expected ratio would have been $1.4 \times 10^{-3}$ moles 3OHKyn / mole CLP. This decrease with concentration may however, simply be due to the decrease in random molecular interactions at lower concentrations.

When 5 mM GSH was included in the incubations, an effect similar to that described in section 2.2.6 was observed, i.e., the onset of incorporation of radiolabel was delayed due to the presence of the antioxidant. At the lower 3OHKyn concentrations however, onset of oxidative modification was delayed to between 48 and 72 hours at the 200 μM level, and for greater than 72 hours at 5 μM 3OHKyn (inset). Once again a relationship between 3OHKyn concentration and labelling was observed, in this case that the rate of GSH oxidation to a critical level where 3OHKyn can be oxidised was seen to be proportional to the concentration of 3OHKyn.

**2.3.8 Interactions of Crystallin Families with 3OHKyn**

This experiment was designed to determine if any differences in binding efficacy existed between the α-, β- and γ-crystallin families. The incubations were as described previously, with equivalent doses of 3O3HKyn taken from a single stock solution.

The results depicted in figure 2.16 show a comparison of label uptake over a 96 hour period for each of the crystallin families as well as the total CLP. From this graph it can be seen that the uptake of label by the β- and γ-crystallins is slightly higher than that shown for the mixture of total CLP. α-Crystallin however was labelled to a
significantly lesser degree than the others, suggesting either that \( \alpha \)-crystallin contains fewer sites for oxidative modification to occur, or that the accessibility to these sites is somewhat limited compared to the \( \beta \)- and \( \gamma \)-crystallins.

**Figure 2.16:** Comparison of the molar-binding ratios of \( \alpha \)-, \( \beta \)- and \( \gamma \)-crystallin to that of total CLP. Incubations were at ambient temperature and excluded from light, except when time point aliquots were removed. All solutions were in 100 mM phosphate buffer pH 7.0, 5 mg/ml protein, and 0.5 mg/ml 3OHKyn. Markers indicate: —- \( \beta \)-crystallin —- \( \gamma \)-crystallin—O— CLP —- \( \alpha \)-crystallin

An inspection of the final (96 hour) molar binding ratios shows, as would be expected, that the average of the \( \alpha \)-, \( \beta \)- and \( \gamma \)-crystallin values is 0.791 mol.mol\(^{-1}\), which indicates that these familial ratios are cumulatively responsible for the CLP ratio (0.789 mol.mol\(^{-1}\)).
The lower molar binding ratio for α-crystallin is reflected in the UV-profiles for the modified crystallins (Fig. 2.17). β- and γ-crystallin show distinct maxima at ~375 nm, as well as non-specific absorbance up to and beyond 800 nm. α-Crystallin however exhibits a different pattern of absorbance. The maxima at 375 nm is absent and the absorbance in this area is lower than for β- and γ-crystallin. Absorbance towards the higher wavelengths however decreases less rapidly than for β- and γ-crystallin, resulting in a shift towards greater absorbance of the wavelengths over 400 nm.

**Figure 2.17:** UV-visible spectra of ethanol-precipitated and washed α-, β-, and γ-crystallins after 96 hours incubation with 3OHKyn at pH 7.0 in the absence of light. Precipitates were resolubilised in 8M urea prior to acquisition of spectra over the range 300 - 800 nm.
2.4 Discussion

In this study, the tryptophan metabolite 3OHKyn, which is continuously biosynthesised in the human lens, has been assessed for its reactivity with bovine lens crystallins, since protein modification is a characteristic feature of age-related nuclear cataract. In particular, we wished to assess the influence of the rate of 3OHKyn oxidation on the rate of binding to crystallins and, since the lens is exposed to UVA, to determine if UV-light of wavelength and intensity similar to that found in vivo could affect the rate of binding. The centre of the lens, where the nuclear cataract begins, is thought to be a region of low oxygen tension so we also sought to examine the influence of aerobic and anaerobic conditions on the rate of protein modification. Finally we hoped to demonstrate that the attachment of 3OHKyn was covalent in nature and intimately associated with the colouration of the treated protein.

Previous investigations in this laboratory have revealed that 3OHKyn levels in vivo are in the region of 5 μM approximately 400 times less than the concentration used throughout the current work. Clearly, the significant lifetime of the lens, and the fact that 3OHKyn is continuously replenished in the human lens as an intermediate in the tryptophan catabolic pathway toward 3HKG production, lead to the proposal that the lens is exposed to large cumulative quantities of 3OHKyn.

In order to ascertain the influence of these conditions on the binding of 3OHKyn to CLP, a series of incubations were carried out under the variants of UV-light, oxygen and pH. At neutral pH, in the presence of air, 3OHKyn is readily oxidised, forming a number of coloured products with broad (400-500 nm) absorption characteristics and it has been shown that, at neutral pH, 3OHKyn can react with polypeptides and lens proteins, predominantly with amino groups. In order to follow the progress of reaction of 3OHKyn with CLP, radiolabelled compound was utilised, and the extent of modification observed over a 96 hr period under a variety of conditions.
3OHKyn, although an intermediate in the formation of the UV filter compound, 3OHKyn glucoside from tryptophan, has also been suggested to act as an antioxidant by inhibition of oxidative covalent crosslinking of lens crystallins. Earlier work had shown 3OHKyn to be an efficient antioxidant. Contrary to this, Tomoda et al. (1990) suggested that 3OHKyn and UV irradiation react photodynamically to form xanthommatin, which could be involved in the pigmentation of human cataractous lenses, although no xanthommatin has been found in the lens. All these observations suggest that oxidation products of 3OHKyn, not 3OHKyn itself, are responsible for colouration of the lens, and may explain why van Heyningen (1973) found no evidence of 3OHKyn in chemical combination with brown material from nuclear cataracts. Initial incubations were carried out under aerobic conditions at either pH 7.0 or 8.4 (to accelerate oxidation of 3OHKyn), with or without UV-light (Fig. 2.3). The results of this incubation show that both pH and UV-light influence the efficacy of covalent modification. Interestingly the faster rate of oxidation at higher pH did not markedly increase the rate of protein modification (Fig 2.11).

UV-visible spectra of CLP at 0 hr and 96 hr incubation (not shown) revealed the formation of protein-bound products with long wavelength absorption, consistent with changes observed in human cataractous protein. Presumably, therefore, oxidation products of 3OHKyn are able to bind to CLP. The SDS-PAGE gels (Fig. 2.5) indicated that exposure to oxygen, particularly at high pH, in the presence of 3OHKyn, resulted in covalent crosslinking. The controls and anaerobic samples did not show evidence of such crosslinking, which indicates that 3OHKyn facilitates this process only under oxidative conditions.

The index of tanning figures for washed and resolubilised 96 hr precipitates (Fig. 2.6), were in general, consistent with the radiolabel experiments (Figs. 2.3 and 2.11). The only exceptions were the oxygen- and sunlight-exposed bicarbonate and phosphate samples. A higher index of tanning correlates with an increased incorporation of 3OHKyn-derived radiolabel. This indicates that the index of tanning
provides a measure of the degree of covalent modification of the protein by 3OHKyn. Sunlight exposure at high pH did not follow this trend, that is, the bicarbonate sample had a higher index of tanning but a lower total incorporation than the phosphate sample, which could be a result of the effect that elevated pH has on the identity and absorption characteristics of the 3OHKyn oxidation products. Little is known about the nature and number of oxidised 3OHKyn species, and it is feasible that exposure to UV-radiation may affect the products to an extent that, in this instance, a higher A$_{370}$ falsely indicates a greater incorporation of labelled product.

The 3OHKyn-tanned protein was found to absorb at wavelengths above 400 nm, and also to develop non-tryptophan fluorescence (Fig. 2.8). Interestingly, with age, the human lens develops an unusual blue fluorescence with emission at wavelengths longer than 400 nm after excitation near 340 nm$^{53,135}$. These fluorophores can oxidise to green fluorescent products with excitation at 450-470 nm and emission at ~520 nm in vivo; similar fluorophores are formed in vitro in the presence of glycine$^{107}$. A correlation has been drawn between increased blue/green fluorescence and the level of insoluble lens protein$^{136}$. In our study, CLP controls incubated without 3OHKyn exhibited no fluorescence (results not shown). CLP in the presence of 3OHKyn, however, showed both an increased intensity of fluorescent products with time, and a development of longer wavelength fluorophores (Fig. 2.8). Confirmation that a covalent modification had occurred was obtained by proteolysis of the 3OHKyn-tanned CLP followed by HPLC separation, which revealed a broad distribution of labelled products indicating heterogeneous protein modification (Fig. 2.9). Digestion of cataract protein from human lenses under the same conditions yielded a similar broad coloured HPLC peak to figure 2.9, eluting at the same retention time (unpublished data).

Many investigators have proposed a photooxidative mechanism for cataract formation$^{118,137-140}$, and our results suggest that this factor could contribute to the
extent of reaction of lens crystallins with the naturally occurring UV filter compound, 3OHKyn (Fig. 1).

In an attempt to assess the role of oxygen in the modification of CLP by 3OHKyn, air was carefully excluded from buffers and samples throughout an experiment utilising a Glovebag™ (Fig. 2.11). Samples placed in the dark and exposed to air incorporated 3OHKyn in a linear fashion at pH 7.0, and to a slightly greater extent, at pH 8.4. Samples from which oxygen was excluded bound little label at pH 7.0, and only slightly more at pH 8.4. It seems that oxidation of 3OHKyn is an imperative condition for major protein modification, and that UV-light enhances this. The fact that some crystallin modification was noted even under anaerobic conditions however, suggests that this process could occur over time even under the low oxygen conditions present in the centre of the lens. Superoxide radical formation may be implicated in these processes, possibly through reaction with one of the autoxidation products of 3OHKyn.\textsuperscript{132}

GSH acts as an oxidant scavenger in the lens, and levels in normal human lenses decrease with age\textsuperscript{89,90}, thus increasing the vulnerability of lens proteins to oxidation\textsuperscript{141}. When GSH was included in oxygen-exposed samples, a lag period was observed prior to the onset of incorporation of 3OHKyn (Fig. 2.13). Placed in the context of an \textit{in vivo} model, this result suggests that GSH has the ability to prevent covalent modification of lens proteins by 3OHKyn, probably by maintaining 3OHKyn in the reduced state. Indeed, reduction of GSH concentration is a biochemical event which is among the earliest detectable in cataract formation\textsuperscript{83,90}. From the results presented in figure 2.13, when the levels of GSH were reduced significantly, oxidation of 3OHKyn occurred, which presumably led to reaction with CLP. It was of interest that significant modification by 3OHKyn was observed, even in the presence of approximately 1 mM GSH. Stutchbury and Truscott (1993)\textsuperscript{67} showed that, \textit{in vitro}, 3OHKyn actually accelerates the rate of GSH oxidation, hence promoting an environment conducive to formation of oxidised 3OHKyn species, which can then become covalently bound to
lens crystallins. Kamei (1993)\textsuperscript{141} concluded that a lowering of GSH levels, as opposed to a complete depletion, can accelerate the oxidation of lens proteins and lipids. This is consistent with our results (Fig 2.13), which indicated that 3OHKyn could be oxidised and covalently incorporated in to CLP while low levels of GSH were still present.

In conclusion, although proposed to be a potent antioxidant in certain biological systems\textsuperscript{29,31}, 3OHKyn, when thus oxidised, also has the propensity to form reactive intermediates which have been shown here to bind covalently to CLP. UV-light augments the reaction. As a consequence of this reaction, aggregate formation, pigmentation and development of non-tryptophan fluorescence occur, all of which have been observed in nuclear cataractous lenses.

It should be noted that the aerobic experiments herein were carried out in atmospheric levels of oxygen giving rise to an oxygen tension in solution of approximately 5 mm Hg (290 µM). The oxygen concentration in the lens has been measured at 43-130 µM\textsuperscript{107} and the nucleus is assumed to have a lower tension. Considering the lifetime of nuclear lens proteins and the fact that high levels of oxidised cysteine and methionine residues are found in crystallins from the nuclei of cataract lenses\textsuperscript{84} oxidation is certainly an important factor. In the current studies, some reaction of 3OHKyn with CLP, albeit a low level, was observed even under anaerobic conditions. Hence, the cumulative effect of long-term, low-level oxygen exposure in the presence of compounds such as 3OHKyn must be considered deleterious with respect to the possibility of damaging oxidative modification reactions occurring particularly in regions where the glutathione concentration falls below 1 mM. The finding of a difference in tryptophan metabolism in cataract patients compared with normal controls\textsuperscript{71} emphasises the need for more research in this area.
Chapter 3  
Reaction of 3-Hydroxykynurenine with the Dipeptide Glycyllysine

CHAPTER 3

Reaction of 3-Hydroxykynurenine with the Dipeptide Glycyllysine

3.1  Introduction

In the mammalian lens 3OHKyn is present as one of a number of long-wavelength, UV-light-absorbing compounds. It is an o-aminophenol, and as such, undergoes facile autoxidation resulting in the formation of products which have been found to react with proteins to produce brown-coloured polypeptides with indistinct long wavelength absorption characteristics. The binding of 3OHKyn oxidation products to lens proteins is accompanied by aggregation to higher molecular weight species and development of non-tryptophan fluorescence. Experiments involving reaction of 3OHKyn with various compounds, including glycine, revealed that 3OHKyn reacts with compounds that have a free amine functionality, and that under oxidative and photolytic conditions, this leads to the production of a mixture of blue and green fluorophores. The mechanism of formation of these products, and the nature of the covalently bound species involved in the evolution of this fluorescence, is not known. However, the use of a single amino acid model system allows for the possibility of Strecker degradation products which complicates the analysis. This is not the case with polypeptides such as the lens crystallins.

Senile nuclear cataract is characterised by the formation of high molecular weight, coloured proteins which are insoluble and which produce opacity and subsequent light-scattering, leading to a decrease in the transmission of light. These aggregates are formed by post-translational modification of pre-existing polypeptides. A comparison of the levels of blue and green fluorescence in cataractous and normal lenses has revealed that a much higher green-to-blue fluorescence ratio exists in the
soluble protein fractions of cataractous lenses compared to clear ones \(^{145}\), indicating that these green products are associated with cataractogenesis.

If 3OHKyn is indeed involved in modification of the proteins \(\textit{in vivo}\), then a valuable marker for this process would be knowledge of the nature of the 3OHKyn-protein adducts. In the present study, 3OHKyn was reacted, under oxidative conditions, with the dipeptide, glycyllysine (GK), which comprises the two N-terminal amino acid residues of the \(\gamma\)-crystallin family of lens proteins \(^{129}\). The aim of this work was to isolate the major fluorescent product from the GK/ 3OHKyn reaction mixture, and to elucidate its structure.
Figure 3.1: Structures of 3OHKyn and related oxidised molecules
3.2 Methods

3OHKyn, Gly-Lys.HCl (GK), xanthurenic acid (XA), DMSO-$d_6$ and trypsin (TPCK treated) were purchased from Sigma (ST. Louis, MO, USA). 2-Methylbenzoxazole was obtained from Fluka Chemicals. $D_2O$ was supplied by Cambridge Isotope Laboratories. All other chemicals were of analytical quality.

3OHKyn ($2 \text{ mg ml}^{-1}$) was reacted with GK ($50 \text{ mg ml}^{-1}$) in a 5 ml solution of 100 mM phosphate buffer pH 7.0. A gentle stream of humidified oxygen was bubbled into the solutions to provide mixing and constant saturation with oxygen. The reaction mixture was maintained at 37 °C in a circulating water bath for five days.

3.2.1 Separation and purification of the major reaction product

The GK reaction mixtures were centrifuged to remove any precipitated oxidation products prior to HPLC purification. Analytical HPLC was carried out on a Beckman Microsorb MV 100Å C18 column (250 x 4.6 mm, 5 µm) with a 0-40% acetonitrile gradient over 30 min at a flow rate of 1 ml min$^{-1}$. UV detection was at 365 nm and 229 nm using a Waters 440 UV detector with an extended wavelength module attached. Fluorescence detection was at excitation and emission wavelengths of 390 nm and 490 nm respectively using an ICI LC1250 on-line fluorescence detector. Purification of the major peak was carried out on the same HPLC system, using a Brownlee C8 semi-preparative column (250 x 10 mm, 7 µm) with a 0-40% acetonitrile gradient over 50 min. 20 mg of material was loaded per run, and collected fractions were pooled and lyophilised for subsequent spectroscopic analysis.

Fluorescence spectra were acquired on a Hitachi F4500 fluorimeter and UV spectra on a Shimadzu UV-2401-PC spectrophotometer. All samples were dissolved in 100 mM phosphate buffer pH 7.0.
3.2.2 Mass Spectrometry

Electrospray ionisation mass spectra were acquired on a VG Quattro II triple quadrupole mass spectrometer (VG Biotech Ltd., Altincham, Cheshire, UK). Samples were dissolved in 50% aqueous acetonitrile, 0.1% formic acid, and delivered by a Harvard Apparatus 22 syringe pump at 5-20 µl min⁻¹, depending on sample concentration. Electrospray conditions were as follows: nitrogen bath gas flow, 350 L/h and nebulising gas to the probe at 10 L/h. Capillary probe tip potential was 3.2 kV, HV lens 0.2 kV and skimmer potentials ranged from 25 to 35 V. All spectra were acquired in positive-ion mode at mass unit resolution by multi-channel analysis. The mass spectrometer was calibrated with NaI.

