Kinetics and mechanism of thermal decomposition of kynurenines and biomolecular conjugates: Ramifications for the modification of mammalian eye lens proteins†

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Thermal degradation reactions of kynurenine (KN), 3-hydroxykynurenine (3OHKN), and several adducts of KN, to amino acids and reduced glutathione (GSH) have been studied at physiological temperature. These compounds are all implicated in age-related mammalian eye lens cataract formation at the molecular level. The main reaction pathway for both KN and 3OHKN is deamination *via* β-elimination to carboxyketoalkenes CKA and 3OHCKA. These reactions show a weak pH dependence below pH values of ~8, and a strong pH dependence above this value. The 3OHKN structure deaminates at a faster rate than KN. A mechanism for the deamination reaction is proposed, involving an aryl carbonyl enol/enolate ion, that is strongly supported by the structural, kinetic, and pH data. The degradation of Lys, His, Cys and GSH adducts of the CKA moieties was also studied. The Lys adduct was found to be relatively stable over 200 h at 37 °C, while significant degradation was observed for the other adducts. The results are discussed in terms of known post-translational modification reactions of the lens proteins and compared to incubation studies involving KN and related compounds in the presence of proteins.

Introduction

The main functions of the mammalian eye lens is to focus light upon the retina and to filter ultraviolet light. The retina is protected from UV damage by low-molecular-weight compounds contained in the lens, which absorb UV light in the region 300–400 nm. The UV-filter compounds are kynurenine (KN) and its derivatives, which originate from the amino acid tryptophan. Scheme 1 shows UV-filter compound formation and transformations. The most abundant UV filters in the human lens are 3-hydroxykynurenine *O*-β-D-glucoside (3OHKG), 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid *O*-β-D-glucoside (AHBG), and glutathionyl-3-hydroxykynurenine *O*-β-D-glucoside (3OHKG-GSH), followed by kynurenine (KN) and 3-hydroxykynurenine (3OHKN).[1–7] The initial steps in Scheme 1 are enzymatic reactions: the formation of *N*-formylkynurenine (not shown) from TrpH is followed by hydrolysis to yield KN. Hydroxylation and glucosylation of the latter produce 3OHKN and 3OHKG.[1,8] The deamination reactions of the "primary" UV filters (KN, 3OHKN and 3OHKG) to carboxyketoalkenes (CKAs) are followed by reduction and linkage to free thiols (glutathione and cysteine). These are spontaneous reactions, resulting in the formation of "secondary" UV filters (such as AHBG, AHBDG, 3OHKG-GSH).[9–14]

The transparency of the lens and its high refractive index are due to the compact packing of fiber cells that constitute the lens nucleus. These cells are protein rich: proteins comprise more than 30% of the wet lens weight. Most of the lens proteins are crystallins—these are water-soluble structural proteins whose main function is to increase the refractive index, while not obstructing light.[15] Once the proteins are produced, they form the lens nucleus and the lens grows throughout the human lifespan by the addition of new cell layers over the pre-existing core. Since there is no protein turnover in the lens nucleus, post-translational modifications, caused by oxidative processes, accumulate with age, resulting in coloration, aggregation, and lower solubility of old lens proteins. Eventually, these processes lead to the development of age-related nuclear (ARN) cataracts.[15–21]

Truscott and others have suggested that the UV-filter compounds shown in Scheme 1 are important in crystallin modification.[22–25] Three of the major UV filters—KN, 3OHKN, and 3OHKG—are intrinsically unstable, and under physiological conditions they undergo spontaneous deamination,[11,26] forming highly reactive carboxyketoalkenes (CKA (IUPAC name: 4-(2-aminophenyl)-4-oxocrotonic acid), 3OHCKA, and 3OHCKAG, respectively in Scheme 1). The CKA molecules can covalently bind to the nucleophilic amino acid residues of proteins (primarily cysteine, histidine, and lysine).[13,27–28] These reactions lead to disruption of protein functionality and increased susceptibility of the protein to UV light absorption. It has been demonstrated that protein-bound kynurenines are better photosensitizers than free UV filters,[29–31] and UV irradiation of modified proteins can lead to further damage either due to direct photoreaction, or through

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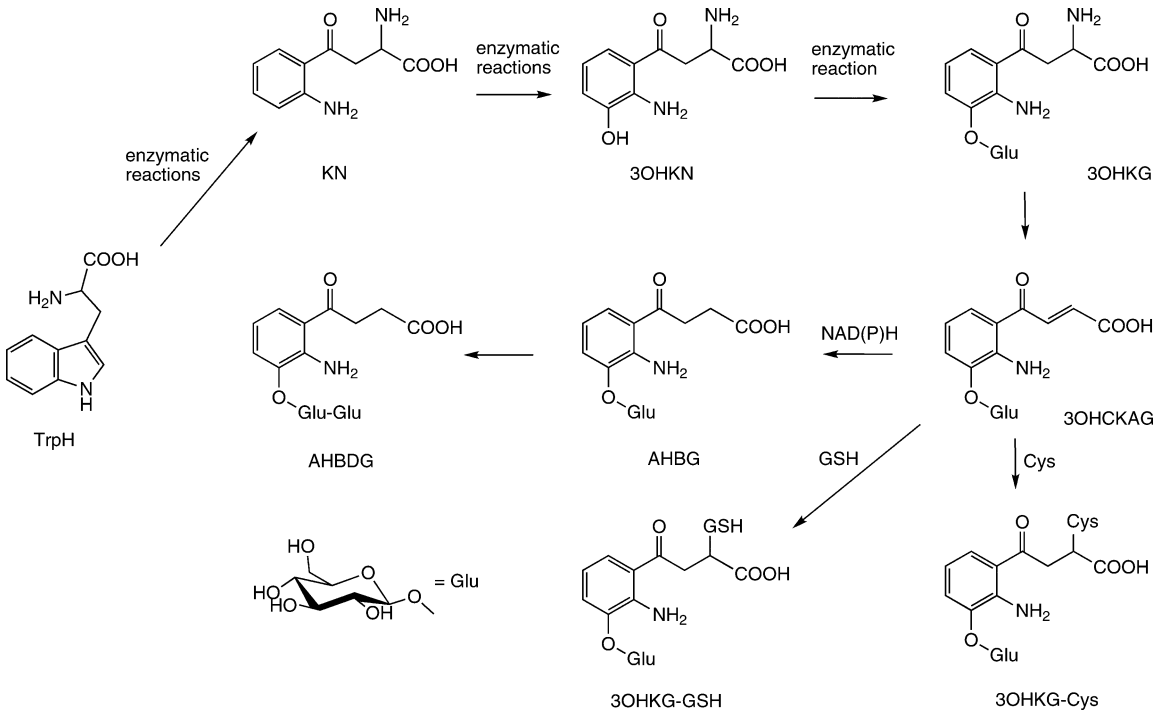


Scheme 1

the formation of reactive oxygen species such as singlet oxygen and superoxide. The protection of proteins from modification by deaminated UV filters is provided by reduced glutathione (GSH),[12,13,32] which can bind CKAs, forming new UV filters such as the 3OHKG-GSH adduct.