Inline HPLC mass spectrometry (LC-MS) was carried out using an Applied Biosystems 140B solvent delivery system and 785A UV detector. Separation of the GK / 3OHKyn reaction mixture was on an Alltech Alltima 300Å C18 column (250 x 2.1 mm, 5 µm) at a flow rate of 200 µl min⁻¹ using a 0-80% acetonitrile gradient (1% CH₃COOH) over 40 min and a column oven temperature of 25°C. All spectra were acquired in centroid mode. Tandem mass spectrometry experiments (MS/MS) were acquired with a collision energy of 25V and an Argon gas collision cell pressure of 3.8 x 10⁻⁶ Bar.

The high resolution time-of-flight (TOF) mass spectrum was acquired on a VG Autospec™ orthogonal acceleration TOF mass spectrometer (Micromass, Wythenshawe, UK), equipped with an electrospray ion source. The sample was continuously injected at 5 ml min⁻¹ in 50% methanol / H₂O, 1% acetic acid. The machine was calibrated with polyethylene glycol 600.

Deuterium exchange was achieved by adding 500 µl of D₂O and 5 µl of DCI to ~ 100 µg of lyophilised sample. Exchange was allowed to proceed under nitrogen for three hours prior to 50% dilution with acetonitrile and subsequent mass spectrometric analysis as above.
3.2.3 **NMR spectroscopy**

NMR spectra were acquired at 400 MHz ($^1$H) and 100 MHz ($^{13}$C) on a Varian Unity-400 NMR spectrometer at 25°C. Samples were dissolved in DMSO-$d_6$ and the spectra were referenced to the residual DMSO methyl resonances at 2.6 ppm for $^1$H spectra, and 43.5 ppm for $^{13}$C spectra. Sample concentrations were quinolinobenzoxamine (QBA) 11 mM, xanthurenic acid (XA) and 2-methylbenzoxazole 30 mM.

The following two-dimensional (2D) NMR experiments were acquired: double-quantum filtered correlation spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY), nuclear Overhauser effect spectroscopy (NOESY), heteronuclear single-quantum coherence spectroscopy (HSQC) and heteronuclear multiple-bond coherence spectroscopy (HMBC). The $^1$H experiments (DQF-COSY, TOCSY and NOESY) were acquired in the phase-sensitive mode using time-proportional phase incrementation \(^{146}\). The mixing time for the NOESY experiment was 300 ms, and the spin lock period for the TOCSY experiment was 70 ms. Typically, 512 $t_1$ increments, with up to 96 scans per increment, were acquired over 2048 data points which were zero-filled to 2048 data points in both dimensions and multiplied by a Gaussian window function prior to Fourier transformation.

For the $^1$H-$^{13}$C correlation experiments (HSQC and HMBC), gradients were used for coherence selection \(^{147}\). Up to 1024 $t_1$ increments with up to 128 scans per increment were acquired over 2048 data points. The data were processed as for the $^1$H 2D experiments. The HSQC and HMBC spectra were acquired in the phase-sensitive and absolute-value modes respectively. The delays in the HSQC and HMBC spectra were set for $J_{CH}$ values of 140 Hz and 9 Hz respectively.
3.3 Results

3OHKyn (Fig. 3.1) is an o-aminophenol and is readily autoxidised at neutral pH to form numerous products. The analytical HPLC chromatogram for the reaction mixture of GK and 3OHKyn, with detection at 365 nm and 229 nm, and fluorescence (ex 390 nm / em 490 nm) is shown in figure 3.2. The major product absorbing at 365 nm, corresponding to the peak indicated by an arrow at 18 min, was not present in oxidised samples of either of the reactants and was collected for spectroscopic analysis.

UV-Vis and fluorescence spectra of the major coloured HPLC fraction are shown in figures 3.3a and 3.3b respectively. The UV-Vis spectrum of GK / 3OHKyn shows two absorption maxima at 245 nm and 367 nm. These maxima are consistent with a kynurenine absorbance ($\lambda_{\text{max}}$ 370 nm), or 3OHKyn-like absorbance ($\lambda_{\text{max}}$ 370 nm), and benzoxazole (Fig. 3.1) which has a $\lambda_{\text{max}}$ at 247 nm. The 3D fluorescence spectrum exhibits a single sharp peak at excitation and emission maxima of 390 nm and 490 nm respectively. Kynurenine has fluorescence maxima of ex 370 / em 490 at pH 11.0, indicating that kynurenine and the unknown product may contain structural similarities.

3.3.1 HPLC purification of the major reaction product

The major reaction product was purified from the reaction mixture by semi-preparative HPLC. Figure 3.4 represents the absorbance profiles for this separation, monitored at 365 nm and 229 nm. In the 365 nm chromatogram (upper) a distinct major peak can be seen from 23 to 24.5 minutes - this was identified as the compound of interest, thus was collected and pooled from subsequent runs.
Figure 3.2: HPLC trace and fluorescence (ex 390 nm / em 490 nm) of the GK / 3OHKyn reaction mixture. The eluant was monitored for absorbance at 365 and 229 nm. The arrow indicates the major peak absorbing at 365 nm which was collected for structural analysis.
Figure 3.3: UV and fluorescence spectra of the major 365 nm-absorbing HPLC peak indicated in figure 2. (A) Two maxima, at 245 nm and 367 nm are evident. (B) The 3D fluorescence spectrum of the same compound. Excitation and emission maxima of 390 nm and 490 nm were obtained.
Figure 3.4: Semi-preparative HPLC chromatograms of the GK / 3OHKyn reaction mixture. The eluant was monitored for absorbance at 365 and 229 nm. The large coloured peak eluting between 23 and 24.5 minutes was collected and pooled for subsequent NMR spectroscopic analysis.
The peak at 14.5 minutes was found to be unreacted 3OHKyn; the other minor peaks were not collected. From a mixture of 10 mg of 3OHKyn and 250 mg of GK, 4.8 mg of purified compound was collected. This represented an 18% yield with respect to 3OHKyn.

3.3.2 Mass spectrometry

The mass spectrum of the major reaction product collected from HPLC (Fig. 3.5a) revealed a singly charged species at 604 \( m/z \), and a doubly charged species at 303 \( m/z \). A molecular mass of 603 Da was significantly higher than that expected for a simple combination of GK (203 Da), and 3OHKyn (224 Da), suggesting that the adduct may be a trimer. This was supported by TOF high resolution MS which yielded a mass of 603.22891 Da and a corresponding calculated empirical formula of \( \text{C}_{26}\text{H}_{33}\text{N}_{7}\text{O}_{10} \). Such a formula could best be rationalised by a structure consisting of two GK and one 3OHKyn moieties.

An MS/MS spectrum of the singly charged molecular ion (\( m/z \) 604) was acquired and is shown in figure 3.5b. Ions at \( m/z \) 84, 101, 130, and 147 confirmed the presence of at least one relatively unmodified GK moiety since they were also found in the MS/MS spectrum of GK (Fig. 3.6). The base peak at \( m/z \) 84 is indicative of loss of ammonia from the lysine immonium ion. A fragmentation scheme based on the MS/MS spectrum of the structure referred to as quinilinobenzoxamine (QBA) is shown in figure 3.7. Assignment of some of the product ions is indicated by bracketing of the bond(s) involved. The product ion at \( m/z \) 476 is attributed to cleavage on the carboxyl side of either the Lys\(_1\) or Lys\(_2\) \( \alpha\)-NH groups.
Figure 3.5: Mass spectra of the major GK/3OHKyn reaction product. (A) Mass spectrum revealing a singly charged ion at \( m/z \) 604.5, and a doubly charged ion at \( m/z \) 303.1. (B) Tandem mass spectrum of the \( m/z \) 604.5 peak and resultant fragment ions. This fragmentation pattern indicated that at least one GK moiety was incorporated into the compound, and formed the basis of the fragmentation mechanism proposed in figure 3.7.
Figure 3.6: Tandem mass spectrum of a standard sample of glycyllysine. The characteristic base peak at m/z 84, along with those at m/z 101, 130, and 147 correspond to those observed in the MS/MS spectrum of the reaction product shown in figure 3.5B. This indicates that at least one GK moiety was involved in the reaction.
Loss of CO and water appeared to be responsible for the ions at \( m/z \) 458 and \( m/z \) 430. This was suggested by the MS/MS of a standard sample of xanthurenic acid (XA), which exhibited this characteristic loss under identical electrospray conditions (Fig. 3.8). Cleavage of the second lysine, again on the carboxyl side of the \( \alpha\)-NH group, gave rise to the fragment ion \( m/z \) 301. Consecutive losses of the remaining amide moieties would account for the peaks \( m/z \) 257 and \( m/z \) 214.

**Figure 3.7:** Proposed structure and fragmentation pattern of the major coloured product from the reaction between 3OHKyn and GK. The compound is referred to as QBA. The thirteen exchangeable protons are indicated in bold.
**Figure 3.8:** Tandem mass spectrum of a standard sample of xanthurenic acid (XA). The fragment ions at m/z 188 and m/z 160 represent a loss of water and CO respectively from the molecular ion. This pattern was observed in the fragmentation of QBA.
**Figure 3.9:** Mass spectrum of QBA after 180 minutes exchange with D$_2$O under acidic conditions. The $m/z$ of the singly charged molecular ion was increased by 12 units and the doubly charged ion by 6.5 units (equivalent to an increase of 13 Da for the molecular ion), compared with the equivalent ions observed in figure 3.5A.
Further evidence to support the proposed structure was obtained from the mass spectrum of a sample of the compound which had been incubated in D$_2$O to allow exchangeable protons to be replaced by deuterium atoms. There are 13 exchangeable protons in the fully protonated (doubly charged) molecule, and 12 such protons in the singly charged species (Fig 3.7). Complete proton-deuteron exchange would therefore realise an increase in mass of 13 Da and 12 Da respectively. The spectrum of the deuterated molecule (Fig. 3.9) contained two major peaks at 309 and 616 m/z, which is consistent with a structure containing 13 exchangeable protons in the doubly charged species, and 12 in the molecular ion.

### 3.3.3 NMR spectroscopy

Purification of a sufficient quantity of material for NMR spectroscopic characterisation involved multiple semi-preparative HPLC separations. Detailed information on the structure of this product was obtained from 1D and 2D $^1$H and $^{13}$C NMR spectra. The aromatic region of the 1D $^1$H NMR spectrum showed only two singlet aromatic protons, H$_A$ and H$_B$, at 7.86 and 6.98 ppm respectively, three $\alpha$-NH protons at 8.17, 8.55 and 9.16 ppm, and four broad, exchangeable NH resonances from 7.1 to 7.8 ppm (Fig. 3.10). The two downfield $\alpha$-NH doublet resonances had $J_{\alpha\text{NH}}$ values of 7.7 and 7.9 Hz respectively, which was consistent with them arising from lysine residues. The upfield $\alpha$-NH resonance had a triplet appearance with $J_{\alpha\text{NH}}$ values of 6.0 and 5.6 Hz, suggesting that it arose from a glycine residue.
Figure 3.10: Aromatic and NH region of the 1D $^1$H NMR spectrum of QBA. Two singlet (isolated) protons, $H_A$ and $H_B$, are shown at 7.86 and 6.98 ppm respectively. Three $\alpha$-NH protons at 9.16, 8.55 and 8.17 ppm, correspond to two Lys residues and one Gly residue, based on the observed coupling constants (see text). Four broad, exchangeable NH resonances can be seen from 7.1 to 7.8 ppm. The arrow indicates an impurity.
3OHKyn has three aromatic protons (Fig 3.1). Thus, the presence of HA and HB in the spectrum of the reaction product suggested that an addition reaction had occurred at the para to the aromatic amino group of 3OHKyn. The CH-CH₂ spin system of the aliphatic sidechain of 3OHKyn was absent from the ¹H spectrum of the product, possibly indicating some form of cyclisation such as that found in the 3OHKyn dimer, xanthomattin ⁵⁶ and also in the transamination product of 3OHKyn, XA (Fig. 3.1).

Cross-peaks in the TOCSY spectrum (Fig. 3.11) from the α-NH protons arose from two lysine residues, referred to as Lys₁ and Lys₂, and one glycine residue (Gly₁). The four broad resonances from 7.1 to 7.8 ppm correlated to the ε-CH₂ resonances of Lys₂, indicating that they arose from the ε-NH₃⁺ group of this lysine. In the NOESY spectrum no exchange cross-peaks were observed between these resonances. The DMSO solvent contained some water, as judged by the presence of a large, broad resonance at 3.58 ppm in the ¹H 1D spectrum. In the NOESY spectrum (Fig. 3.12), the four Lys₂ ε-NH₃⁺ resonances each exhibited an exchange cross-peak with this water resonance. Normally the ε-NH₃⁺ protons of a lysine residue give rise to a single broad resonance due to rapid chemical and solvent exchange. The observation of four individual non-chemically-exchanging ε-NH₃⁺ resonances for Lys₂ suggests that the sidechain of Lys₂ is restrained, i.e., the ε-NH₃⁺ protons experience different environments. One possibility is that this group is restricted in conformation due to salt bridge interaction with the carboxyl group at C9, although it was not possible to confirm this via nOe interactions because of the absence of protons in the vicinity of C9. In contrast, Lys₁ produced no ε-NH₃⁺ to ε-CH₂ cross-peaks in the TOCSY spectrum, probably because of broadening due to chemical exchange with the water. A cross-peak was observed, however, between the α-NH and ε-CH₂ resonances of Lys₁.
Figure 3.11: TOCSY spectrum showing cross-peaks between the α-NH and α-CH₂ groups of a single glycine residue and the α-NH to α-CH and sidechain groups of two lysine residues. The absence of a second Gly α-NH to α-CH₂ cross-peak suggested that the amino terminus of one GK dipeptide was involved in an oxidative reaction with 3OHKyn.
**Figure 3.12:** NOes from the aromatic and NH region to the aliphatic region in the 2D $^1$H NOESY spectrum of QBA. An nOe from Lys$_1$ to Gly$_1$ indicated the residues were linked. An nOe from H$_B$ to Gly$_1$ α-CH$_2$ suggested that a GK dipeptide was bound to the aromatic ring of 3OHKyn, proximal to H$_B$. The broad cross-peaks at F1 = 3.58 ppm arise from exchange between the water and the Lys$_2$ ε-NH$_3^+$ resonances.
Figure 3.13: Aromatic and NH region of the 2D $^1$H NOESY spectrum of QBA. Observed nOes from $H_B$ to the $\alpha$-NH groups of Lys$_1$ and Gly$_1$ suggested that the dipeptide had attached to C12 of the structure (see Fig. 3.1), isolating it from the ortho aromatic proton and remaining in close proximity to the $\alpha$-NH groups. No cross peaks were observed for $H_A$ at 7.86 ppm, indicating that it was isolated from both the peptide residues and other aromatic protons.
Chapter 3 Reaction of 3-Hydroxykynurenine with the Dipeptide Glycyllysine

NOESY correlations

(a)

![Diagram of NOESY correlations]

HMBC correlations

(b)

![Diagram of HMBC correlations]

Figure 3.14: Summary of the (a) 2D $^1$H NOESY correlations, and (b) 2D $^1$H-$^{13}$C HMBC correlations for QBA.
Figure 3.15: (A) Aliphatic region of the 2D $^1$H-$^1$C HSQC spectrum of QBA.

The $^1$H shifts from the TOCSY spectrum (Fig. 3.11) were used to assign the $^1$C shifts of the Gly$_i$, Lys$_i$ and Lys$_j$ CH$_n$ groups.
Figure 3.15: (B) Aromatic region of the HSQC spectrum of QBA. Only two aromatic protons were observed in the 1D $^1$H spectrum ($H_A$ and $H_B$), and these peaks were used to assign the corresponding $^{13}$C resonances.
**Figure 3.16:** HMBC spectrum of cross-peaks between the $^{13}$C resonances and $^1$H resonances of QBA.

(A) This section of the HMBC spectrum was used to assign the cyclised 3OHKyn sidechain $^{13}$C resonances, i.e., C6 - C9, as well as the carboxyls of Gly$_1$ and C15.

(B) (over) Cross-peaks were observed between the Lys$_1$ and Lys$_2$ $^{13}$C carbonyl resonances of the carboxylic acids and the $\alpha$-CH groups.

(C) (over) The $^{13}$C resonance of C13, to which H$_b$ is attached, was assigned from a cross-peak to Gly$_1$, $\alpha$-NH.