Recently, it has been revealed that kynurenine adducts to individual amino acids are also unstable: they decompose, restoring the CKA.[28,33,34] Thus, the modification of the lens proteins by deaminated UV filters is a complex, dynamic process in which the same CKA molecule can reversibly add several times to different amino acid residues and/or antioxidants present in the lens. The dynamics of UV-filter evolution are governed by many factors. The UV filters and antioxidants are transported into the lens nucleus from the cortex and surrounding humor, and the efficiency of the protective systems strongly depends on the transport of antioxidants and oxidation products to and from the lens. It has been reported that an internal barrier is formed between the lens cortex and nucleus in middle age.[21,35,36] The existence of such a barrier slows down the movement of antioxidants to the lens center, exposing the lens nucleus to additional oxidative stress.

The chemical equilibrium between free and covalently bound UV filters is determined by the rates of non-enzymatic reactions: kynurenine deamination, addition of CKAs to amino acid residues and antioxidants, and finally, CKA-adduct decomposition. The rate constants for CKA addition to nucleophilic amino acids and antioxidants present in the human lens have been measured.[13] The rates for CKA addition to the thiols cysteine and glutathione, are several orders of magnitude higher than the rates of addition to other molecules, including amino acids. In the same study it was noted that at the physiologically relevant temperature of 37 °C, the rate constant of the reaction of CKA with cysteine is almost 20 times higher than that with glutathione. This result demonstrates the importance of steric factors in the addition reactions. Furthermore, it implies that the addition of CKAs to the amino acid residues in proteins may be much slower than their addition to free amino acids. While a consistent picture of the molecular level reactions related to cataract development is emerging, there are still several missing pieces of information. For example, the rates of formation of CKA adducts are known for many compounds,[13] but their rates of decomposition and eventual disposition have not been determined. The mechanism of the deamination reactions of KN and 3OHKN has yet to be elucidated, nor have there been any quantitative measurements of the kinetics of this important reaction as a function of pH.

In this paper we report the results of a study on the stability of the "primary" UV filters, KN and 3OHKN, and on the stability of deaminated KN adducts to several amino acids and GSH. The results presented below establish the pH dependence for the reactions of KN and 3OHKN deamination. A mechanism for the deamination reaction is proposed that is consistent with these results. Additionally, we have determined the rate constants for the decomposition of CKA adducts to glutathione, cysteine, histidine, and lysine under physiological conditions. These reactions will be discussed and compared with measurements of similar reactions involving the lens proteins themselves.

Before presenting the results it is important to note the following with regard to nomenclature in these systems: the adducts to amino acids and GSH are labeled throughout this paper as KN-Cys, KN-GSH, *etc.* However, it is actually the deamination product (CKA or 3OHCKA) that is covalently binding to the amino acids or antioxidant. We are following the convention of acronyms for these adducts first put forward by the research group of R. J. W. Truscott (Sydney), to avoid confusion, and to facilitate comparisons of our results with theirs. The structures of all of these adducts are known and have been previously published.[28,33,34]

Results

Decomposition of KN and 3OHKN as a Function of pH

The kinetics of KN decay observed under anaerobic incubation (37 °C) of 1 mM kynurenine in aqueous buffered solutions at different pH values are shown in Fig. 1. In a control experiment at pH 7.0, the incubations were performed in non-buffered solution. The pH value was adjusted by dropwise addition of a NaOH solution. These solutions give essentially the same result as that measured in buffered solution. We can conclude that the buffer components do not contribute to the decomposition reactions, therefore all further measurements were performed in buffered solutions. The kinetic data were treated as monoexponential functions and the solid lines in Fig. 1 correspond to calculated curves. The pH dependence of the deamination rate constant is plotted in Fig. 2.

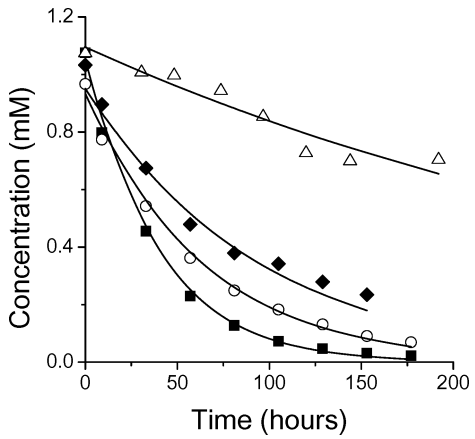


Fig. 1 Kinetics of KN thermal decomposition under anaerobic conditions for different pH values: △ - pH 6.6; ◆ - pH 8.5; ○ - pH 9; ■ - pH 9.7 during the incubation at $T = 37$ °C. Solid lines show monoexponential fits.

It was reported earlier[11,26] that the major pathway of KN thermal decomposition is deamination, which we have confirmed by analysis of the HPLC profile of our incubated samples. The major products found after KN incubation are carboxyketoalkene CKA (the direct deamination product), KN yellow from CKA cyclization, and the KN dimerization product (Scheme 2). The identity of these products was confirmed by HPLC with UV–VIS and mass spectrometric detection.

We made similar measurements to study the degradation of 3OHKN. The HPLC analysis revealed the formation of 3OHCKA and 3OHKN yellow. Kinetic curves for 3OHKN decomposition are shown in Fig. 3, and the pH dependence of the 3OHKN deamination rate constant is plotted in Fig. 4. The pH dependences for KN and 3OHKN have similar shapes: the deamination rate is almost pH independent below pH 8, above which it steeply increases. Note that the absolute rate increase for 3OHKN at high pH is much higher than for KN.

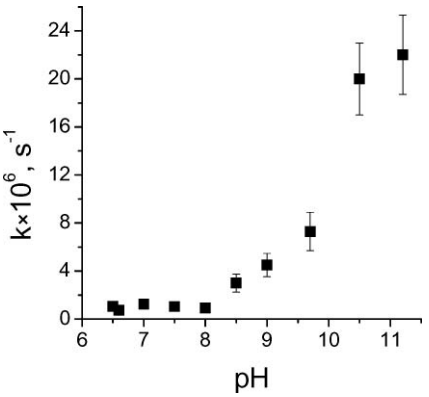


Fig. 2 pH dependence of the first-order rate constant of KN decomposition during the incubation at $T = 37$ °C.