![Diagram of HMBC spectrum](image)

- H$_a$ \(\rightarrow\) C$_4$
- Lys$_1$ $\alpha$-NH \(\rightarrow\) Gly$_1$, C=O
- Lys$_1$ $\alpha$-NH \(\rightarrow\) C$_{15}$
Chapter 3  Reaction of 3-Hydroxykynurenine with the Dipeptide Glycyllysine
Thus it appeared that two GK moieties had been incorporated into the structure with a second glycine residue either being cleaved, or involved in the formation of a ring. The former process however was unlikely due to the preserved mass of the reactants.

The $^1$H NMR spectrum was assigned via a combination of through bond (DQF-COSY and TOCSY) and through-space (NOESY) spectra. Further insight into the structure of QBA (Fig. 3.7) was obtained from the NOESY spectrum, the NH and aromatic region of which is depicted in figure 3.13. An nOe from Gly$_1$ NH to Lys$_1$ NH was present, indicating that these two residues were linked. A strong nOe from Lys$_1$ NH to Gly$_1$ α-CH$_2$ was also observed (Fig 3.12) which confirmed this conclusion. The downfield aromatic proton (H$_A$) had no cross-peaks arising from it, suggesting that it was isolated. Its reduced intensity compared to H$_B$ in the 1D $^1$H NMR spectrum (Fig. 3.10) implies that H$_A$ has a longer $T_1$ value than H$_B$, which is also consistent with it not having many protons nearby.

In contrast to H$_A$, H$_B$ had three correlations in the NOESY spectrum; to α-CH$_2$ (Fig 3.12), and Gly$_1$ NH and the Lys$_1$ α-NH (Fig. 3.13). This suggested that only one aromatic proton i.e., H$_B$ remained on the 3OHKyn ring after reaction, and that a GK dipeptide had attached para to the aromatic amino group of 3OHKyn. The observed nOe correlations are summarised in figure 3.14a.

The protonated $^{13}$C resonances were assigned from an HSQC spectrum (Figs. 3.15) and are presented in Table 3.1 along with the $^1$H assignments. The majority of the non-protonated $^{13}$C resonances were assigned from HMBC spectra (Figs. 3.16). The observed HMBC correlations are presented in figure 3.14b. Based on the lack of nOes from H$_A$, cyclisation to a XA-like structure was most likely. Thus, a standard sample of XA (Fig. 3.1) was prepared and HSQC, HMBC and $^{13}$C 1D NMR spectra were acquired to assist with the assignments of the non-protonated carbons of QBA (i.e. C4, C5, C6, C11, C12 and C14).
Table 3.1: Summary of $^1$H assignments, and the protonated and non-protonated $^{13}$C resonances assigned for the major reaction product, QBA, from a combination of HSQC and HMBC experiments.

### $^1$H assignments

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### $^{13}$C assignments

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* Not observed, possibly due to broadening associated with resonance stabilisation (see text)
Cross-peaks from the aromatic protons in the HMBC spectrum of the reaction product (QBA) and XA are shown in figures 3.17a and 3.17b. In combination with the HSQC spectrum (Fig. 3.15), a definitive assignment of all the carbon resonances of XA was determined. Interestingly, the pattern of the C2-C6 ring spin system in the HMBC spectrum of XA revealed a correlation in which cross-peaks across three bonds were stronger than those across two bonds. This characteristic was used to assign the $^{13}$C shifts of C4 and C11-14 in QBA. The chemical shift for C5 was assigned to a $^{13}$C resonance at 129.0 ppm by analogy with the XA HMBC results, as no cross-peak was observed to it from HA or HB in the HMBC spectra.

In order to propose a structure for the cyclic N-terminal attachment of Gly$_2$ at C4 and C14, we referred to the empirical formula, C$_{26}$H$_{33}$N$_7$O$_{10}$ from the high resolution MS experiment. Based on the number of nitrogens in the formula, the most likely structure was deemed to be a heterocyclic benzoxazole ring. The comparable $^{13}$C chemical shifts of C4 and C14 assigned for 2-methylbenzoxazole (Fig. 3.18), 145.1 and 154.3 ppm are in close agreement with those assigned from the HMBC spectrum of QBA, thus supporting the structure shown in figure 3.1.

Interestingly, no $^{13}$C resonance was observed for C2 of QBA. The molar concentration of QBA was approximately 37% compared with the standard of 2-methylbenzoxazole. The intensities of the quaternary carbons in figure 3.18 are weak, thus it was assumed that the corresponding carbon resonances for QBA were within the noise of the spectrum (not shown). In particular, C2 in QBA has no protons nearby, which would lead to a very long $T_1$ value for this resonance and hence a weak signal. Likewise, cross-peaks to C2 in the HMBC spectrum of QBA were unlikely to be observed due to the absence of neighbouring protons.
Figure 3.17: HMBC spectra of the $^1$H-$^{13}$C cross-peaks from the aromatic and NH regions of (A) QBA, and (B) XA. In both spectra the ortho and meta carbon cross-peaks to the equivalent aromatic protons in each molecule ($H_B$ and $H_C$) have been labelled. It is clear that in both spectra, the cross-peaks across three bonds (meta) are stronger than those across two (ortho). This pattern was most readily apparent for XA, and by analogy was used to assign the $^{13}$C shifts for C4, C11, C12 and C14 in the spectrum of QBA.
Figure 3.18: $^{13}$C spectrum of a standard sample of 2-methylbenzoxazole. (Fig. 3.1), showing the comparable shifts of C4 and C9, correlating to C4 and C14 of QBA. This spectrum exhibited a peak at 167.8 ppm, which was assigned to C2. This resonance was not observed for QBA (see text).
3.3.4 **HPLC-MS of the total reaction mixture**

In an attempt to identify some of the minor compounds generated by the reaction of GK with 3OHKyn, inline HPLC-MS was employed. A sample of the reaction mixture was separated by reverse-phase HPLC, the eluant of which was introduced directly into the source of the mass spectrometer. Figure 3.19 represents the portion of the chromatogram acquired between 10 and 20 minutes across a scan window of 200 to 700 m/z. The lower profile, (a), is the total ion chromatogram for the separation, and shows that a number of products within the scan window were present in the reaction mixture, however a particularly prominent peak was observed at 15.15 minutes. This peak had a very strong absorbance at 360 nm, as indicated in chromatogram (b), and corresponds to the elution of QBA. Note the lag time between the UV-detector and the mass analyser (14.88 min. cf. 15.15 min.). Chromatogram (c) is a single ion chromatogram scanning for m/z 604.5 only. The peak at 15.15 minutes confirms that this is indeed QBA.

The doublet of peaks at 13.90 and 14.09 minutes (a) gave rise to a similar doublet in the UV-profile (b). The combined spectra within this doublet exhibited a major peak at m/z 406.4. Chromatogram (d) represents the single ion chromatogram for this mass and again a doublet peak shape was observed. As 3OHKyn is a racemic mixture and only the L-lysine isomer was used in the reaction, this doublet probably was due to lysine binding to both stereoisomers of 3OHKyn. This implies that, unlike in QBA, the sidechain of 3OHKyn was not cyclised in this reaction product. Furthermore, the mass of 406.4 suggests that only one GK dipeptide was involved in the formation of this product.

Other minor coloured products were observed with m/z values of 243, 358 and 418, none of which exhibited stereoisomeric characteristics in the chromatogram. Their identities were not investigated further.
Figure 3.19: Chromatograms acquired during the inline HPLC-MS separation of the GK / 3OHKyn reaction mixture. (a) Total ion chromatogram for the m/z range 200 - 700. (b) UV-absorbance profile monitored at 365 nm. (c) Single ion-monitoring chromatogram for m/z 604.5. (d) Single ion-monitoring chromatogram for m/z 406.4.
3.4 Discussion

3OHKyn has been shown to be a powerful antioxidant, which may be utilised as a local defence against oxidative stress in inflammatory diseases through the up-regulation of indoleamine 2,3-dioxygenase. This antioxidative property, however, means that 3OHKyn itself is irreversibly oxidised to products which, it has been shown, bind readily to proteins. Furthermore, a by-product of 3OHKyn oxidation, hydrogen peroxide, is thought to be responsible for neuronal cell death observed in a number of neurodegenerative disorders.

It was, however, an interest in the intracellular damage caused by direct covalent modification of proteins, by the oxidation products of 3OHKyn, which formed the basis of this study. At present we have no way of detecting such post-translational modifications, and since tryptophan metabolites have been suspected of being cataractogenic, the role of 3OHKyn in the lens is of particular interest. 3OHKyn has been shown to bind to lens crystallins to produce coloured proteins similar to those observed in nuclear cataract. Thus, to determine if 3OHKyn is involved in this process, biochemical markers are required. This study involved the reaction of the dipeptide GK with 3OHKyn to produce a covalent adduct, and consequently, is the first identification of such a bio-marker. It was found that a product absorbing strongly at 365 nm, which also exhibited fluorescence, was formed during oxidative reaction of GK and 3OHKyn. The yield of this compound was 18% relative to 3OHKyn.

Mass spectrometry and NMR spectroscopy were used to elucidate the structure of a novel polypeptide crosslink incorporating 3OHKyn. The highly modified product, which was named quinilinobenzoxamine (QBA), contained two GK moieties. This compound was a product of the in vitro reaction between the α-amino groups of GK and 3OHKyn. Under neutral conditions the reactivity of α-amino groups is greater...
than that of ε-amino groups due to the latter's higher pKa values, and this may explain the lack of apparent involvement of the lysine sidechain in the adduct. Any product which results from Lys ε-NH₂ addition may also be less stable.

In the presence of excess peptide substrate, 3OHKyn will react with peptides as well as autoxidise to xanthomattin and DHQCA (Fig. 1.5). Several pathways for the formation of QBA are possible, one of which is depicted in figure 3.20. Initially, 3OHKyn 1 is oxidised to the corresponding o-quinonimine 2, which is susceptible to a nucleophilic Michael addition by the glycine α-amino group. The resultant iminophenol 3 is attacked by a second glycine α-amino group to form a Schiff base intermediate 4. The vinyl form of 4 once again allows for an intramolecular Michael addition which, via a keto-enol tautomerisation (not shown), followed by oxidation, gives a substituted quinoline carboxylic acid 5. Compound 5 is further oxidised to a Schiff intermediate, which is subject to an intramolecular nucleophilic attack on the imine by the aromatic hydroxyl group, resulting in ring closure and formation of the benzoazole moiety, which upon oxidation yields QBA 6.

The reactivity of oxidised 3OHKyn towards amino acid functional groups is similar to that of DOPA, catecholamines, catechol and other aminophenols. Hegedus and Nayak (1994) found that when these compounds were incubated with human plasma samples, melanins of identical fluorescence characteristics to those found in vivo, were produced in vitro 155. The fluorescence spectrum of QBA revealed a distinct ex/em which lay within the broad bands described by many researchers as being characteristic of the aging human lens 60, 145, 156-158. This fluorescence has been shown to increase with age, and is accompanied by a concomitant decrease in lens transmittance 159 which, given time, leads to the development of senile nuclear cataract.
Figure 3.20: Possible mechanism of reaction of 3OHKyn with the dipeptide GK, leading to the formation of the product QBA (6).
Spector, et al., detected blue fluorescence in the soluble and insoluble fractions of older human lenses, which resulted exclusively from a 43 000 dalton polypeptide, and suggested that it consisted of two crystallin subunits which had been cross-linked. This protein was shown to possess no N-terminal amino groups, and hydrolysis yielded a fluorescent, low molecular weight compound. Interestingly, Casey, et al., found that the most susceptible site for glycation of γB-crystallin was the N-terminal α-amino group, which played an important role in the glycation-mediated cross-linking of this protein.

In conclusion, the results presented in this chapter describe the structure of an oxidatively generated cross-linked product between 3OHKyn and the N-termini of two GK dipeptides. Evidence from an inline HPLC-MS experiment suggested that was the major product of the reaction, however a number of minor products, including a single GK/3OHKyn adduct were observed in the chromatogram. Chapter 5 will present an investigation into the relationship between QBA and the reactivity of the γ-crystallin N-termini, to determine if 3OHKyn is responsible for the cross-linking of these crystallins within the lens.
CHAPTER 4

Reaction of 3-Hydroxykynurenine with the Dipeptide Glycylglycine

4.1 Introduction

In the previous chapter it was demonstrated that reactive quinonimine oxidation products of 3-hydroxykynurenine can bind to protein amino groups, and further, that under oxidative conditions, 3-hydroxykynurenine can function to cross-link polypeptide chains. Mass spectrometry and NMR spectroscopy were used to elucidate the structure of a cross-linked product using the dipeptide glycyllysine. The structure was found to possess a benzoxazole moiety which linked two GK groups and was named quinilinobenzoxamine (QBA). The crosslink, which was both coloured and fluorescent, involved the peptide α-amino groups reflecting the fact that under neutral conditions the reactivity of the α-amino group is greater than that of the ε-amino group of lysine due to the latter's higher pKa value.

Further consideration of the presence of ε-amino groups led to the postulate that the charged hydrophilic sidechain of lysine may have influenced the mechanism of formation of QBA. The observation of a restrained lysine sidechain in the NMR data of QBA, which suggested a restricted conformation, did indeed implicate these charged groups in the reaction / stabilisation process.

In this chapter, the dipeptide glycylglycine (GG) was incubated under the same conditions as those described in Chapter 3 to ascertain if the ε-amino group of GK played a significant role in the formation of a product involving the α-amino group of glycine. The products were isolated by HPLC and the structure of the major product was elucidated by a combination of NMR spectroscopy and mass spectrometry.
4.2 Methods

Refer to section 3.2 for a description of the conditions under which GG and 3OHKyn were oxidatively reacted, and the methods used for subsequent structural analysis of the isolated products.

Additional chemicals, glycylglycine and 2-hydroxybenzimidazole were obtained from Sigma Aldrich.

4.3 Results

The analytical HPLC chromatogram for the GG / 3OHKyn reaction mixture is shown in figure 4.1. The 365 nm absorbance profile (Fig. 4.1a) exhibited three large coloured peaks labelled P1 – P3. Of these peaks, only P2 and P3 were matched by corresponding fluorescent peaks (Fig. 4.1b). By comparison with the retention time of a standard on HPLC, and the ESI-MS of 3OHKyn, P1 was shown to be unreacted 3OHKyn. Of peaks P2 and P3, P2 gave rise to a much larger peak when monitored at 229 nm, (chromatogram not shown) implying that the amount of product eluting was substantially greater than that of P3. Fractions corresponding to the aforementioned peaks were collected for initial analysis by ESI-MS.

The mass spectra for the collected fractions P2 and P3 are shown in figures 4.2a and 4.2b respectively. The collected peak P2 had a \( m/z \) 335.2, while the molecular ion of P3 was observed at \( m/z \) 462.1. These masses, along with the peaks being fluorescent, suggested that adducts had formed between an indeterminate number of 3OHKyn and GG moieties.
Figure 4.1  UV and fluorescence elution profiles of the GG / 3OHKyn reaction mixture following 96 hours incubation at 37 °C. (a) Absorbance at 365 nm, (b) fluorescence at ex 390 nm / em 490 nm. The labels refer to 3OHKyn (P1), 335 m/z product (P2), and the 462 m/z product (P3).
Figure 4.2  Mass spectra of the two major coloured products collected from the reaction between GG and 3OHKyn, labelled in figure 4.1. (a) Mass spectrum of fraction P2, revealing a singly charged ion at m/z 335.2, and (b) spectrum of fraction P3, revealing a singly charged ion at m/z 462.1.
4.3.1 Preparative HPLC Purification of the Major Peak, P2

In order to collect sufficient material for structural analysis by NMR spectroscopy, semi-preparative HPLC was carried out on the remaining reaction mixture. A typical chromatogram is shown in figures 4.3a and 4.3b. In these figures, the doublet peak eluting at 4 to 6 minutes was attributed to buffer salts, unreacted GG and 3OHKyn autoxidation products. The major 229 nm absorbing peak at 14 minutes was determined to be P2, (m/z 335), and was collected over multiple runs and pooled for NMR spectroscopic analysis. A total of 3.9 mg of product was collected.

The major 365 nm absorbing peak at 24 min correlated to P3, (m/z 462), from the analytical HPLC chromatogram. An insufficient quantity of this product was collected from the semi-preparative runs to enable NMR analysis to be carried out, however further mass spectrometric analysis was possible. The smaller coloured peak at 17.5 minutes, designated by an arrow (Fig. 4.3) was found to have a m/z of 287, and was also collected for subsequent tandem mass spectrometry.

4.3.2 NMR Spectroscopy of the Major Reaction Product P2

P2, (m/z 335), obtained from semi-preparative HPLC was dissolved in 700 µl of DMSO-d6 to a concentration of approximately 13 mM for NMR spectroscopic characterisation.