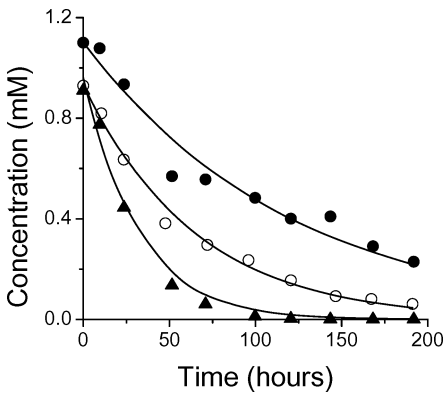
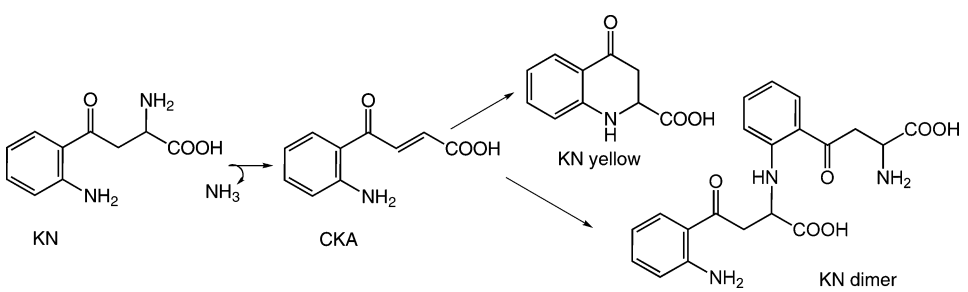


Fig. 3 Kinetics of 3OHKN thermal decomposition under anaerobic conditions at different pH values: ● - pH 5.5; ○ - pH 6.8; ▲ - pH 8.5 during the incubation at $T = 37$ °C. Solid lines show exponential fits.

Decomposition of K-Lys, KN-His, KN–Cys, and KN-GSH Adducts

The kinetics of the decomposition of *deaminated* KN adducts to lysine, histidine, cysteine, and glutathione during incubation under



Scheme 2

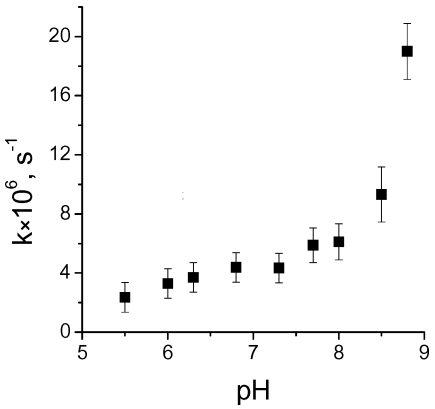


Fig. 4 pH dependence of the first-order rate constant of 3OHKN decomposition during the incubation at $T = 37$ °C.

physiological conditions ($T = 37$ °C, pH 7.0) are shown in Fig. 5. The solid lines in Figs. 5B, 5C, and 5D are good monoexponential fits, and the calculated rate constants of the adduct decompositions are listed in Table 1.

After 200 h of incubation, the KN-Lys adduct showed only about 7% loss of the starting material (from 7×10^{-5} to 6.6×10^{-5} M, Fig. 5A), which is within the experimental error. For the KN-His adduct, the level of decomposition was somewhat higher, and after 460 h of incubation, almost half of the starting material had decomposed (Fig. 5B). It has been reported[34] that the protection of the side-chain amino group of adducts by the *tert*-butoxycarbonyl (*t*-Boc) group significantly raises adduct stability twofold to threefold.

To verify this, we incubated an additional adduct in which the α-amino group of the His side chain was protected with an *N*-acetyl group, KN-NAc-His. The kinetics of KN-NAc-His decomposition was essentially the same as that for the unprotected

Table 1 Rate constants of adduct decompositions (k_{dec}) and addition of amino acids to deaminated kynurenine addition to amino acids (k_{add}) under physiological conditions ($T = 37$ °C, pH 7.0)

Adduct	k_{dec}/s^{-1}	$k_{add}/M^{-1}s^{-1}$ [a]
KN-Lys	$<10^{-7}$	$(2.3 \pm 0.6) \times 10^{-4}$
KN-His	$(3.4 \pm 1.0) \times 10^{-7}$	$(1.2 \pm 0.3) \times 10^{-4}$
KN-NAc-His	$(3.8 \pm 1.2) \times 10^{-7}$	—
KN-Cys	$(1.7 \pm 0.4) \times 10^{-5}$	36 ± 4
KN-GSH	$(5.8 \pm 0.7) \times 10^{-6}$	2.1 ± 0.2

[a] from ref. 13.

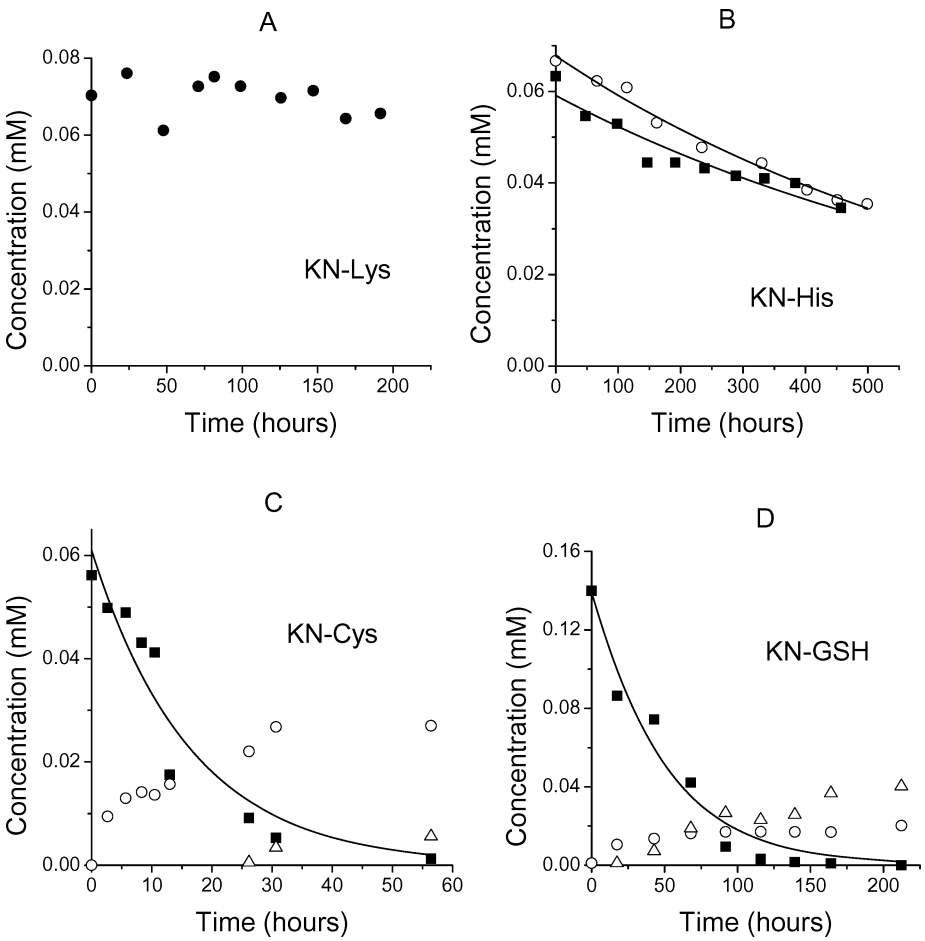


Fig. 5 Kinetics of adduct decomposition during the incubation under physiological conditions ($T = 37$ °C, pH 7.0). A) Decay of KN-Lys adduct. B) Decay of KN-His (■) and KN-NAc-His (○) adducts. C) Decay of KN-Cys (■); formation of CKA (○), and formation of KN yellow (Δ). D) Decay of KN-GSH (■); formation of CKA (○), and formation of KN yellow (△). In all plots, solid lines show exponential fits. See text at the end of the Introduction for definition of acronyms and structures.

KN-His adduct (Fig. 5B). It is likely that the bulky *t*-Boc group sterically hinders the hydrolysis of the KN side chain, and thus decelerates the overall rate of adduct decomposition.[34] The smaller *N*-acetyl group does not significantly influence the rate of adduct decay.

The fastest decomposition was observed for cysteine and glutathione adducts of KN (KN-SR) (Figs. 5C and 5D): the calculated decomposition rate constants for these compounds are higher than those for the KN-His adducts by more than an order of magnitude (Table 1). It is interesting that the rate constant for KN-Cys decay is almost threefold higher than that for KN-GSH. Perhaps this effect can be explained in the same way as the influence of the *t*-Boc group: the bulkier GSH moiety obstructs the access of hydroxyl ions to the reaction center in the adduct.

The HPLC profiles of the incubated samples demonstrate that the major products of adduct decomposition are carboxyketoalkene (CKA), KN yellow, and KN dimer; some minor unidentified KN-derived products have also been observed. The kinetics of CKA and KN yellow formation, observed during the incubation of KN-Cys and KN-GSH adducts, are shown in Figs. 5C and 5D, respectively. The formation of KN yellow is much slower than that of CKA, which suggests that the formation of these compounds is stepwise: KN yellow is the product of CKA cyclization,[11,37,38] occurring at 37 °C with the rate constant $k_{cycl} = (1.6 \pm 0.4) \times 10^{-6}$ s^{-1} (Scheme 3).[13]

KN-SR ⟶ CKA ⟶ KN yellow

Scheme 3

It is logical to suggest that the decomposition of the adducts results in the formation of CKA and free amino acids. However, thiols Cys and GSH add to the unsaturated double bond of CKA at fast rates (Table 1), restoring the starting adducts in both cases. Thus, the quasi-equilibrium concentration of CKA during incubation is actually much lower than observed in the experiment. We have assumed that the decomposition of adducts KN-Cys and KN-GSH occurs with the formation of the oxidized thiols, Cys-Cys and GSSG. The presence of free thiols in KN-Cys and KN-GSH solutions after incubation was examined with the use of the Ellman's reagent DTNB.[39] No free thiol groups were detected in the incubated adduct solutions. However, examination of the incubated KN-GSH solutions using a MALDI-TOF mass spectrometer revealed the presence of oxidized glutathione, GSSG. The mechanism of formation of the oxidized thiols is unclear. In a recent paper by Parker *et al.*[34] it was reported that the addition of GSH to KN-*t*-Boc-Cys solutions promotes the decomposition of the adduct. It is possible that reduced thiols (RSH), formed due to breakdown of the starting adducts, can react with the adducts, yielding CKA and the oxidized thiols, RSSR. However, the effect of GSH on the rate of adduct breakdown became pronounced only with GSH concentrations above 10 mM,[34] which is much higher than we would ever observe *via* adduct breakdown under our experimental conditions.

Discussion

pH Dependence of KN and 3OHKN degradation

The degradation of UV-filter compounds in neutral and basic solutions has been studied in a qualitative fashion by Taylor *et al.*, where measurements were made at two pH values.[11] Under physiological conditions (37 °C, pH 7) they found that after 7 days of incubation, the losses of starting material of KN, 3OHKN, and 3OHKG were 58, 79, and 70%, respectively, which roughly corresponds to deamination rate constants of 1.4×10^{-6} s^{-1}, 2.7×10^{-6} s^{-1}, and 2×10^{-6} s^{-1}. In basic solution (pH 9) the rate of decomposition for all three UV-filter compounds increased.[11] The deamination rate constants for neutral solutions, obtained in the present quantitative study (1.2×10^{-6} s^{-1} for KN and 4.3×10^{-6} s^{-1} for 3OHKN), are in a good agreement with these previous results.

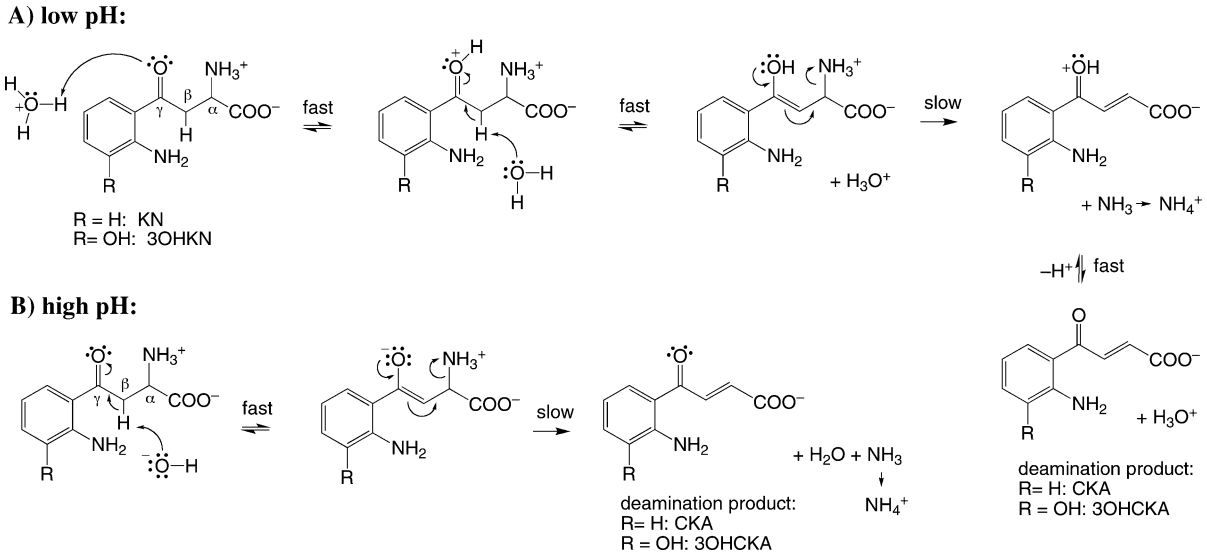
For both kynurenines studied here, the pH dependences of the deamination rate constants show two regions: below pH 8 the deamination rate is weakly pH-dependent, while in basic solutions the rate constants of deamination continuously grow with pH (Figs. 2 and 4). This indicates that the deamination reactions of UV filters at low and high pH values proceed by different mechanisms. It should also be noted that, for all pH values, the deamination of 3OHKN is faster than that of KN by a factor of three to five.