The 1D ^1H NMR spectrum (Fig 4.4) has three aromatic protons, two doublets at 7.50 and 7.85 ppm, and one triplet (doublet of doublets) at 7.80 ppm. This pattern of resonances is consistent with a 3OHKyn-like ring configuration (Fig 4.5), and indicated that no addition reaction had occurred at the aromatic proton positions of 3OHKyn. The single α-NH resonance at 9.80 ppm had a triplet appearance (doublet of doublets) suggesting that it arose from a single glycine residue. Thus, it appeared
Figure 4.3  UV-absorbance traces of the C8 semi-preparative HPLC purification of the GG / 3OHKyn reaction mixture with detection at (a) 365 nm and (b) 229 nm. 250 μl of reaction mixture was loaded per run and eluted at 4.0 ml min⁻¹ on a 1% acetonitrile per minute gradient. P2 was collected and pooled for subsequent NMR spectroscopic analysis.
Figure 4.4: 1D $^1$H NMR spectrum of the major reaction product P2. Two doublet aromatic protons, $H_A$ and $H_C$, are shown at 7.85 and 7.50 ppm respectively. One triplet proton was also observed at 7.80 ppm. A single $\alpha$-NH proton, triplet in nature, is indicated at 9.80 ppm corresponding to one Gly residue. An aliphatic spin system is observable around 4 ppm, downfield of the large water peak. Some impurities present in the sample gave rise to peaks upfield of the DMSO peak at 2.6 ppm.
that a reaction had occurred between the GG dipeptide and 3OHKyn, however as was seen with the quinolinobenzoxamine product in chapter 3, only one glycine α-NH resonance was observed.

The mass of this molecule suggested that only one GG dipeptide was involved in the reaction, and once again, ring formation involving the N-terminal glycine residue had possibly occurred. Unlike QBA, the CH-CH$_2$ sidechain resonances of 3OHKyn are clearly observed in the 1D $^1$H NMR spectrum at around 4 ppm, indicating that cyclisation of this aliphatic chain had, in this case, not occurred.

![Structures of 3OHKyn, 2-hydroxybenzimidazole, and the proposed structure of the major product (P2), isolated from the GG / 3OHKyn oxidation reaction mixture. These compounds are referred to in the subsequent NMR section 4.3.2.](image)

**Figure 4.5:** Structures of 3OHKyn, 2-hydroxybenzimidazole, and the proposed structure of the major product (P2), isolated from the GG / 3OHKyn oxidation reaction mixture. These compounds are referred to in the subsequent NMR section 4.3.2.
The TOCSY spectrum (Fig. 4.6) shows a cross-peak from the glycine α-NH at 9.80 ppm to a resonance at 4.17 ppm which corresponds to the α-CH$_2$ of this residue. Interestingly there was no evidence of a second α-CH$_2$ from another Gly, which implied that this portion of the N-terminal Gly was also involved in the reaction with 30HKyn.

Referring to the notation for 30HKyn in figure 4.5, assignments for H$_A$ and the sidechain CH$_2$ were made from the NOESY experiment shown in figure 4.7 where a cross-peak was observed between these two groups. A cross-peak was also observed between a Gly α-NH and its α-CH$_2$ reinforcing the finding from the TOCSY spectrum. The remaining proton assignments were made from the through bond DQF-COSY spectra shown in figures 4.8a & b, i.e., the aliphatic sidechain CH proton, and the aromatic protons H$_B$ and H$_C$. Table 4.1 summarises the $^1$H chemical shifts.

The protonated $^{13}$C resonances were assigned from one-bond correlations to their attached protons via an HSQC spectrum which is shown in figures 4.9a (aromatic region) and 4.9b (aliphatic region). The aliphatic region (Fig. 4.9b) contained some cross-peaks from impurities, however the assignment of the remaining protonated carbons was possible, and these assignments are summarised in table 4.1.

Non-protonated $^{13}$C resonances were assigned from the HMBC spectra shown in figures 4.10a & b. The HMBC correlations, along with the NOESY correlations, are summarised in figure 4.11. Referring to the numbering in this proposed structure, the cross-peaks from H$_B$ and H$_C$ protons to their corresponding ortho carbons revealed shifts of 128.3 (C9), 143.3 (C4) and 158.0 ppm (C5). The $^{13}$C chemical shifts for C4 and C9 suggested that they remained as quaternary carbons after oxidative reaction with GG$_{16}$. However, the absence of resonances from the N-terminal Gly α-NH$_3^+$ and CH$_2$ groups suggested that they were involved in reaction with resonances from 30HKyn. Since the NMR data showed that the aliphatic sidechain and the three aromatic protons of 30HKyn were not involved in the reaction, then the most likely site of reaction was at the C4 and/or C9 position. If this were so, and considering that both
Figure 4.6: TOCSY spectrum showing a cross-peak between the \( \alpha \)-NH and \( \alpha \)-CH\(_2\) groups of a single glycine residue at 4.17 and 9.80 ppm. The absence of a second Gly \( \alpha \)-NH to \( \alpha \)-CH\(_2\) cross-peak suggested that the amino terminus of the GG dipeptide was involved in an oxidative reaction with 3OHKyn.
Figure 4.7: NOEs from the aromatic and NH protons in the 2D $^1$H NOESY spectrum of P2. An nOe from H$_a$ to an aliphatic CH$_3$ group indicated that attachment of the sidechain of 3OHKyn (see Fig. 4.5) remained unaffected by the reaction with GG. An nOe from a Gly $\alpha$-NH to its $\alpha$-CH$_3$ confirmed the observation from the TOCSY spectrum that only one Gly residue maintained its integrity after reaction with 3OHKyn.
Figure 4.8a: Aliphatic region of the 2D $^1$H DQF-COSY spectrum of P2. This spectrum indicates that a spin-system between neighbouring CH$_2$-CH groups exists. The non-equivalent protons of the sidechain CH$_3$ are indicated at 3.73 and 4.03 ppm. The sidechain CH can be seen at 4.10 ppm.
Figure 4.8b: Aromatic region of the 2D $^1$H DQF-COSY spectrum of P2.
The complex of cross-peaks around 7.80 ppm indicates that none of the protons are isolated on the ring, as observed in 3OHKyn (see Fig. 4.5).
Doublets at 7.85 and 7.50 ppm correspond to protons $H_A$ and $H_C$, while the multiplet at 7.80 arises from the central $H_B$ proton.
Figure 4.9a: Aromatic region of the 2D $^1$H-$^{13}$C HSQC spectrum of P2. The $^1$H shifts from the DQF-COSY spectrum permitted a straightforward assignment of carbons C6, C7 and C8 (Fig. 4.5), corresponding to protons $H_A$, $H_B$ and $H_C$. 
Figure 4.9b: Aliphatic region of the 2D 1H-13C HSQC spectrum of P2. Cross-peaks giving the 13C shifts for the aliphatic CH and CH$_2$ as well as the Gly α-CH$_2$ are indicated. The cross-peak at 2.6 and 43.5 ppm is from the DMSO solvent.
Figure 4.10a: HMBC spectrum of the \(^1\)H-\(^{13}\)C cross-peaks from the aliphatic region of P2. Referring to the labelling in figure 4.5 for P2, cross-peaks were observed for the sidechain carboxylic acid (COOH) from the CH$_3$ and CH groups at 177 ppm. No cross-peak to CO$_\alpha$ was observed. Cross-peaks from the Gly $\alpha$-CH$_2$ to COOH$_\alpha$ and CO$_\beta$ were observed at 175 ppm and 167 ppm respectively.
Figure 4.10b: $^1$H-$^{13}$C cross-peaks from the aromatic region of P2 in the HMBC spectrum. Cross-peaks were observed across three bonds from each aromatic proton to their corresponding meta carbons. This pattern of correlations was used to assign $^{13}$C shifts for the quaternary carbons C4, C5 and C9 and is summarised in table 4.1.
Table 4.1 Summary of $^1$H and $^{13}$C Shifts for the Reaction Product P2

### $^1$H ASSIGNMENTS

<table>
<thead>
<tr>
<th></th>
<th>$\alpha$-NH</th>
<th>$\alpha$-CH$_2$</th>
<th>CH$_2$</th>
<th>CH</th>
<th>HA</th>
<th>HB</th>
<th>HC</th>
</tr>
</thead>
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<tr>
<td>Gly1</td>
<td>9.80</td>
<td>4.17</td>
<td>3.73,</td>
<td>4.10</td>
<td>7.85</td>
<td>7.80</td>
<td>7.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.03</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### $^{13}$C ASSIGNMENTS

- C2: n.o.*
- C4: 143.3
- C5: 128.3
- C6: 118.7
- C7: 134.5
- C8: 120.0
- C9: 158.0
- C=Oa: n.o.*
- C=Ob: 167.0
- COOH$_a$: 177.0
- COOH$_b$: 175.0
- CH$_2$: 38.5
- CH:  56.0
- $\alpha$-CH$_2$: 45.0

*Cross-peaks were not observed to these carbon resonances in the HMBC experiment and thus they could not be assigned.
Figure 4.11: Summary of the (a) 2D $^1$H NOESY correlations, and (b) 2D $^1$H-$^{13}$C HMBC correlations for P2.
the N-terminal Gly \( \alpha\)-NH\(_2\) and CH\(_2\) groups had reacted, then a likely product of this reaction was a five membered heteroaromatic ring structure involving a combination of amino and/or a hydroxyl group(s).

A molecular mass of 334.098380 Da obtained from the high resolution MS experiment was consistent with a structural formula \( \text{C}_{14}\text{H}_{14}\text{N}_4\text{O}_6 \). The most likely configuration based on this formula was a substituted benzimidazole molecule with the N-terminal Gly \( \alpha\)-NH\(_3^+\) and CH\(_2\) groups forming part of the imidazole ring (Fig. 4.5).

A \(^{13}\text{C}\) 1D NMR spectrum of our sample exhibited no resolved peaks due to a lack of material. The HMBC spectra revealed no cross-peak from C2 in this proposed structure, thus a standard sample of 2-hydroxybenzimidazole (Fig. 4.5) was examined to see if a C2 resonance was observable at higher concentrations. At a concentration of 230 mM in DMSO-\(d_6\), a cross-peak was observed between the imidazole NH and C2 in the HMBC spectrum at 10.67 and 159.3 ppm (Fig. 4.12). The imidazole NH resonance was not observed in the \(^1\text{H}\) NMR spectra of the unknown, P2. It must be noted that the concentration of 2-hydroxybenzimidazole was approximately 18 times that of the unknown (P2); thus, the relatively large amount of water in the unknown (Fig. 4.5) had a significant effect on the observability of this resonance. It is proposed that the proton exchange with water was responsible for the absence of an imidazole NH resonance, and thus a cross-peak between the imidazole NH and C2. Indeed, this resonance is never observed in \(^1\text{H}\) spectra of imidazole derivatives (e.g., histidine peptides) in protic solvents.\(^{162}\) Similarly, the kynurenine sidechain NH\(_3^+\) was not observed due to rapid exchange with water in the DMSO solvent.
Figure 4.12: 2D $^1$H-$^{13}$C HMBC spectrum of the NH and aromatic region of a standard sample of 2-hydroxybenzimidazole (Fig. 4.5). This spectrum revealed a cross-peak at 159.3 and 10.67 ppm corresponding to a correlation between C2 and the imidazole NH group. This cross-peak was not observed in P2.
4.3.3 Tandem Mass Spectrometry (ESI-MS/MS)

Product P2 (335 m/z)

In order to find further evidence to support the substituted benzimidazole (SB) structure (P2), proposed in section 4.3.2 and illustrated in figure 4.11, fragmentation studies were carried out on the molecule using collisionally-induced dissociation MS of the molecular ion. Figure 4.13a shows the MS/MS spectrum of the singly charged SB molecular ion (note a slight difference in the calibration of MS2 which gave a measured m/z of 335.1 compared to 335.2, as shown in figure 4.2a) showing the product ions after collision with argon.

The first major fragment appears at m/z 289.5 corresponding to a possible loss of formic acid (Fig. 4.14), consistent with the 179.1 m/z fragment in the MS/MS spectrum of 3OHKyn (Fig. 4.15). The peak at m/z 262.4 corresponds to loss of 73 mass units from the molecular ion. Similarly, in figure 4.15, a loss of 73 mass units from the 3OHKyn molecular ion gave rise to a 151.9 m/z fragment, which corresponds to a structure with a carboxymethyl group as the sidechain of 3OHKyn (Fig. 4.14). The fragment ion at m/z 74.1 supports this structure as it correlates to a loss of iminoacetic acid from the sidechain. The peak at m/z 216.3 may indicate a loss of formic acid from the Gly sidechain of the 262.4 m/z fragment.

Evidence for the presence of a glycylglycine at C2 is provided by the equivalent losses of 103 from the 289.5 and 262.4 m/z peaks (Fig. 4.14). If the N-terminal Gly α-NH₂ and CH₂ groups were involved in imidazole ring formation as proposed, a loss of the remaining portion of the glycylglycine would account for this 103 amu difference described. This portion of the GG molecule gave rise to a peak at 104.1 m/z in the MS/MS spectrum of P2 (Fig. 4.13a) Rationalisation of the smaller fragments provided no further insight into the structure. This may not be surprising considering that benzimidazoles have a high resistance to fragmentation \(^{163}\). The proposed
Figure 4.13: Tandem electrospray mass spectra of peaks collected from the semi-preparative HPLC separation of the GG/3OHKyn reaction mixture. 
a) The major reaction product P2 (m/z 335.1), b) the major 365 nm absorbing product, P3 (m/z 462.5), and c) the minor coloured peak indicated by an arrow in figure 4.3 (m/z 287.1).
Figure 4.14: Suggested identities of the major tandem mass spectral fragments (Fig. 4.13a) for the major reaction product 2-DIK. The identical loss of 103 mass units from the 289 m/z and 262 m/z ions is in accordance with cleavage of the remaining aliphatic portion of the glycyglycine molecule.
Figure 4.15: Tandem electrospray mass spectrum of 3OHKyn.
molecular ion structure for P2, represented in figure 4.14 at \( m/z \) 335 is referred to henceforth as 2-diglycylimidazolekynurenine (2-DIK).

**Product P3 (462 m/z)**

Figure 4.13b shows the MS/MS spectrum of fraction P3 from the semi-preparative HPLC chromatogram (Fig. 4.3), corresponding to a 462.1 \( m/z \) molecular ion. Interestingly this spectrum does not exhibit the 3OHKyn sidechain losses shown in figure 4.13a, possibly indicating cyclisation. The major loss of 103 amu to give 359.4 \( m/z \) suggests a GG moiety as described above. Several formic acid losses can be seen, indicating the presence of at least three carboxylic acid groups. Considering the higher mass of this product it was assumed that, as in the previous chapter, a dimeric species had formed in the reaction. A possible structure for this product is illustrated in figure 4.16a.

**287 m/z product**

The other coloured peak to be examined by MS/MS was the 287 \( m/z \) product indicated by an arrow in figure 4.3. The spectrum is shown in figure 4.13c. Once again a loss of 103 amu was observed to give the 183.9 \( m/z \) ion. The major ions at 155.9 and 129 \( m/z \) were also observed in the ESI-MS/MS spectrum of xanthurenic acid (Ch.3) as loss of CO and HCN. This suggested that the aliphatic sidechain of 3OHKyn had cyclised, however to account for the molecular ion mass, the carboxylic acid moiety must have been lost. A structure for this molecule is proposed in figure 4.16b.
Figure 4.16: Proposed structures for the reaction products described in figures 4.13b and 4.13c respectively. 

a) A dimeric molecule of $m/z$ 462.5 consisting of two GG molecules and one 3OHKyn, analogous to the GK dimer QBA described in chapter 3.

b) A benzimidazole linked GG/3OHKyn product where the aliphatic sidechain of 3OHKyn has cyclised and lost the carboxylic acid group.
Figure 4.17: Electrospray mass spectrum of P2 after 90 minute of deuterium exchange in a 1% DCl/D$_2$O solution. The peak at $m/z$ 342.4 indicates that seven protons had been substituted by deuterons, i.e., the molecule contained seven exchangeable protons.
Chapter 4 Reaction of 3-Hydroxykynurenine with the Dipeptide Glycylglycine

4.3.4 Deuterium Exchange Mass Spectrometry

In order to further examine the benzimidazole structure for the 335 m/z product, a sample of the compound was incubated in D$_2$O and the exchangeable protons replaced by deuterium atoms. The mass spectrum of the resultant product is shown in figure 4.17. After 90 minutes of exchange under acidic conditions, the molecular ion had gained 7 mass units, indicative of a molecule containing 7 exchangeable protons. Referring to the proposed structure (Fig. 4.5) this would be the case: three protons of the charged free amino group, two carboxylic acid protons, the Gly α-NH and the single proton on the imidazole ring. This result provided some evidence for the presence of an imidazole moiety in the structure.

4.3.5 Fluorescence Spectroscopy

In figure 4.1b, it was seen that, of the collected peaks, P2 had the greatest fluorescence at ex 390 / em 490 fluorescence. These wavelengths were chosen, based on the 3D fluorescence spectrum of the collected 335 m/z peak from the analytical HPLC separation, which is shown in figure 4.18a. The spectrum exhibits a single sharp peak at excitation and emission maxima of 390 nm and 490 nm respectively. It was of interest to compare this fluorescence to that of the proposed structure, 2-DIK. Thus a standard of benzimidazole was analysed and the 3D fluorescence spectrum is shown in figure 4.18b. It can be seen that benzimidazole, when prepared in the same buffer solution as our product, exhibits similar fluorescence maxima, the excitation of 390 m being identical, and only a slight upward shift in the emission maximum from 490 nm to 510 nm. As pointed out in chapter 3, kynurenine has fluorescence maxima of ex 370 / em 490 at pH 11.0, indicating that potentially, our product may contain structural similarities to both kynurenine and benzimidazole.
Chapter 4  Reaction of 3-Hydroxykynurenine with the Dipeptide Glycylglycine

Figure 4.18  3D Fluorescence spectra of a) P2, the proposed substituted benzimidazole, and b) a standard sample of benzimidazole. Samples were prepared in 100 mM phosphate buffer, pH 7.0, at a concentration of approximately 10 μM.

Figure 4.19  UV-Visible spectrum of P2.
Figure 4.19 portrays the UV-vis spectrum of 2-DIK. A single peak of maximum absorbance at 335 nm is the dominant feature of this spectrum, which is in contrast to the spectrum of QBA in chapter 3, which exhibited two absorption maxima at 245 nm and 367 nm. This difference in absorbance characteristics suggested that the cross-linking moiety for 2-DIK was not the same as that elucidated for QBA, which supports a benzimidazole rather than benzoxazole-based structure.
4.4 Discussion

When the dipeptide glycylglycine was reacted oxidatively at neutral pH with the tryptophan metabolite, 30HKyn, a number of coloured and fluorescent products were observed in the HPLC elution profiles. The masses of the three major products (as judged by UV-absorbance at 229 nm and 365 nm) were determined by electrospray mass spectrometry to be 335, 462 and 287 m/z. The major product at 335 m/z was collected by semi-preparative HPLC to give a yield of approximately 26% relative to 3OHKyn.

Using a combination of mass spectrometry and NMR spectroscopy, sufficient information was obtained to permit an insight into the nature of the structure. From the 1D ¹H NMR spectrum it was clear that the aliphatic sidechain of 3OHKyn remained unreacted, and the three existing aromatic protons of 3OHKyn were present. Reaction therefore appeared to have occurred through the amino and/or phenol ring substituents. The inclusion of glycylglycine in the reaction product was determined from distinct α-NH and α-CH₂ resonances in the 1D ¹H spectrum. The absence of second α-NH and α-CH₂ resonances, however, implied that the amino-terminal glycine was involved in the cross-link. It was suggested that an imidazole ring had formed, which was in agreement with the mass from the high resolution mass spectrum and the number of exchangeable protons from the deuterium exchange experiment (Fig. 4.17).

This result is in contrast to the cross-link observed in the previous chapter where a benzoxazole moiety was formed via the oxidative reaction of glycyllysine with 3OHKyn. Figure 4.20 represents a possible mechanism for the formation of the imidazole-based product. The preferential formation of a benzimidazole cross-link (rather than benzoxazole) may result from the initial nucleophilic attachment of the α-amino group of GG at the carbonyl of oxidised 3OHKyn. In chapter 3 it was proposed that the aromatic imine was substituted to form a Schiff base intermediate after a Michael addition of GK at H₈ of 3OHKyn. It may be that it is sterically more
Figure 4.20  Proposed mechanism for the formation of 2-DIK from the oxidative reaction of 3OHKyn and GG.
favourable for GK to add in at H₈, due to the presence of the Lys sidechain, which then predisposes the intermediate to Schiff base formation at the aromatic amino position.

Conversely, the relatively small size of GG may experience no steric hindrance near the oxidised amino/phenol groups of 3OHKyn 2, thus, a nucleophilic attack of the α-NH of GG at the aromatic carbonyl is proposed. A loss of water to give 3 and subsequent tautomerisation results in the ortho-substituted glycylglycinekynurenine 4. Further oxidation of 4 to a Schiff intermediate permits a nucleophilic attack of the aromatic amine at the α-CH group of glycine resulting in ring closure. Further oxidation of this intermediate leads to the formation of a substituted benzimidazole, the proposed reaction product between 3OHKyn and glycylglycine 5.

It was observed in chapter 3 that the major product from the reaction of glycyllysine (GK) and 3OHKyn was QBA, a dimer incorporating two GK dipeptides. A monomeric substituted benzimidazole species (m/z 406), homologous in mass to 2-DIK, was observed in this reaction, however the proportion of this species to the dimer was only 18%, calculated from LC-MS single ion-monitoring peak areas.

Conversely, the major reaction product between GG and 3OHKyn (2-DIK) was shown to be monomeric, i.e., only one dipeptide was incorporated. Two less abundant coloured products were isolated by HPLC, one of which, P3, was shown to have a mass of 462 m/z and corresponded to a structure homologous to the benzoxazole, QBA, only with GG rather than GK as the cross-linked peptide. Thus the proportion of monomer (benzimidazole based) to dimer (benzoxazole based) appears to be dependent upon the type of peptide(s) involved in the oxidation reaction.

As explained above, the presence of a lysine in the dipeptide may be responsible for the preferential production of a benzoxazole cross-link due to steric hindrance against initial reaction near the oxidised amino / phenol groups. GG would experience no such steric inhibition, thus the initial reaction sites of GG at the aromatic carbonyl, and GK at H₈ may dictate the type of product obtained; an addition at H₈ giving rise to a dimeric benzoxazole, and a substitution at the hydroxyl position yielding a monomeric product.
CHAPTER 5

Quinilinobenzoxamine and γ-Crystallin: Evidence Towards a Possible Lens Protein-Benzoxazole Cross-link

5.1 Introduction

Both the human and bovine γ-crystallin families contain identical N-terminal dipeptides; namely, glycyllysine. As was observed in chapter three, 3OHKyn, when exposed to oxygen, acts to cross-link two GK moieties resulting in a structure which was named quinilinobenzoxamine (QBA). This molecule was both coloured and fluorescent, and involved the glycine α-amino groups.

Other studies have shown that under oxidative and photolytic conditions 3OHKyn reacts with the free amine functionality of various compounds, which leads to the production of mixed fluorophores. These products however, have not been characterised. The γ-crystallins are the only family of lens structural proteins with free α-amino groups. As such, it would be expected that γ-crystallin subunits can react with 3OHKyn under oxidative conditions to produce a cross-linked product containing a benzoxazole moiety.

Senile nuclear cataract is characterised by the formation of high molecular weight, coloured proteins which produce opacity and subsequent light-scattering, leading to a decrease in the transmission of light. These aggregates are formed by post-translational modification of pre-existing polypeptides, and have been shown by SDS-PAGE analysis to include cross-linked crystallin subunits.

In the studies presented in this chapter, total γ-crystallin isolated from bovine lenses was reacted with 3OHKyn under the same conditions as those described for GK in chapter three. From this reaction mixture it was hypothesised that a cross-link of two γ-crystallin subunits may be formed through their free N-terminal α-amino groups,
and thus be evident in a tryptic digest of the reaction mixture. Trypsin is known to cleave on the carboxyl side of lysine and arginine residues, which represent residues 2 and 9 in the γ-crystallins. A synthetic nonapeptide representing the N-terminus of all bovine γ-crystallins was also reacted with 3OHKyn. This was employed to assist in the elucidation of the reaction and digestion mechanism, in the event that trypsin was unable to cleave at the Lys2 residues. Thus it was the aim of this chapter to search for evidence of QBA (or an analogous product of the nonapeptide reaction mixture) in the products isolated from the oxidative reaction of 3OHKyn with total γ-crystallin.

5.2 Methods

3OHKyn and trypsin (TPCK treated) were purchased from Sigma (St. Louis, MO, USA). The synthetic γ-crystallin N-terminal nonapeptide, H-GKITFYEDR-OH, was obtained from Chiron Technologies Pty. Ltd., Clayton, Victoria, Australia. All other chemicals were of analytical quality.

Lyophilised total calf lens protein was obtained as previously described in section 2.2.1. The crystallin families were separated on a Sephacryl S-300 size exclusion chromatography column (1200 x 25 mm) in 100 mM phosphate buffer, 0.02% NaN₃, pH 7.0. The γ-crystallin fractions were collected, dialysed and lyophilised, then frozen at -20 °C until required.

3OHKyn (2 mg ml⁻¹) was reacted with γ-crystallin (20 mg ml⁻¹) or nonapeptide (20 mg ml⁻¹) in 1.5 ml solutions of 100 mM phosphate buffer pH 7.0. A gentle stream of humidified oxygen was bubbled into the solutions to provide mixing and constant saturation with oxygen. The reaction mixtures were held at 37 °C for 96 hours and 20 μl daily additions of n-octanol were made to prevent frothing of the solutions.

The γ-crystallin reaction mixture was separated on a Pharmacia Superdex 75 size exclusion column (600 x 16 mm) with a 200 mM NH₃HCO₃ buffer, pH 8.1 as the
eluant. Data collection, pumping and fraction collection were controlled by a BioRad, BioLogic FPLC system. Flow rate was 1.0 ml min⁻¹, and 2 ml fraction were collected throughout the run.

The isolated γ-crystallin / 3OHKyn reaction products were lyophilised, then made up to a concentration of 10 mg ml⁻¹ in 200 mM NH₄HCO₃, 0.1 mM CaCl₂ buffer, pH 8.1. The solutions were heated to 95 °C for 5 minutes to completely denature the protein, prior to digestion for 24 hours with trypsin (1:50 w/w). The digest mixtures were frozen at -20 °C prior to analysis by inline HPLC mass spectrometry analysis (LC-MS). The conditions and methods for LC-MS were the same as those described in section 3.2.2.

Prior to tryptic digest, a small portion of the lyophilised product was taken for analysis by SDS-PAGE and mass spectrometry. SDS-PAGE was performed on 15% homogeneous gels according to the method of Laemmli and the gels were scanned with a BioRad densitometer. Fluorescence and UV-spectra were acquired as described in section 3.2.1.

5.3 Results

The γ-crystallin family of lens proteins are monomeric and have an average molecular weight of approximately 20 kD. As such, the γ-crystallin subunits electrophorese as a broad single band on SDS-PAGE gels, when separated from the α- and β-crystallin families (Fig. 2.2). With such a homogenous mass range, the formation of any dimerised subunits is easily detected by the appearance of a band at ~40 kDa, indicating a product of greater molecular mass. After 96 hours of oxidative reaction, the 3OHKyn / γ-crystallin mixture was initially examined by SDS-PAGE (Fig. 5.1). Lane 1 shows a control sample of γ-crystallin, with an average mass of approximately 20 kDa, oxidised in the absence of 3OHKyn. Lanes 2-4 show
decreasing loads of the γ-crystallin / 3OHKyn reaction mixture. In these lanes, it is evident that a species of molecular weight greater than 40 kDa has been produced, when compared to the standard band of ovalbumin (45 kDa) on the left side of the gel. The presence of this band suggested that a dimerisation had occurred between two γ-crystallin subunits, possibly through an oxidation product of 3OHKyn.

**Figure 5.1:** 15% homogeneous SDS-PAGE gel of γ-crystallin (20 mgml⁻¹) incubated for 48 hr with 3OHKyn (1 mgml⁻¹). Lane 1 is a control sample of γ-crystallin incubated in the absence of 3OHKyn, and lanes 2 - 4 are decreasing loads of the γ-crystallin / 3OHKyn mixture. With reference to the ovalbumin standard (45 kD), it is clear that a product has been formed with a molecular weight slightly less than 45 kD. This product presumably was a result of a dimerisation reaction between the γ-crystallin subunits (~ 20 kD) and at least one 3OHKyn moiety.
A Pharmacia Superdex 75 FPLC column was used to separate the 3OHKyn / γ-crystallin reaction products, as this media has an effective mass separation range of 3000 Da to 70 kDa, which encompasses the expected product masses. Figure 5.2 displays a portion of the elution profile obtained when 10 mg of γ-crystallin was loaded on to the column. Two peaks were collected from this separation, the large peak at 72 minutes, and the small peak eluting at 64 minutes. Based upon the bands observed by SDS-PAGE, it was assumed that the large peak was non-cross-linked γ-crystallin and the smaller, earlier eluting peak, was a product of greater mass, possibly a dimer of γ-crystallin subunits.

**Figure 5.2:** Superdex S-75 FPLC separation of the crude 3OHKyn / γ-crystallin reaction mixture. The peak indicated by an arrow eluted first and was thought to be a dimer of γ-crystallin subunits.
A total of three separations of these two peaks were pooled and freeze-dried for subsequent analyses. The product from the first small peak was labelled dimer, the second large peak referred to as monomer.

5.3.1 Digestion and HPLC-MS of 3OHKyn-modified γ-Crystallin

The monomeric and dimeric products were heat-denatured (Section 5.2) and digested separately with trypsin for 24 hours, after which time all of the protein had gone into solution. The resultant digest mixtures were separated using inline HPLC-MS. Figure 5.3 represents the total ion current (TIC) chromatogram for the tryptic digest of the γ-crystallin dimer. The complexity of the chromatogram is indicative of a digest mixture and numerous sequence-predicted tryptic fragments were identified.

![TIC chromatogram for the HPLC separation of the tryptic digest products of the proposed γ-crystallin / 3OHKyn dimer. Some of the matching fragment sequences are indicated.](image)

**Figure 5.3:** TIC chromatogram for the HPLC separation of the tryptic digest products of the proposed γ-crystallin / 3OHKyn dimer. Some of the matching fragment sequences are indicated.
Chapter 5  Quinilinobenzoxamine and γ-Crystallin

If dimerisation had occurred through a benzoxazole linkage of the N-terminal amino groups, then it would be expected that a QBA-like moiety would be present in the mixture. Since trypsin digests preferentially on the carboxyl side of lysine and arginine residues, and residue two in all γ-crystallin subunits is lysine, a likely product of digestion was QBA itself. Figure 5.4a shows the selected ion chromatogram (SIC) of a standard sample of QBA, scanning for $m/z$ 604.5, the mass of QBA as described in chapter 4. A single sharp peak was observed at 12.20 minutes, representing the elution of a species of $m/z$ 604.5 at this time. Figure 5.4b represents the SIC for the same mass, in the chromatogram of the tryptic digest of the γ-crystallin / 3OHKyn reaction mixture shown in figure 5.3.

![Figure 5.4](image)

**Figure 5.4:** Single mass, total ion chromatograms scanning for 604 $m/z$ ion in (A) standard QBA sample, and (B) tryptic digest of γ-crystallin.
**Figure 5.5:** Tandem mass spectra of (A) a standard QBA sample, and (B) m/z 604.5 tryptic digest fragment of γ-crystallin eluting at 12.09 minutes in figure 5.4b.
The major peak in this chromatogram coincides with that for QBA, i.e., the retention times, given inter-run variations for the HPLC system, were the same. This result suggested then, that QBA was present in the digest of the γ-crystallin / 3OHKyn reaction mixture, possibly as a cross-linking moiety via the γ-crystallin N-termini. In order to show that this peak did indeed represent the elution of QBA, evidence was sought through tandem MS fragmentation of the respective species to compare the fragment ions contained in the summed spectra under the peaks shown for \( m/z \) 604.5. These spectra are shown in figure 5.5. Unfortunately, fragments associated with the tandem mass spectrum of QBA (Fig 5.5a) are not present in the MS/MS spectrum of the \( m/z \) 604.5 peak from the γ-crystallin dimer tryptic digest (Fig. 5.5b). This result suggested that the product eluting at 12.09 minutes in figure 5.4b, with \( m/z \) 604.5, was in fact a compound of different structure.

A survey of potential tryptic digest fragments with an uncharged mass of 603.5 +/- 0.5 Da was conducted using the BioLynx™ module of Micromass' MassLynx™ instrument software. Sequences of the five major bovine γ-crystallin subunits (γA, γB, γD, γE and γF) were downloaded from the SWISS-PROT database and theoretically 'digested' with trypsin using the BioLynx™ software. None of the predicted tryptic fragments had a mass within 0.5 Da of QBA, even when doubly and triply charged ions were taken into account. When some chymotryptic activity was attributed to the trypsin however, two identical, potential fragments were observed in the γB- and γE-crystallin subunits as shown.

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Residues</th>
<th>Sequence</th>
<th>Mass</th>
<th>Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>γB_BOVINE</td>
<td>72-76</td>
<td>(F) NDSIR (S)</td>
<td>603.30</td>
<td>-0.19</td>
</tr>
<tr>
<td>γE_BOVINE</td>
<td>72-76</td>
<td>(F) NDSIR (S)</td>
<td>603.30</td>
<td>-0.19</td>
</tr>
</tbody>
</table>
The theoretical fragments from the collision-induced dissociation of this pentapeptide are listed in table 5.1. Four fragments, marked in bold, were observed to match the MS/MS spectrum of the m/z 604.5 product presented in figure 5.5b. Although not definitive evidence that the pentapeptide of residues 72-76 was responsible for the peak at 12.09 minutes (Fig. 5.4b), this result further suggested that the two closely eluting peaks in figure 5.4 were due to different molecules.