Mechanism of deamination

The deamination reactions of KN and 3OHKN take place by simultaneous loss of ammonia from the α-carbon and a proton from the β-carbon (Scheme 4). The overall driving force for these reactions may arise from the fact that the final products are α,β-unsaturated keto-acids containing an extended π-bond system not present in the starting materials. There are three major factors that can influence the rate of the deamination reaction: 1) the acidity of the β-proton; 2) the strength of the base removing this proton, and 3) the leaving group's base strength (weaker bases make better leaving groups). At most of the pH values studied in this work, the α-amino group is protonated. This makes it a reasonable choice as the leaving group (as NH_3) in the elimination step. Deamination is not possible by an elimination pathway from the deprotonated structure, because NH_2^- is a very strong base and therefore a very poor leaving group.

It should be noted that NH_3 still has significant base strength and really isn't an ideal leaving group for an elimination reaction, but it is a much better leaving group than NH_2^- so it is through this pathway that the reaction must proceed. The "tepid" leaving-group ability of NH_3 is most likely why the overall reaction rates for deamination are quite slow for these compounds. Of course, the advantage of having an NH_3 leaving group is that it will immediately be protonated to NH_4^+ ($pK_a = 9.3$) in most of the solutions studied here. The positively charged ion is completely non-nucleophilic, making the back reaction impossible. This is an important factor in the kinetic stability of the deamination products. An additional important point is that the leaving group is the same for deamination of KN and 3OHKN, therefore the observed differences in reactivity between these compounds must be based on other criteria. We will discuss this issue in more detail below.

Base strength is a major factor in these reactions because at high pH there is a strong base present (HO^-), while at low pH only water is present, a weak base. This by itself may explain the acceleration in the deamination rate seen for both KN and 3OHKN above pH 8, *i.e.* we are observing generalized base catalysis. A question then arises as to the more precise nature of the elimination mechanism. Obviously we can rule out the



Scheme 4

E1 extreme limit as we are in basic solution where carbocations will not form. An E2 mechanism is unlikely at all pH values because we expect the leaving group ability of the amino group to be very different in its protonated *vs.* deprotonated states. For these reasons, we have drawn the mechanism in Scheme 4B as "$E1_{CB}$", that is, deprotonation at the β-position takes place before the leaving group leaves, creating the enolate (conjugate base, CB). Of course it is possible to have an "$E1_{CB}$-like" transition state in an E2 reaction, which cannot be ruled out. Either way, this deprotonation step is the basis for the observed general base catalysis (acceleration of KN decomposition from pH 8 to pH 10.5).

The observed rate constant for deamination is not directly proportional to the HO^- concentration in solution: the increase in the rate of decay is only a factor of 20 even though the HO^- concentration has increased by a factor of 300 (2.5 pH units). Furthermore, the rate constants for KN decomposition at pH 10.5 and pH 11.2 are almost identical, meaning that the acceleration of KN decomposition tails off (Fig. 2, last data point). We attribute this to the deprotonation of the amino group, rendering it a much poorer leaving group. Both of these observations point to an E2 mechanism where the transition state is more like an $E1_{CB}$ reaction, *i.e.*, deprotonation at the β-carbon is almost complete before the leaving group on the α-carbon begins to leave. An aqueous solution of pH 11.2 is a rather harsh environment for any organic molecule, therefore it should be noted that other degradation processes may also be taking place under these conditions. Still, the data in Fig. 2 strongly support every aspect of the mechanism proposed in Scheme 4B.

Concerning the acidity of the β-protons of KN and 3OHKN, the additional carbonyl group in the γ-position may greatly influence the rate of deprotonation at the β-site. At low pH values, with water as the base, no direct β-deprotonation is expected. However, excess hydronium ions can protonate the carbonyl oxygen (Scheme 4A, first step). The protonated carbonyl, through an inductive effect, makes the β-protons much more acidic and water can participate in the second step of the mechanism to make the enol. The enol can then undergo the electron-pair shift illustrated in the third step (slow), leading to loss of NH_3 and reformation of the protonated carbonyl. Loss of a proton from the carbonyl oxygen is the last (fast) step, leading to the deamination product. Although the initial protonation is not highly favored, because of the low H_3O^+ concentration, it should be remembered that our overall reaction rates are very slow compared to "normal" organic flask reactions. On the time scale of our observations (sometimes hundreds of hours) it seems likely that even moderately acidic solutions will eventually lead to protonation of the carbonyl groups in KN and 3OHKN, with subsequent generalized acid catalysis of the E2 reaction.

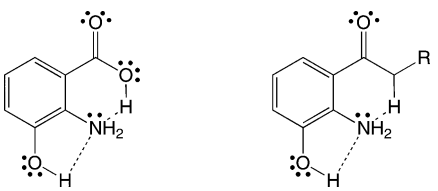
At high pH values, deprotonation to make the enolate anion directly from the γ-carbonyl can be expected when there is a significant concentration of HO^- (Scheme 4B). The elimination reaction can proceed immediately from the enolate anion, although in aqueous solution the enol may also be present in a rapid equilibrium with the anion. Since the final product is the same for either structure, the elimination step is shown at the bottom of Scheme 1B only for the enolate anion. The elimination reaction produces exclusively the *E*-isomer of the deamination product, as determined from NMR data (see the ESI†), where the vinyl region shows two doublets at 6.65 ppm and 7.57 ppm, with a typical *trans* ^2J coupling constant of 15.8 Hz.

We propose that at all pH values, either the enol or enolate anions of KN and 3OHKN are involved in their deamination reactions. Formation of the enols is catalyzed by excess acid (hydronium ion) and formation of the enolate anions is catalyzed by excess base (hydroxide ion). Scheme 4 summarizes the mechanism at A) low pH values and B) high pH values. The proposed mechanism accounts for two very important features of the kinetic data: first and foremost, it gives clear reasoning for the acceleration of the deamination reaction at high pH values, *i.e.* when there is a significant concentration of strong base and there is an enolizable proton present. Second, the mechanism accounts for the fact that at pH values below 8, deamination is still possible but is slower and must involve the enol of the γ-carbonyl because water is not basic enough to participate directly in the elimination reaction. Since the leaving group is non-ideal at either pH value, base strength is the

determining factor in the observed trend in the rates of reaction as a function of pH.