<table>
<thead>
<tr>
<th>Table 5.1</th>
<th>Major predicted fragment ions of the peptide NDSIR</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>87.1   202.1  289.1  402.2  -</td>
</tr>
<tr>
<td>b</td>
<td>115.1  230.1  317.1  430.2  -</td>
</tr>
<tr>
<td>c</td>
<td>132.1  247.1  334.1  447.2  -</td>
</tr>
<tr>
<td>Asn</td>
<td>-      516.2  401.2  314.2  201.1</td>
</tr>
<tr>
<td>Asp</td>
<td>-      490.3  375.2  288.2  175.1</td>
</tr>
<tr>
<td>Ser</td>
<td>-      473.2  358.2  271.2  158.1</td>
</tr>
<tr>
<td>Ile</td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td></td>
</tr>
</tbody>
</table>

Confirmation of this was obtained in an experiment where the tryptic digest of the γ-crystallin dimer was spiked with pure QBA. In this experiment, QBA was found not to co-elute with the γ-crystallin dimer, m/z 604.5 product (Fig. 5.6). This was determined by looking at the single-ion chromatograms for ions distinctive to each compound which were observed in the spectra under the major peaks in figure 5.4.

Since QBA did not appear to be present in the digest, it was proposed that a possible reason for the result above, was the inability of trypsin to access the peptide bonds on the carboxyl side of Lys2 in the γ-crystallin subunits, due to steric hindrance. The N-termini of the both the human and bovine γ-crystallin families are the same up to Arg9, apart from position 5 in human γD-crystallin (table 5.2). Thus, if trypsin were
unable to digest at Lys2, the next labile bond along the peptide chain is adjacent to Arg9.

**Figure 5.6:** Chromatograms of an LC-MS separation of the γ-crystallin / 3OHKyn reaction mixture, spiked with QBA. (A) 360 nm UV-absorption profile showing a peak resulting from QBA. (B) SIC for an ion characteristic to QBA. (C) SIC for an ion characteristic to the m/z 604.5 product in figure 5.4b. (D) TIC chromatogram.
Table 5.2: N-terminal sequences of the \( \gamma \)-crystallin subunits showing homology from the N-terminal Gly to Arg9.

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Sequence at N-terminus</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \gamma_{A} _BOVINE )</td>
<td>Gly-Lys-Ile-Thr-Phe-Tyr-Glu-Asp-Arg</td>
</tr>
<tr>
<td>( \gamma_{B} _BOVINE )</td>
<td>Gly-Lys-Ile-Thr-Phe-Tyr-Glu-Asp-Arg</td>
</tr>
<tr>
<td>( \gamma_{D} _BOVINE )</td>
<td>Gly-Lys-Ile-Thr-Phe-Tyr-Glu-Asp-Arg</td>
</tr>
<tr>
<td>( \gamma_{E} _BOVINE )</td>
<td>Gly-Lys-Ile-Thr-Phe-Tyr-Glu-Asp-Arg</td>
</tr>
<tr>
<td>( \gamma_{F} _BOVINE )</td>
<td>Gly-Lys-Ile-Thr-Phe-Tyr-Glu-Asp-Arg</td>
</tr>
<tr>
<td>( \gamma_{A} _HUMAN )</td>
<td>Gly-Lys-Ile-Thr-Phe-Tyr-Glu-Asp-Arg</td>
</tr>
<tr>
<td>( \gamma_{B} _HUMAN )</td>
<td>Gly-Lys-Ile-Thr-Phe-Tyr-Glu-Asp-Arg</td>
</tr>
<tr>
<td>( \gamma_{C} _HUMAN )</td>
<td>Gly-Lys-Ile-Thr-Phe-Tyr-Glu-Asp-Arg</td>
</tr>
<tr>
<td>( \gamma_{D} _HUMAN )</td>
<td>Gly-Lys-Ile-Thr-Phe-Tyr-Glu-Asp-Arg \textbf{Leu}</td>
</tr>
</tbody>
</table>

The mass of the N-terminal nonapeptide, GKITFYEDR, is 1128.3 Da, compared with 203.2 for GK, a difference of 925.1 Da. Thus a dimer of \( \gamma \)-crystallin subunits which were cleaved at Arg9 instead of Lys2 would have a mass 2 \( \times 925.1 \) Da greater than that of QBA. Since the singly charged mass of QBA was shown to be 604.5 in chapter 4, this would result in a dimer of \( m/z \) (604.5 \( + 2 \times 925.1 \)) or, 2454.7. A peptide of this size would be expected to carry more than one charge; a list of predicted \( m/z \) ions is presented in table 5.3.
Table 5.3: Multiply-charged ion series predicted for the nonapeptide dimer linked by a benzoxazole moiety

<table>
<thead>
<tr>
<th>Number of charges</th>
<th>m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2454.5</td>
</tr>
<tr>
<td>2</td>
<td>1227.8</td>
</tr>
<tr>
<td>3</td>
<td>818.8</td>
</tr>
<tr>
<td>4</td>
<td>614.4</td>
</tr>
<tr>
<td>5</td>
<td>491.7</td>
</tr>
</tbody>
</table>

5.3.2 Reaction of the Synthetic Peptide, GKITFYEDR, with 3OHKyn

In order to determine if steric hindrance of trypsin at Lys2 was the reason that QBA was not evident in the γ-crystallin dimer digest, the N-terminal nonapeptide was purchased and reacted with 3OHKyn under the conditions described (section 5.2). After 96 hours the mixture was separated on a semi-preparative HPLC system and the coloured peaks collected for mass spectrometric analysis (Fig. 5.7). The large peak at 8 minutes was found to be unreacted 3OHKyn. The peaks of interest can be seen at 19 min, as a single sharp spike on a rising broadly eluting peak, and a triplet, indicated by an arrow, from 20.5 - 21.5 minutes. These peaks were collected for subsequent analyses. Initial analysis was carried by direct injection into the spectrometer. The spectra acquired are shown in figure 5.8.
Figure 5.7: Semi-preparative HPLC chromatograms of the nonapeptide / 3OHKyn reaction mixture. The eluant was monitored for absorbance at 365 nm. The coloured peaks eluting at 19.0 min, and between 20.5 and 21.5 minutes as indicated by black bars, were collected and pooled for subsequent mass spectrometric analysis.
Figure 5.8: Mass spectra of the coloured peaks eluting at (A) 19.0 min and, (B) between 20.5 and 21.5 minutes. It is evident from the number of peaks in each spectrum that the samples from the HPLC were not pure. Some major peaks have been marked with symbols denoting the number of charges (e.g., **** = +4) on the ion and the ion series they belong to.

Initial analysis of the HPLC samples revealed that a number of different products were within the collected peaks, as expected from the HPLC profile. The
mass spectrum of the 19 minute peak (Fig. 5.8a) contained two multiply charged ion series arising from molecular ions at \( m/z \) 1715.5(* series), and \( m/z \) 1332.0(# series). The other peaks could not be assigned to ion series, thus their identities were not pursued. The \( m/z \) 1715.5 ion may be a cleavage product of a dimerised nonapeptide, as the mass is larger than that expected from a simple combination of 30HKyn and the nonapeptide. This ion was not characterised further. The other ion noted had a calculated non-protonated mass of 1331.0 Da. This mass is 202.7 Da higher than the mass of the native nonapeptide, thus implying attachment of a 30HKyn autoxidation product to the peptide. Interestingly, it was observed in chapter 4 that the mass of the compound, 2-diglycyl-imidazolekynurenine (2-DIK) was 334.2 Da, which corresponded to an addition of 202.2 Da to the native dipeptide glycylglycine. This similarity in modification mass suggested that a covalent addition reaction had occurred at the N-terminal glycine of the nonapeptide, possibly the same as that described for the dipeptide glycylglycine in chapter 4.

Figure 5.8b depicts the mass spectrum of the HPLC triplet-like peak collected between 20.5 and 21.5 minutes (Fig 5.7). As in figure 5.8a, there appears to be a number of compounds within this sample. Once again, some multiply-charged members of ion series have been marked. Of the ions shown, only one matches those calculated in table 5.3, that being \( m/z \) 614.4 (++++). In a spectrum acquired over a larger mass range however (not shown), it was noted that an \( m/z \) 818.8 (+++ ) ion was present, which indicates that they arose from the \( m/z \) 2454.5 (+) molecular ion, the predicted nonapeptide dimer.

The major ions observed in the spectrum at \( m/z \) 564.5 and 752.0 arise from a molecular ion of \( m/z \) 2254.0. This is ~200 Da less than the mass of the predicted nonapeptide dimer, and may be due to the loss of an arginine and CO\(_2\). The ions at \( m/z \) 672.2 and 647.5 were noted to be doubly charged from analysis of their peak shape in MassLynx™.
5.3.3 Tandem Mass Spectrometry of Selected Nonapeptide \(3OHkyn\) Reaction Products

In order to confirm that the \(m/z\) 1332.0 (\#) and \(m/z\) 2454.5 (+) ions described in the previous section represent analogous molecules to those described in chapters 3 and 4, it was necessary to do fragmentation studies using tandem mass spectrometry.

**MS/MS of the \(m/z\) 666.6 ion**

An MS/MS spectrum of the \(m/z\) 666.6 ion is depicted in figure 5.9a. Based on the structure of 2-DIK, (Fig. 4.11), it is only possible for carboxy-terminal fragments to be produced in MS/MS as the N-terminus is effectively blocked by the benzimidazole moiety. A comparison of the predicted \(y''\) fragments listed in table 5.4 with the MS/MS spectrum of \(m/z\) 666.6 (Fig. 9a) reveals four matching ions, indicated in bold.

| Table 5.4: Predicted fragment ions of the unmodified peptide GKITFYEDR |
|-----------------|---|---|---|---|---|---|---|---|---|---|---|---|
|                | a | b | c | | | | | | | | | |
| Gly             | - | 58.0 | 73.0 | 1097.5 | 969.4 | 856.3 | 755.3 | 608.2 | 445.2 | 316.1 | 201.1 |
| Lys             | - | 30.0 | 201.1 | 943.5 | 830.4 | 729.3 | 582.3 | 419.2 | 290.1 | 175.1 |
| Ile             | - | 158.1 | 299.2 | 314.2 | 271.2 | 372.3 | 519.3 | 682.4 | 811.4 | 926.5 | - |
| Thr             | - | 271.2 | 400.3 | 415.3 | 372.3 | 519.3 | 682.4 | 811.4 | 926.5 | - |
| Phe             | - | 372.3 | 547.3 | 415.3 | 372.3 | 519.3 | 682.4 | 811.4 | 926.5 | - |
| Tyr             | - | 519.3 | 710.4 | 562.3 | 725.4 | 725.4 | 854.4 | 969.5 | - |
| Glu             | - | 682.4 | 839.4 | 725.4 | 725.4 | 854.4 | 969.5 | - |
| Asp             | - | 811.4 | 839.4 | 854.4 | 854.4 | 969.5 | - |
| Arg             | - | 926.5 | 954.5 | 969.5 | - | | |

- 147 -
These matching ions imply that an intact nonapeptide is part of the molecule, particularly due to the presence of a $y_7''$ ion in the spectrum.

\[
y_7''
\]

Gly - - Lys - - Ile - - Thr - - Phe - - Tyr - - Glu - - Asp - - Arg

Further evidence is provided by the doubly charged ion at $m/z$ 629.4. The corresponding molecular ion ($m/z$ 1257.8) is analogous to the $m/z$ 262 ion in the interpretation of the MS/MS of 2-DIK (Fig. 4.14), reproduced below. In the case of the nonapeptide, residues KITFYEDR have replaced the glycylglycine of 2-DIK. Therefore an additional mass of $1128.3 - 132.1 = 996.2$ would be expected. This would result in a mass of $996.2 + 262 = 1258.2$.

\[
\text{H}_3\text{C} \quad \begin{array}{c}
\text{N} \\
\text{C-C—KITFYEDR}
\end{array} \\ 1258
\]

\[
\text{H}_3\text{C} \quad \begin{array}{c}
\text{N} \\
\text{C-C—NHCH}_2\text{COOH}
\end{array} \\ 262
\]

Thus it appeared that an analogous product was isolated using a longer peptide, i.e., 9 residues cf. 2 residues. Other ions, such as those at $m/z$ 428.6 and 315.5 were attributed to cleavages on the peptide backbone closer to the imidazole ring.

**MS/MS of the $m/z$ 614.4 ion**

The MS/MS spectrum of the $m/z$ 614.4 ion from figure 5.8b, is shown in figure 5.9b. It was proposed in the previous section that this is the quadruply charged ion of a benzoxazole-linked, dimeric nonapeptide, analogous to QBA. Referring to
Figure 5.9: Tandem mass spectra of selected ions from the spectra shown in figure 5.8. (A) The doubly charged, \( m/z \) 666.6 ion, and (B) the quadruply charged, \( m/z \) 614.4 ion. These ions were selected as they were thought to be analogous to the structures elucidated in chapters 4 and 3, namely, 2-diglycylimidazole-kynurenine and quinilinobenzoxamine respectively.
table 5.4, it can be seen that the entire \( y'' \) series of ions is present. This indicates that the nonapeptide has been modified by simple addition of 3OHKyn, that is, one in which the N-terminal glycine has not been involved in ring formation. This would be the case in a simple covalent modification of the glycine NH group, as was observed in chapter 3, where a second glycyllysine was attached to the aromatic ring of the benoxazole moiety in QBA.

The most significant ion in the spectrum, which links \( m/z \) 614.4 to QBA, is that at \( m/z \) 301.2. It was observed in chapter 3 that cleavages on the carboxyl sides of both lysines gave rise to this ion which contained the core tricyclic structure of the molecule.

The lack of ions of higher mass to charge ratio is due to the fact that at such masses, multiple charges usually prevail, suggesting that peaks at \( m/z \) 670.7, 755.6 and 883.1 are actually doubly charged ions of larger mass molecular ions.

### 5.3.4 UV-Visible and Fluorescence Spectroscopy

UV-visible and fluorescence spectra were acquired on the 20.5-21.5 minute peak from the semi-preparative HPLC (Fig. 5.7), and are shown in parts A and B of figure 5.10 respectively. The UV-visible spectrum of this sample shows a maximum at 367 nm,
which is consistent with a 3OHKyn-like absorbance (\(\lambda_{\text{max}} 370\) nm) and the same as the maximum observed in the UV-spectrum of QBA (Fig. 3.3). The other maximum observed in the UV-spectrum of QBA, at 245 nm was not observed in figure 5.10a, however the greater relative proportion of low-wavelength (200 - 300 nm) absorbing species may be responsible for obscuring such a peak. This was not unexpected considering the sample was not pure.

**Figure 5.10:** UV and fluorescence spectra of the 20.5 - 21.5 min peak in figure 5.7.

(A) A maximum at 367 nm is evident. (B) The 3D fluorescence spectrum of the same sample. An excitation and emission maximum of 380 and 490 nm is indicated with an arrow.
The 3D fluorescence spectrum of the sample (Fig. 5.10b) exhibits what appears to be four emission maxima at an excitation maximum of 380 nm. Once again, the heterogeneous nature of the sample would explain the number of emission maxima. QBA (Fig. 3.3) was shown to have maxima at ex/em 390/490 nm, which correspond closely to one of those observed for the sample above, namely, ex/em 380/490, which is marked with an arrow. Together, the UV-visible and 3D fluorescence spectra suggested that a QBA-like structure was present in the mixture of compounds collected from the HPLC.

5.3.5 Searching For the Nonapeptide Dimer in a γ-Crystallin / 3OHKyn Dimer Tryptic Digest

In the previous section it was shown that a dimeric compound consisting of nonapeptides, apparently incorporating the same cross-linking moiety as QBA, was present in the nonapeptide / 3OHKyn oxidation reaction mixture. With this in mind the γ-crystallin / 3OHKyn dimer digest was examined; the rationale being that the aromatic portion of the molecule may prevent tryptic cleavage at the two lysine residues. Thus if a dimer of γ-crystallin subunits had formed, the smallest N-terminal digest product may be a dimer of nonapeptides.