The difference in reactivity between KN and 3OHKN is worthy of discussion. Because the OH group in 3OHKN is *meta* to the γ-carbonyl substituent, the lone pairs on the OH oxygen are unable to donate into the phenyl ring. For this reason the OH lone pairs cannot participate in any stabilization or destabilization *via* resonance effects in the reactants, products or reactive intermediates. However, there may be an inductive effect of the phenolic oxygen atom through the σ-bond framework, pulling electron density away from the γ-carbonyl carbon. This in turn will make the neighboring β-protons slightly more acidic, meaning that, for the same reaction conditions, 3OHKN should degrade faster than KN. This is exactly what is observed experimentally.

It should be noted, however, that the pK_a of 2-aminobenzoic acid (4.96) is lower than that of 2-amino-3-hydroxybenzoic acid (5.19), which would seem to contradict our argument for an inductive effect. However, the benzoic acids may not be good models for the elimination (or enolization) reaction because there may be significant intramolecular H-bonding to the amino group through a six-membered-ring transition state. This H-bonding will be competitive between the OH on the benzoic acid and OH on the phenyl ring, as shown in Scheme 5. Such competition will reduce the ability of the amino group to assist in the deprotonation reaction, which would explain the rather significant difference in pK_a values of the two benzoic acids.



Scheme 5

Competition for H-bonding is expected to be a minor effect in the right hand structure (Scheme 5) leading to the enolate in kynurenine degradation chemistry. Because of the large difference in pK_as of the acids and ketones, one can use the Hammond postulate to predict a much later transition state for deprotonation of the ketone. The amino group will therefore play a smaller role in the deprotonation reaction, rendering intramolecular H-bonding effects much less important. Correspondingly, the electron withdrawing inductive effect of the phenolic OH oxygen may be more pronounced in our system. This interplay of two intramolecular effects is an interesting problem that should be of interest from a computational perspective in future work.

Kinetic analysis

Table 1 summarizes the data for KN adduct stability, as well as the rate constants for adduct formation, which occurs *via* the Michael addition reaction[40] of CKA to amino acids. Analysis of the data shows that KN adducts to thiols—Cys and GSH—are significantly less stable than those to His and Lys; at the same time, the rate constants of Cys and GSH addition to CKA are several orders of magnitude higher than those of other amino acids/antioxidants. These results help to explain the results of protein incubation in the presence of KN. They also help to understand the analysis of post-translational modifications of the lens proteins by UV filters.[23,24,28,33,34,41,42] It has been demonstrated that the initial attachment sites of deaminated KN and deaminated 3OHKN to proteins are Cys residues.[24] However, other studies showed that crystallins isolated from human lenses contain significant levels of bound kynurenine, and the modifications occur mostly *via* His and Lys residues, with only minor levels of kynurenine attached to Cys residues.[33] Thus, the covalent attachment of deaminated kynurenines to antioxidants such as GSH, amino acids, and proteins appears to be a reversible reaction, and the stabilities of the adducts play a more important role in this chemistry than the rate constants of the addition reactions.

The concentrations of KN-Lys and KN-His adducts in human lens proteins significantly increase with age.[28] This impact of crystallin modification by UV filters is manifested in lens coloration, increases in fluorescence intensity, and opacification. It is interesting that the levels of protein-bound kynurenines in the nuclei of cataractous lenses are much lower than in normal ones.[42] This probably means that protein-bound UV filters are in dynamic equilibrium with free ones, and the decrease in concentration of protein-bound kynurenines reflects the total decrease of UV filter levels in cataractous lenses.[42]

Comparison with KN-protein incubation studies

In a previous study we measured the rate constants of deamination product (CKA) linkage to nucleophilic amino acids and antioxidants, and showed that at physiological conditions the rate constant of CKA binding to Cys is 5 orders of magnitude higher than its value for His or Lys.[13] An earlier study of the incubation of crystallins in the presence of KN and KN derivatives has been reported,[34] and the Cys residue in these proteins was determined to be initial site of chemical modification. Despite lower rate constants for CKA binding to Lys and His, modifications of these residues were also revealed during incubated protein analysis. According to Vazquez *et al.*,[28] the KN-Cys adduct decomposes under physiological conditions, yielding deaminated KN and KN yellow, and our experimental data match these findings.

Experiments on KN incubation with crystallins have been reported[27,28] and tryptic digestion analysis of these proteins showed Cys, His and Lys residues as the preferred sites of KN modification. Longer incubation of KN with crystallins results in an increase in the presence of the more stable KN-His and KN-Lys adducts; this correlates with our findings on adduct stability. Vazquez *et al.*[28] have reported that KN-His and KN-Lys concentrations appear to be a function of time and increase during aging. The concentration of Cys-modified residues decreases with age according to adduct instability, however modification on His residues accumulates.

It is interesting to note that in tryptic digests of aged lens crystallins, modified Cys residues were detected in lesser concentration than those for Lys or His, despite higher rate constant values for KN-Cys adduct formation.[28] This was explained by proposing that the release of CKA molecules may allow them to react with other amino acid residues, causing additional modification in proteins. It is expected that CKA bound to Cys residues will be released during prolonged thermolysis, and modifications on His and Lys will accumulate. However, even after the termination of incubation, a significant amount of CKA remained bound to Cys residues. From

these results it appears that CKA attached to Cys residues may be hidden in the protein tertiary structure and become inaccessible for hydrolysis. This result certainly confirms that there is a strong protein environmental effect on the properties of KN derived compounds. It is possible to assume that during aging, the process of KN-Cys adduct cleavage may shift the CKA adduct distribution in favor of the formation of KN-Lys and KN-His adducts. This possibility will be explored in future experiments.

It has been proposed that another nucleophile, GSH, is the major antioxidant in the lens-inhibiting CKA binding to the lens proteins.[5,12,32,43] The rate constant for the addition of CKA to GSH is rather high compared to the formation of KN-His or KN-Lys.[13] However, in the work presented here, we have found that the KN-GSH adduct turned out to be less stable than other CKA adducts. Unbound GSH may act as a target for CKA attack, but taking into consideration the rate constant for KN-GSH adduct cleavage (Table 1) and the decrease of GSH concentration in the lens during aging, reactions involving GSH diminish lens protection overall. In fact, insufficient levels of GSH in the lens will lead to an accumulation of Lys- and especially His-modifications in crystallins.[28] It was recently established[5] that GSH attached to 3OHKG is the main UV-filter-derived compound present in the lens, which is definitive proof of the protective function of this antioxidant.