The γ-crystallin / 3OHKyn dimer was separated by LC-MS as described in section 5.3.1, however the eluant was monitored for the m/z 614.4 ion only so that MS/MS fragmentation could be carried out. Figure 5.11a represent the TIC for the m/z 614.4 ion. Two peaks corresponding to the elution of species with this mass to charge ratio are evident at 17.43 and 19.77 minutes. The summed product-ion spectra underneath these peaks are shown in figures 5.11b and 5.11c respectively. When compared with the MS/MS spectrum of the nonapeptide dimer (Fig. 5.9b), it is clear that the γ" series of ions are absent from both spectra. In fact none of the ions in the
Figure 5.11: (A) Total-ion-chromatogram for the MS/MS product ions of the quadruply-charged $m/z$ 614.4 peak in the LC-MS separation of the $\gamma$-crystallin / 3OHKyn dimer tryptic digest. (B) Summed product-ion spectra under the peak at 17.43 minutes in (A). (C) Summed product-ion spectra under the peak at 19.77 minutes in (A).
spectra of the γ-crystallin dimer digest correspond to those in figure 5.9b, suggesting that the m/z 614.4 ions eluting in figure 5.11a are not products of the nonapeptide dimer of mass 2453.5. A survey of the theoretical tryptic fragments of each of the native bovine γ-crystallins indicated that spectrum 5.11c may correspond to the C-terminal pentapeptide (residues 169-173) of γF-crystallin, AVDFY.

5.3.6 Assessment of the Stability of QBA and the Specificity of Tryptic Cleavage Under Conditions used for Tryptic Digestion of the γ-Crystallin Dimer

In the preceding sections it was found that neither the synthetic dipeptide or nonapeptide dimers could be found in the digest of the γ-crystallin / 3OHKyn dimer. Two possible reasons for this were considered. One, that the trypsin possessed some chymotryptic activity and was digesting at sites other than Arg and Lys, or secondly, that the digestion conditions employed were harsh enough to affect the stability of the benzoxazole moiety. Considering that the protein was boiled in ammonium bicarbonate buffer prior to tryptic digestion, it seemed that the latter possibility may have been the case. Thus, an experiment to assess the stability of QBA under the conditions used for the digestion of the γ-crystallin / 3OHKyn dimer was carried out. QBA was dissolved in buffer and heated to 95° C for 5 minutes prior to digestion with trypsin for 24 hours. The pH of the solution was monitored and found to rise to 9.10 after heating, and to stabilise at 9.40 pH units after an hour at 37° C. LC-MS chromatograms were acquired at 0, 1 and 24 hours to assess the integrity of QBA under these conditions. No observable degradation of the sample occurred, even after 24 hours in solution at 37° C.
Since the stability of QBA appeared not to be responsible for the absence of the model dimers in the tryptic digest of the γ-crystallin / 3OHKyn dimer, then the specificity of tryptic cleavage was investigated. The mixture containing the nonapeptide dimer was digested with trypsin as per the γ-crystallin / 3OHKyn dimer to see if QBA was released from the molecule (see Fig 5.12). An ideal tryptic cleavage would be expected to result in one molecule of QBA and two ITFYEDR peptides for each nonapeptide dimer in the mixture. LC-MS was employed to monitor the digestion of the nonapeptide dimer.

The TIC and two SICs of the nonapeptide dimer mixture prior to tryptic digestion are shown in figure 5.13. The quadruply charged \( m/z \) 614.4 ion, corresponding to the dimer eluted at 17.52 minutes as shown in the SIC for this ion. Thus, as expected it was clearly present in the reaction mixture which was loaded onto the column. The top SIC for the \( m/z \) 543.7 ion was included in this figure due to the nature of the post-digest results (see Fig. 5.14).

The sample was digested for 24 hours with trypsin after which time a portion was once again separated and analysed by LC-MS, from which selected chromatograms are shown in figure 5.14. The same SICs are depicted as for the pre-digest sample. Major changes in all three profiles occurred as a result of digestion. In the TIC (lower) a large peak eluted at 18.37 minutes and the peaks which were in the
vicinity of 17 minutes (Fig 5.13) were no longer evident. In the $m/z$ 614.4 SIC (middle), no evidence of this ion remained, suggesting that digestion of the peptide chains had occurred. As noted above (Fig. 5.12), two products, namely QBA and the peptide ITFYEDR, of $m/z$ 604.5 and 943.5 respectively, would be predicted from a standard tryptic digest of the nonapeptide dimer. Only the SIC for the $m/z$ 604.5 ion (QBA) is depicted, as the scan range did not include $> m/z$ 850 (Fig. 5.14). There is evidence of this ion in the chromatogram, co-eluting with the large peak at 18.3 minutes in the TIC.

![Figure 5.13](image)

**Figure 5.13:** LC-MS chromatograms of the nonapeptide dimer mixture prior to digestion with trypsin. The quadruply charged dimer ion eluted at 17.52 minutes in the middle SIC. The SIC for $m/z$ 543.7 is shown as a comparison to the post-digest chromatogram (Fig. 5.14).
Chapter 5  Quinolinobenzoxamine and γ-Crystallin

Figure 5.14: LC-MS chromatograms of the nonapeptide dimer mixture after digestion with trypsin. The quadruply charged dimer ion is not present in the central SIC. The SIC for \( m/z \) 543.7 exhibits a marked peak at 18.37 minutes (compare figure 5.12).

From the upper SIC profile, it could be seen that this peak was due predominantly to an \( m/z \) 543.7 ion. The ion current figures on the right of the chromatograms suggest that only 1% of the area under the peak could be an ion corresponding to the mass of QBA.

The scans under the 18.3 minute peak were summed and the resultant spectrum is depicted in figure 5.15. A survey of the nonapeptide residues revealed a match between \( m/z \) 543.7 and the mass of the internal peptide chain, ITFY. Small peptides fragment easily, even at low cone voltages, thus the smaller ions in the spectrum were examined to provide some sequence information. In the spectrum it can be seen that the predicted b and y* ions for the sequence ITFY were assignable. The ion at \( m/z \) 344.3 could be assigned the tripeptide fragment KIT, residues 2-4 of the nonapeptide.
Figure 5.15: Summed spectra under the 18.37 minute peak in the TIC of figure 5.14. In-source fragmentation has resulted in the formation of product ions which were used to assign the sequence of the base peak at m/z 543.7.

5.3.7 Comparison of the Tryptic Digest Fragments from the Nonapeptide Dimer and γ-Crystallin Dimer

It was observed in the previous section, that the major product after tryptic digestion of the nonapeptide dimer was a tetrapeptide of m/z 543.7. A tryptic digest of the γ-crystallin dimer was examined for evidence of this peptide of sequence ITFY. Figure 5.16a represents the TIC (lower) and the SIC for the m/z 543.7 ion (upper), of the γ-crystallin dimer tryptic digest. The SIC of m/z 543.7 has one peak at 18.06 minutes, indicating that an ion eluted at this time. This mass is the same as that
observed for a product of the nonapeptide dimer tryptic digest, the proposed peptide, ITFY (Sec. 5.3.6).

A comparison of the spectra for the $m/z$ 543.7 ion for the nonapeptide dimer, (Fig. 5.15) and the γ-crystallin dimer (Fig. 5.17) revealed that they have four matching peaks in their common acquisition range, suggesting that they arose from the same peptide.

**Figure 5.16:** LC-MS chromatograms of the γ-crystallin / 3OHKyn dimer after digestion with trypsin. The TIC (lower) reflects a complex mixture of tryptic peptides. The SIC ($m/z$ 543.7) displays a prominent peak at 18.06 minutes.
Figure 5.17: Summed spectra under the 18.06 minute peak in the SIC of figure 5.16. Product ions matching those of the peptide in figure 5.15 suggested that the sequence of the base peak at $m/z$ 543.7 was ITFY.
5.4 Discussion

The purpose of this chapter was to search for evidence of a benzoxazole cross-link in the reaction mixture of \( \gamma \)-crystallin and 3OHKyn. In chapter three it was shown that such a cross-link occurred between two GK dipeptides, which resulted in the elucidation of the structure of QBA. A mixture of \( \gamma \)-crystallin and 3OHKyn was incubated for 96 hours, and initially analysed by SDS-PAGE, which showed that a dimer of \( \gamma \)-crystallin subunits was present in the mixture. The products of the incubation were separated by FPLC into \( \gamma \)-crystallin dimer and monomer components and digested with trypsin for subsequent LC-MS analysis.

The LC-MS chromatogram was scanned for a component of \( m/z \) 604.5, the same as that of QBA. A peak was observed in the SIC of the \( \gamma \)-crystallin / 3OHKyn dimer digest at a similar elution time as that observed for QBA (Fig. 5.4). MS/MS analysis of the ion responsible for this peak, however, revealed that the fragment ions did not match those observed in the MS/MS spectrum of QBA (Fig. 5.5). Thus it was concluded that QBA was not present in the digest of the \( \gamma \)-crystallin / 3OHKyn dimer that was isolated by FPLC. A closer analysis of the fragments in the MS/MS spectrum of the \( \gamma \)-crystallin / 3OHKyn dimer \( m/z \) 604.5 peak (Fig. 5.5b) suggested they came from a peptide fragment of sequence NDSIR. This peptide was identified as residues 72-76 in \( \gamma B \)- and \( \gamma E \)-crystallins, apparently arising due to some chymotryptic activity in the digest.

Since QBA could not be detected in the digest, it suggested that trypsin may be sterically hindered at the Lys2 residues of \( \gamma \)-crystallin. In order to model this, a synthetic peptide consisting of the nine N-terminal residues of \( \gamma \)-crystallin was used. This peptide was chosen since Arg9 is the next trypsin-labile residue in the \( \gamma \)-crystallins. The synthetic nonapeptide was incubated with 3OHKyn under the same conditions as for GK (chapter 3) and \( \gamma \)-crystallin. Mass spectral analysis of the coloured components of the reaction mixture, separated by HPLC (Fig. 5.8), revealed...
that a number of components of higher mass than the nonapeptide were present. Two ions, one doubly charged at \( m/z \) 666.6 (Fig. 5.8a), and the other quadruply charged at \( m/z \) 614.4 (Fig. 5.8b) were investigated further.

MS/MS of the \( m/z \) 666.6 ion, (Fig. 5.9a), suggested that a nonapeptide had been modified at the N-terminus by a benzimidazole moiety. Some fragment ions were assigned which were analogous to those observed for 2-DIK, the structure identified in chapter 4 as the major product arising from the reaction between the dipeptide GG and 30HKyn.

Similarly the presence of an \( m/z \) 301.2 ion in an MS/MS spectrum of the \( m/z \) 614.4 ion, (Fig. 5.9b), suggested that it arose from a structure analogous to QBA (chapter 3), i.e., two nonapeptides cross-linked through their N-terminal glycine residues via a benzoxazole moiety.

A sample of the compound eluting in the 20.5 - 21.5 minute HPLC peak (Fig. 5.7) which gave rise to the \( m/z \) 614.4 ion was taken for UV-visible and fluorescence spectroscopy (Fig. 5.10). The spectra obtained, although not from a homogeneous sample, exhibited features which were consistent with UV-visible and fluorescence spectra of QBA (Fig. 3.3).

With evidence to suggest that the synthetic nonapeptide was cross-linked to form a compound of 2453.5 mass units, the digest of the \( \gamma \)-crystallin / 30HKyn dimer was investigated for evidence of the same molecule. If \( \gamma \)-crystallin were cross-linked in the same manner as QBA, and digestion occurred at Arg9 but not Lys2, then a compound identical to the nonapeptide dimer would be expected to be in the digest. The LC-MS chromatogram was scanned for the quadruply charged \( m/z \) 614.4 ion. It revealed two distinct peaks (Fig. 5.11). MS/MS of the compounds responsible for these peaks showed that the product ions did not match those of an MS/MS of the nonapeptide dimer. In fact one of the spectra (Fig. 5.11c) matched the fragments predicted for the C-terminal pentapeptide of \( \gamma F \)-crystallin. Thus, it appeared that either
the digest conditions were destabilizing the benzoxazole moiety, or trypsin was not cleaving at the predicted sites.

In order to test the integrity of the cross-link, QBA was subjected to identical digestion conditions as the γ-crystallin / 3OHKyn dimer. Although it was observed that the pH rose to 9.40 over the period of digestion, no significant breakdown of QBA was observed, suggesting that the γ-crystallin / 3OHKyn dimer cross-link would be stable.

The tryptic specificity was assessed by digesting the nonapeptide dimer mixture, which was expected to yield only two cleavage products; QBA and the peptide ITFYEDR (Fig. 5.12). LC-MS of the digest products revealed that the major product was the tetrapeptide ITFY, corresponding to a chymotryptic fragment. No appreciable product of $m/z$ 604.5 was observed in the chromatogram (Fig. 5.14). The appearance of the ITFY fragment implied that digestion at Lys2 had occurred and that some chymotryptic activity resulted in digestion at Tyr6. The peptide, ITFY, was also present in the γ-crystallin / 3OHKyn dimer LC-MS chromatogram (Fig. 5.16).

The remaining tripeptide EDR was not observed, presumably eluting in the void volume of the LC-MS separation. The apparent absence of QBA in the products suggested that the digest had not been limited to cleavage at the Lys2 (and Tyr6) residues. Combinations of possible digest products such as those shown in figure 5.18 were considered, and the digest chromatogram was examined for multiply charged ions which fell within the acquisition mass range.

No matching ions for the products depicted above were present in the digest chromatograms. This finding suggested that the process of digestion, although not detrimental to QBA itself, may have affected the nature of the products through the mechanism of cleavage. That is, no cleavage was observed in the control digest of QBA (Sec. 5.3.6), however when longer peptides are cross-linked, trypsin is required to complex to the 'peptide arms' of the molecule to effect digestion. It is proposed that this act of complexing / digestion at Lys2 in the nonapeptide and γ-crystallin dimers,
may destabilize the benzoxazole, resulting in a structurally altered product, the characteristics of which are not known.

![Diagram of peptide sequence and mass](image)

**Figure 5.18:** Predicted digest products of the nonapeptide dimer as a result of incomplete digestion and/or chymotryptic activity of trypsin.
CHAPTER 6

A General Mechanism of Polypeptide Cross-linking by 3-Hydroxykynurenine

6.1 Introduction

3OHKyn reacts with compounds that have a free amine functionality and was shown in chapter 3 to cross-link two peptides containing an N-terminal glycine residue to form the compound, quinilinobenzoxamine (QBA). Glycine is a unique amino acid in that it has a single proton as its sidechain and this was initially considered necessary for the mechanism of formation of the cross-link (Fig. 3.20). Oxidation of the covalently attached glycyllsine to a Schiff intermediate in compound 5 of this figure results in a loss of hydrogen, which in amino

![Tuftsin and 3-Hydroxykynurenine](image)

Tuftsin 3-Hydroxykynurenine
acids apart from glycine, would leave a sidechain attached to the $\alpha$-CH. It was thought that subsequent cyclisation to a benzoxazole would be unlikely to occur or that the product would vary according to the identity of the N-terminal residue.

Here, the reaction product of the tetrapeptide, Thr-Lys-Pro-Arg (named tuftsin), with 30HKyn is examined. Evidence is presented in support of a general mechanism of formation of a benzoxazole cross-linked peptide dimer by 3OHKyn, i.e., the reaction is not restricted to the peptides with a glycine residue at their N-terminus.

6.2 Methods

Procedures were as described in section 3.2 except for the following. Tuftsin (sequence TKPR) was purchased from Sigma. 3OHKyn (0.5 mgml$^{-1}$) was reacted with tuftsin (10 mgml$^{-1}$) in 5 ml of 100 mM phosphate buffer pH 7.0 for 24 hours. A gentle stream of humidified oxygen was bubbled into the solutions to provide mixing and constant saturation with oxygen. The crude reaction mixture was separated by semi-preparative HPLC on a Brownlee C8 column using a water / acetonitrile gradient containing 20 mM phosphate buffer, pH 7.0. A yellow product was collected and further purified by analytical HPLC on a Brownlee C8 column using a water / acetonitrile gradient containing 0.05% TFA. A total of 7 mg of material was collected.

$^1$H NMR spectra were acquired at 400 MHz on a Varian Unity-400 NMR spectrometer. The HSQC and HMBC NMR spectra were acquired at 500 MHz ($^1$H frequency) on a Bruker DRX500 NMR spectrometer at Bruker, Australia.
Figure 6.1  Elution profiles monitored at 365 nm of the tuftsin / 3OHKyn reaction mixture following 24 hours incubation at 37 °C. (a) Crude separation by semi-preparative HPLC, and (b), isolation of the highly purified compound for subsequent spectroscopic analysis.
6.3 Results

HPLC chromatograms for the crude semi-preparative and the analytical separations are shown in figures 6.1a and 6.1b respectively. The shaded peak at 38 minutes (Fig. 6.1a), which was the highest absorbing at 365 nm, was collected and rechromatographed to yield a single, sharp peak at 28 minutes (Fig. 6.1b). This product was purified over successive runs until approximately 7 mg of material was collected.

The mass spectrum of the purified reaction product revealed a doubly charged species (determined by peak shape) at \( m/z \) 577.8 (Fig. 6.2a), corresponding to a molecular mass of 1153.6 Da. Tuftsin is a tetrapeptide which has a monoisotopic mass of 500.6 Da, and 30HKyn has a mass of 224.2 Da. The mass 1153.6 Da suggested that, as was observed in the case of QBA (Ch. 3), two peptides had been cross-linked by an oxidation product of 3OHKyn.

An MS/MS spectrum was acquired on the \( m/z \) 577.8 peak and is shown in figure 6.2b. Ions which corresponded to those observed in a standard tuftsin MS/MS spectrum are labelled with the appropriate fragment type. The presence of these ions suggested that tuftsin had been incorporated into the compound. The ion at \( m/z \) 301.3 was considered significant as it was present in the MS/MS spectrum of QBA (Fig. 3.5b), where it was thought to arise from cleavages on the carboxyl side of the \( \alpha \)-NH groups both lysine residues.
Figure 6.2: Mass spectra of the major tuftsin / 3OHKyn reaction product. (a) Mass spectrum revealing a doubly charged ion at $m/z$ 577.8. (b) Tandem mass spectrum of the purified 3HK / tuftsin reaction product and resultant fragment ions. The peaks corresponding to tuftsin are denoted by fragment type.
More detailed structural information was obtained from NMR spectroscopy. Figure 6.3a shows the α-NH region of the TOCSY spectrum. Cross-peaks from the α-NH protons arose from two lysine, two arginine and one threonine residues, indicating that two tuftsin peptides were present in the sample. Cross-peaks were also observed from Arg, ε-NH but not from Arg2 ε-NH. Proline, which is the third residue in tuftsin, does not have an α-NH proton, thus cross-peaks from it were not observed in this region of the spectrum. In the aliphatic region of the TOCSY spectrum (Fig. 6.3b), correlations are observed for seven amino acids. These have been denoted as Thr, Lys1, Lys2, Pro1, Pro2, Arg1 and Arg2. The presence of only one Thr residue in the TOCSY spectrum implied that the N-terminus of one of the tuftsin peptides had been modified, as was observed for an N-terminal glycine residue in QBA (Ch. 3).

In the aromatic and α-NH region of the NOESY spectrum (Fig. 6.4a), cross-peaks were observed from an aromatic proton of 3OHKyn (Hb) to the Thr and Lys1 α-NH. 3OHKyn has three aromatic protons, but only one was observed in the NOESY spectrum, i.e., Hb is close in space to these two α-NH protons.

Assignments were made for all cross-peaks observed in the aromatic and NH region to the aliphatic region in the 2D 1H NOESY spectrum (Fig 6.4b). These nOes enabled an unequivocal assignment of which tuftsin peptide each residue belonged to, and a summary of the observed correlations is presented in figure 6.5. The residues subscripted with a one belong to the tuftsin which is proximal to Hb, and contains an
intact threonine residue. Table 6.1 summarises the $^1$H assignments for the tuftsin / 3OHKyn reaction product.

The protonated $^{13}$C resonances were assigned from an HSQC spectrum (Fig 6.6), and are presented in table 6.1 along with the $^1$H assignments. A second aromatic proton was observed in the aromatic region of the HSQC spectrum (not shown) and is referred to as $H_A$ (Fig 6.5). The presence of this proton, and the fact that no nOes to tuftsin were observed, further reflected the similarities between this product and QBA.

The HMBC spectrum (Fig. 6.7) revealed the identity of only four non-protonated $^{13}$C resonances, C12, C4, C11 and the threonine carbonyl (see Fig 6.5). The three former resonances gave rise to cross-peaks with $H_B$. A complete assignment of the quaternary carbons was therefore not possible.
Figure 6.3a: TOCSY spectrum showing cross-peaks between the α-NH and α-CH and sidechain groups of two lysine and two arginine residues, and one threonine residue. Cross-peaks from Arg₁ ε-NH are also present.
Figure 6.3b: TOCSY spectrum showing cross-peaks between the $\alpha$-CH and sidechain groups of two lysine, proline and arginine residues, and one threonine residue.
Figure 6.4a: Noes in the aromatic and NH region in the 2D $^1$H NOESY spectrum of the tuftsin / 3OHKyn reaction product. Cross-peaks between Thr and Lys$_1$ residues indicate they are linked, and near in space to H$_B$ on the aromatic ring of 3OHKyn.
Figure 6.4b: NOes from the aromatic and NH region to the aliphatic region in the 2D $^1$H NOESY spectrum of the tuftsin / 3OHKyn reaction product. Cross-peaks between adjacent residues (sequential nOes) permitted a distinction between tuftsin chains one and two. The large cross-peak arises from exchange between the sidechain lysine and arginine NH$_3^+$ and NH groups and the water resonance around 3.7 ppm.
Figure 6.5: Summary of the correlations observed in the 2D $^1$H NOESY spectrum based on the QBA model of a benzoxazole cross-link (Ch. 3). It was clear from the $\alpha$-NH region of the TOCSY spectrum that two tuftsin peptides were present, however, only cross-peaks from one threonine residue were observed. This was confirmed in the NOESY spectrum which exhibited sequential nOes between each neighbouring residue except for a threonine in tuftsin$_2$. 
Figure 6.6: Aliphatic region of the 2D $^1$H-$^1$C HSQC spectrum of the tuftsin dimer. The $^1$H shifts from the TOCSY spectrum (Fig. 6.3) were used to assign the $^1$C shifts of the tuftsin CH$_2$ groups.
Figure 6.7: HMBC spectrum the tuftsin dimer. Only four additional $^{13}$C resonances compared to the HSQC were observed, those being C12, C4, C11 and the threonine carbonyl.
Table 6.1  Summary of the $^1$H and $^{13}$C Shifts for the Tuftsin / 3OHKyn Dimer

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$^{13}$C ASSIGNMENTS

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| C4 | 141.9 | C7 | 111.6 | C11 | 126.7 |
| C12 | 155.2 | C13 | 93.1 | Thr C=O | 178.0 |

- 179 -
6.4 Discussion

A product of the oxidative reaction of tuftsin and 3OHKyn was found to have a mass of 1153.6 Da and include two tuftsin peptides. The NMR data suggested that a threonine residue of one of the peptides was either absent or modified.

It was observed in chapter 3 that the major product of the oxidative reaction of the dipeptide, glycyllysine, and 3OHKyn was a compound composed essentially of two dipeptides and one 3OHKyn molecule. The aromatic amino and hydroxyl groups of 3OHKyn had formed an oxazole ring with the α-amino and α-CH₂ groups of one of the glycine residues. The resultant structure had a mass of 603.2 Da as observed by high resolution mass spectrometry. The combined masses of two GK and one 3OHKyn moieties is 630.4 Da, thus the net result of the dimerisation reaction was the loss of approximately 27.2 Da. If this net loss is applied to the combined units of a theoretical tuftsin / 3OHKyn dimer, the expected product mass would be:

\[ 2 \times 500.3 \text{ Da (tuftsin)} + 224.2 \text{ Da (3OHKyn)} - 27.2 \text{ Da} = 1197.6 \text{ Da} \]
The mass of this dimer was shown to be 1153.6 Da (Fig. 6.2a), that is, 44 Da less than the calculated mass for the equivalent benzoxazole-linked dimer described as QBA in chapter 3. The structures of threonine and glycine are depicted above. An inspection of their respective sidechains reveals that glycine has one proton, while threonine is composed of CH(OH)CH$_3$, a mass of 45 Da. The net difference in the masses of the two sidechains is 44 Da, the same mass difference described above between QBA and a theoretical tuftsin / 3OHKyn dimer. This difference implies that the sidechain of threonine has been eliminated in forming the tuftsin / 3OHKyn complex.

Figure 6.8 Proposed general reaction mechanism for the formation of 3OHKyn cross-linked peptides.
A reaction mechanism for the formation of QBA was proposed in chapter 3 (Fig. 3.20) which incorporated the α-NH and α-CH₂ of a glycine into the oxazole ring. Initially, it was thought that this mechanism was unique to glycine due to the absence of a sidechain in this amino acid, i.e., elimination of either of the protons of the α-CH₂ group would permit the formation of the oxazole ring.

The result for tuftsin reacting with 3OHKyn has led to the proposal of a general mechanism of oxidative cross-linking through free N-terminal amino groups. This mechanism is illustrated in figure 6.8. Essentially, the first oxidation step results in the loss of either the R group (sidechain), or the proton of the α-CH, i.e., a combination of the two products may be formed (the ratio of which presumably is dependent upon the reaction conditions). The resulting Schiff intermediate is then prone to intramolecular attack by the aromatic hydroxyl group as previously described (Fig. 3.20).

In conclusion, tuftsin, a tetrapeptide with threonine as the N-terminal residue, has been shown to form a dimer via the same mechanism as QBA (Ch. 3). This result suggests that 3OHKyn can react with any peptide that has a free N-terminus, regardless of the identity of the amino acid (except proline). This finding, that the previously proposed mechanism applies to all amino acids, suggests that the ubiquity of this cross-link in disease states such as cataract is potentially much greater than previously thought.
Conclusions

The aim of this project was to gain some insight into the mechanism of reaction of 3OHKyn with crystallins (lens structural proteins) as well as some model synthetic peptides. 3OHKyn is present in the human lens as one of the tryptophan derived UV-filter compounds, however it is unique amongst them in that it a free phenolic group. As such 3OHKyn can form a highly reactive quinonimine intermediate under oxidising conditions. In the presence of oxygen, 3OHKyn has previously been found to react with bovine crystallins, to give brown-coloured products. In the initial stages of this study the roles of UV-light, pH, glutathione and oxygen were examined, with the objective of determining how these factors may possibly affect the binding of 3OHKyn to crystallins under the conditions found within the lens itself.

The presence of oxygen was found to be an important parameter for determining the extent to which 3OHKyn reacts with protein, and when it was totally excluded from reactions, little modification was observed. UV-light was not required for activation, but was found to augment the extent of modification and crosslinking, while an elevated pH, which is known to accelerate the rate of 3OHKyn oxidation, did not markedly increase the extent of reaction with the crystallins. 3OHKyn binding was accompanied by crystallin aggregation, pigmentation, and development of non-tryptophan fluorescence, all of which have been associated with cataract formation.

The inclusion of glutathione, a ubiquitous antioxidant, in reaction mixtures resulted in a delayed onset of crystallin modification. This effect was apparent at concentrations of glutathione greater than 1 mM. When glutathione levels fell below 1 mM however, the antioxidant protection offered by GSH was diminished and the crystallins became modified by 3OHKyn. Since lens glutathione concentrations decrease with age, and are
known to be lower in the lens nucleus than the cortex, the nuclear region appears particularly vulnerable to modification by this UV-filter. Thus 3OHKyn, when oxidised, has the ability to form reactive intermediates which have been shown to bind covalently to bovine lens crystallins. 3OHKyn can modify crystallins in the absence of light, under conditions of low oxygen tension, and in the presence of glutathione, at concentrations found in the nucleus of an aged lens. Its reactivity is increased in the presence of both light and oxygen. The identity of the modifying species was not determined in this work, and in order to investigate this aspect, 3OHKyn was incubated with the dipeptides glycyllysine and glycylglycine in order to isolate modified compounds.

The major product of the reaction between GK and 3OHKyn was isolated, and its structure determined using mass spectrometry and NMR spectroscopy. It was found that the product was both coloured and fluorescent. The mass of the product suggested that an oxidation product of 3OHKyn had cross-linked two GK dipeptides and this was confirmed by NMR. The highly modified product contained only one glycine and it was found that the other glycine had been incorporated into the formation of an oxazole ring at the C-2 and C-3 positions of the 3OHKyn aromatic ring. The second GK was covalently attached through its α-amino group at the C-5 position of 3OHKyn. The complete structure of the cross-linked product elucidated and was named quinilinobenzoxamine (QBA). It is thought that QBA represents the first structure that has been elucidated from the reaction between 3OHKyn and a peptide, and as such may prove a valuable biomarker. A mechanism of formation was proposed.

The NMR data for QBA suggested that one of the lysine sidechains was restrained, possibly due to salt bridge interaction with an aromatic carboxyl group. Glycylglycine has no such sidechains, thus it was interest to see if the absence of a
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A hydrophilic sidechain on the peptide influenced the nature of the products formed from reaction with 3OHKyn.

A number of coloured and fluorescent products resulted from the incubation of GG with 3OHKyn. The structure of the major product was elucidated using mass spectrometry and NMR spectroscopy. In contrast to the compound observed for the peptide GK, an imidazole rather than a benzoxazole ring was formed by an N-terminal glycine. Furthermore, there was no peptide addition at C-5, and the 3OHKyn sidechain was not cyclised, differing in this respect from QBA. The compound was named 2-diglycylimidazolekynurenine (2-DIK). It was argued that a different major product was obtained for GG due to the fact that this peptide experienced less steric hindrance than GK proximal to the quinonimine functional groups of oxidised 3OHKyn. It was proposed that GK, on the other hand, was preferentially bound at C-5 of 3OHKyn, which then predisposed the formation of a Schiff base intermediate at the aromatic imino position (compare figures 3.20 and 4.20). This difference in initiating nucleophilic attacks may be the reason for oxazole formation in the case of two peptides being cross-linked, and imidazole formation in the case of a single peptide modification.

Two minor products of GG and 3OHKyn were identified by mass spectrometry; the analogous form of QBA with cross-linked GG rather than GK, and a structure similar to 2-DIK where the aliphatic sidechain of kynurenine was cyclised and lacking the carboxyl group.

The dipeptide GK was chosen as a model peptide as it represents the two N-terminal residues of all subunits of bovine and human γ-crystattins. The γ-crystattin subunits all possess unblocked N-terminal α-amino groups, thus it was of interest to look for evidence of a benzoxazole cross-link in a reaction mixture of γ-crystallin and 3OHKyn. SDS-PAGE and size exclusion chromatography indicated that a 3OHKyn had dimerised γ-crystallin. LC-MS of a tryptic digest of this product revealed a digest
Conclusions

fragment of the same mass and similar retention time to QBA, however MS/MS analysis of
the product indicated that it was not QBA. A closer analysis of the fragments in the
MS/MS spectrum suggested they came from a peptide fragment of sequence NDSIR.
This peptide was identified as residues 72-76 in γB- and γE-crystallins, apparently arising
due to some chymotryptic activity in the digest.

To determine if steric hindrance at the Lys2 residues of γ-crystallin had prevented
tryptic cleavage and the formation of the GK dimer in the γ-crystallin digest, a synthetic
peptide consisting of the nine N-terminal residues of γ-crystallin was reacted with
3OHKyn. LC-MS analysis of the reaction mixture suggested that products analogous to
QBA and 2-DIK had formed between the nonapeptide and 3OHKyn. An inspection of the
LC-MS chromatogram of the γ-crystallin / 3OHKyn dimer tryptic digest for evidence of
these products proved unsuccessful, thus, it appeared that either the digest conditions were
destabilizing the benzoxazole moiety, or trypsin was not cleaving at the predicted sites.

QBA was subjected to identical digestion conditions as that used for the γ-
crystallin / 3OHKyn dimer. No significant breakdown of QBA was observed, suggesting
that the γ-crystallin / 3OHKyn dimer cross-link would be stable. Tryptic digestion of the
nonapeptide analogue of QBA revealed a major product corresponding to a chymotryptic
activity. This product, the tetrapeptide ITFY was also present in the γ-crystallin / 3OHKyn
dimer LC-MS chromatogram. The apparent absence of QBA in the digests suggested that
the nature of the products may have been affected, even though no degradation was
observed in the control digest of QBA.

In the case where longer peptides are cross-linked, trypsin is required to complex to
the 'peptide arms' of the molecule to affect digestion. It was proposed that the act of
trypsin complexing and digesting at Lys2 in the nonapeptide and γ-crystallin dimers, may
destabilize the benzoxazole, resulting in a structurally altered product, the characteristics of
which are not known.
The peptides GK, GG, GKITFYEDR and the γ-crystallins have in common a glycine as their N-terminal residue. Glycine is unique amongst the amino acids in that it has a single proton as its sidechain. It was proposed that this property may have made glycine prone to nucleophilic attack during formation of the oxazole and imidazole rings of QBA and 2-DIK (Figs. 3.20 and 4.20).

In order to test this hypothesis, the major reaction product of the tetrapeptide tuftsin (TKPR) and 3OHKyn was examined. The mass of this product combined with NMR spectroscopic data suggested that a cross-linked dimer of tuftsin had been produced and that a threonine residue of one of the peptides was either absent or modified. A comparison of the mass of this product with the mass of QBA suggested that the sidechain of one of the threonine residues had been eliminated to form an oxazole ring, as observed in the formation of QBA.

Thus, tuftsin, a peptide with threonine as the N-terminal residue, was able to form a dimer via the same mechanism as QBA. This result suggests that 3OHKyn can react with any peptide that has a free N-terminus, regardless of the identity of the amino acid (with the exception of proline). The work presented in this thesis has led to the identification of modified species in the reaction of 3OHKyn with the free N-terminal residues of proteins. In the lens where 3OHKyn is known to be continually synthesised, these may provide valuable biomarkers in disease states such as age-related nuclear cataract.


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