Conclusions

The pH dependences for the rate constants of KN and 3OHKN deamination show two distinct ranges—strongly pH-dependent above pH 8 and weakly pH-dependent below pH 7. The deamination reaction of these compounds accelerates in basic solution, and the rate constants for 3OHKN are higher than that for KN. The deamination mechanism is proposed to proceed through the formation of the enol (low pH) or enolate anion (high pH) at the aryl carbonyl group of KN and 3OHKN. Amino acid and antioxidant adducts formed after deamination of KN cleave with drastically different rates: KN-Cys and KN-GSH are less stable adducts compared to KN-His or KN-Lys. Under physiological conditions, modifications at Lys and His residues accumulate in the lens, however the mechanisms when crystallin proteins are involved are not clearly understood and remain under active investigation.

Experimental

Materials

D,L-Kynurenine (KN), D,L-kynurenine sulfate salt, 3-hydroxykynurenine (3OHKN), D,L-histidine (His), *N*-acetyl-L-histidine (NAc-His), D,L-lysine (Lys), D,L-cysteine (Cys), reduced L-glutathione (GSH), 5,5′–dithio-bis-(2-nitrobenzoic acid) (DTNB), trifluoroacetic acid (TFA) and α-cyano-4-hydroxycinnamic acid (CHCA) were purchased from Sigma-Aldrich and used as received. Water was doubly distilled. Organic solvents (HPLC grade, Cryochrom, Russia) were used as received.

Adduct synthesis

KN adducts to Lys, His, NAc-His, Cys, and GSH were synthesized according to the procedure described in ref. 28 The KN sulfate salt solutions (5.4 mM) containing a tenfold molar excess of Lys, His, NAc-His, Cys, or GSH were prepared using phosphate buffer of pH 9.5. The pH value was adjusted by drop-wise addition of NaOH if required and controlled by an Orion Research pH meter with a glass electrode. The pH value remained constant during the entire incubation period. The solution (volume 20 mL) was placed in a glass vial, capped, bubbled with argon, sealed, and incubated in a thermostatted water bath at 37 °C for 48 h. After incubation, the resulting mixture was filtered, the pH value adjusted to a value between 4 and 5 by addition of 1 M hydrochloric acid. Sample separation was performed by HPLC. Collected fractions were lyophilized and weighed. After lyophilization the KN-Lys (yield 60%), KN-His (yield 40%), KN-NAc-His (yield 40%), KN-Cys (yield 50%), and KN-GSH (yield 80%) adducts were stored at 4 °C.

Incubation

KN, 3OHKN, KN-Lys, KN-His, KN-NAc-His, KN-Cys, and KN-GSH solutions were prepared in phosphate buffer (0.1 M). The pH values were controlled by an Orion Research pH meter with a glass electrode and adjusted by drop-wise addition of NaOH or hydrochloric acid if required. The solutions were placed into glass vials (1 mL), capped, bubbled with argon, sealed and incubated at 37 °C. For each pH value a fresh solution was prepared, and for each incubation time an individual vial was used. All vials were placed in the thermostat simultaneously and removed at various intervals. After incubation, the samples were stored at 4 °C and then analyzed by HPLC.

Separation and analysis

HPLC separation of the amino acid and antioxidant adducts to deaminated KN was performed with the use of an Agilent LC 1200 chromatograph equipped with an automatic gradient pump and a multiple wavelength UV–Vis detector. A 9.4 × 250 mm ZORBAX Eclipse XBD-C18 Semi-Preparative column was eluted with an acetonitrile/0.05% (v/v) TFA in H_2O gradient. The acetonitrile percentage in the gradient was 0–30% (0–2 min), 30–55% (2–32 min), 55–100% (32–34 min), 100% (34–40 min). The flow rate was 0.9 mL min^{-1}, and the detection was performed simultaneously at five wavelengths—254, 290, 315, 360 and 410 nm.

HPLC analyses of the products of thermal degradation of KN and 3OHKN were performed using an Agilent LC 1100 chromatograph equipped with an quaternary pump, an autosampler, and a diode array detector. Separations were performed on a 4.6 × 150 mm ZORBAX Eclipse XBD-C8 column using a methanol/0.1% (v/v) TFA in a H_2O gradient. The methanol percentage in the gradient was 10–40% (0–15 min), 40% (15–20 min), 40–90% (20–25 min). The flow rate was 0.9 mL min^{-1}, and detection was performed simultaneously at five wavelengths—254, 290, 314, 360 and 406 nm. Chromatograms were recorded and the peak areas were integrated with the use of the Agilent ChemStation for Windows.

Free thiol determination (Ellman's reaction)

Ellman's reagent (5,5′–dithio-bis-(2-nitrobenzoic acid), DTNB) forms an adduct (2-nitro-5-mercaptobenzoic acid, TNB) in the presence of free thiols. The TNB ionizes to the TNB^- anion in

neutral and basic aqueous solutions, with a relatively intense absorbance at 412 nm (extinction coefficient 1.4×10^4 $M^{-1}cm^{-1}$.[44,45] The reaction is rapid and stochiometric, and the threshold of sensitivity is 0.6 nmol mL^{-1}.[46] Determination of the quantity of free GSH or Cys released during the incubation of KN-GSH and KN-Cys adducts was performed by addition of 2×10^{-3} M of DTNB. The solutions (pH 7) were kept for 20 min at room temperature, after which the optical absorption at 412 nm was measured.

Mass spectrometry, NMR, and UV–Vis analysis

Mass spectra of the adducts were obtained on an Agilent 1100 Series LC/MS instrument with a photodiode array and mass detectors. Data analysis was carried out using the Agilent ChemStation program. For the detection of oxidized glutathione (GSSG) in incubated solutions of KN-GSH adducts, mass spectra were acquired on an Autoflex II Bruker Daltonics MALDI-TOF Spectrometer in positive-ion reflector mode. The samples were prepared with the use of a saturated solution of CHCA matrix (70% acetonitrile, 0.1% TFA). ^{1}H NMR spectra were recorded on an Avance-300 Bruker NMR Spectrometer. UV–Vis spectra were obtained on an Agilent 8453 UV-visible Spectroscopy System.

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References

1 R. van Heyningen, *Ciba Found. Symp.*, 1973, **19**, 151–171.
2 A. M. Wood and R. J. W. Truscott, *Exp. Eye Res.*, 1993, **56**, 317–325.
3 A. M. Wood and R. J. W. Truscott, *Vision Res.*, 1994, **34**, 1369–1374.
4 R. J. W Truscott, A. M. Wood, J. A. Carver, M. M. Sheil, G. M. Stutchbury, J. Zhu and G. W. Kilby, *FEBS Lett.*, 1994, **348**, 173.
5 L. M. Bova, M. H. Sweeney, J. F. Jamie and R. J. W. Truscott, *Invest. Ophthalmol. Visual Sci.*, 2001, **42**, 200–205.
6 I. M. Streete, J. F. Jamie and R. J. W. Truscott, *Invest. Ophthalmol. Visual Sci.*, 2004, **45**, 4091–4097.
7 J. Mizdrak, P. G. Hains, D. Kalinowski, R. J. W. Truscott, M. J. Davies and J. F. Jamie, *Tetrahedron*, 2007, **63**, 4990–4999.
8 F. Moroni, *Eur. J. Pharmacol.*, 1999, **375**, 87.
9 L. M. Bova, A. M. Wood, J. F. Jamie and R. J. W. Truscott, *Invest. Ophthalmol. Visual Sci.*, 1999, **40**, 3237–3244.
10 L. M. Taylor, J. A. Aquilina, R. H. Willis, J. F. Jamie and R. J. W. Truscott, *FEBS Lett.*, 2001, **509**, 6–10.
11 L. M. Taylor, J. A. Aquilina, J. F. Jamie and R. J. W. Truscott, *Exp. Eye Res.*, 2002, **75**, 165–175.
12 L. M. Taylor, J. A. Aquilina, J. F. Jamie and R. J. W. Truscott, *Exp. Eye Res.*, 2002, **74**, 503–511.
13 L. V. Kopylova, O. A. Snytnikova, E. I. Chernyak, S. V. Morozov and Yu. P. Tsentalovich, *Exp. Eye Res.*, 2007, **85**, 242–249.
14 O. A. Snytnikova, A. Zh. Fursova, E. I. Chernyak, V. G. Vasiliev, S. V. Morozov, N. G. Kolosova and Yu. P. Tsentalovich, *Exp. Eye Res.*, 2008, **86**, 951–956.
15 H. Bloemendal, W. W. de Jong, R. Jaenicke, N. H. Lubsen, C. Slingsby and A. Tardieu, *Prog. Biophys. Mol. Biol.*, 2004, **86**, 407–485.
16 K. J. Dilley and A. Pirie, *Exp. Eye Res.*, 1974, **19**, 59.
17 S. Lerman and R. Borkman, *Ophthalmic Res.*, 1976, **8**, 335–353.
18 R. J. W. Truscott and R. C. Augusteyn, *Biochim. Biophys. Acta*, 1977, **492**, 43–52.
19 N. T. Yu, C. B. Barron and J. F. R. Kuck, *Exp. Eye Res.*, 1989, **49**, 189.
20 J. J. Harding, *Cataract, Biochemistry, Epidemiology and Pharmacology*, Chapman and Hall , London, 1991.
21 R. J. W. Truscott, *Exp. Eye Res.*, 2005, **80**, 709–725.
22 B. D. Hood, B. Garner and R. J. W. Truscott, *J. Biol. Chem.*, 1999, **274**, 32547–32550.
23 B. Garner, D. C. Shaw, R. A. Lindner, J. A. Carver and R. J. W. Truscott, *Biochim. Biophys. Acta*, 2000, **1476**, 265–278.
24 J. A. Aquilina and R. J. W. Truscott, *Biochim. Biophys. Acta*, 2002, **1596**, 6–15.
25 R. J. W. Truscott, *Int. J. Biochem. Cell Biol.*, 2003, **35**, 1500–1504.
26 Yu. P. Tsentalovich, O. A. Snytnikova, M. D. E. Forbes, E. I. Chernyak and S. V. Morozov, *Exp. Eye Res.*, 2006, **83**, 1439–1445.
27 J. A. Aquilina and R. J. W. Truscott, *Biochem. Biophys. Res. Commun.*, 2000, **276**, 216–223.
28 S. Vazquez, J. A. Aquilina, J. F. Jamie, M. M. Sheil and R. J. W. Truscott, *J. Biol. Chem.*, 2002, **277**, 4867–4873.
29 N. R. Parker, J. F. Jamie, M. J. Davies and R. J. W. Truscott, *Free Radical Biol. Med.*, 2004, **37**, 1479.
30 P. S. Sherin, Yu. P. Tsentalovich, O. A. Snytnikova and R. Z. Sagdeev, *J. Photochem. Photobiol., B*, 2008, **93**, 127.
31 J. Mizdrak, P. G. Hains, R. J. W. Truscott, J. F. Jamie and M. J. Davies, *Free Radical Biol. Med.*, 2008, **44**, 1108.
32 B. Garner, S. Vazquez, R. Griffith, R. A. Lindner, J. A. Carver and R. J. W. Truscott, *J. Biol. Chem.*, 1999, **274**, 20847–20854.
33 S. Vazquez, N. R. Parker, M. M. Sheil and R. J. W. Truscott, *Invest. Ophthalmol. Visual Sci.*, 2004, **45**, 879–883.
34 N. R. Parker, A. Korlimbinis, J. F. Jamie, M. J. Davies and R. J. W. Truscott, *Invest. Ophthalmol. Visual Sci.*, 2007, **48**, 3705–3713.
35 M. H. Sweeney and R. J. W. Truscott, *Exp. Eye Res.*, 1998, **67**, 587–595.
36 B. A. Moffat, K. A. Landman, R. J. W. Truscott, M. H. J. Sweeney and J. M. Pope, *Exp. Eye Res.*, 1999, **69**, 663–669.
37 T. Tokuyama, S. Senoh, Y. Hirose and T. Sakan, *J. Chem. Soc. Jpn.*, 1958, **79**, 752–761.
38 T. Tokuyama, S. Senoh, T. Sakan, K. S. Brown and B. Witkop, *J. Am. Chem. Soc.*, 1967, **89**, 1017–1021.
39 G. L. Ellman, *Arch. Biochem. Biophys.*, 1959, **82**, 70–77.
40 M. B. Smith, and J. March, *March's Advanced Organic Chemistry: Reactions, Mechanisms, and Structure*, Wiley-Blackwell , New York, 2007, p. 612 ff.
41 A. Korlimbinis and R. J. W Truscott, *Biochemistry*, 2006, **45**, 1950–1960.
42 A. Korlimbinis, J. A. Aquilina and R. J. W. Truscott, *Exp. Eye Res.*, 2007, **85**, 219–225.
43 V. N. Reddy and F. J. Giblin, *Ciba Found. Symp.*, 1984, **106**, 65–87.
44 P. W. Riddles, R. L. Blakeley and B. Zerner, *Anal. Biochem.*, 1979, **94**, 75–81.
45 P. W. Riddles, R. L. Blakeley and B. Zerner, *Methods Enzymol.*, 1983, **91**, 49–60.
46 C. K. Riener, G. Kada and H. J. Gruber, *Anal. Bioanal. Chem.*, 2002, **373**, 266–276.

