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Actions of ultraviolet light on cellular structures

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Summary. Solar radiation is the primary source of human exposure to ultraviolet (UV) radiation. Overexposure without suitable protection (i.e., sunscreen and clothing) has been implicated in mutagenesis and the onset of skin cancer. These effects are believed to be initiated by UV-mediated cellular damage, with proteins and DNA as primary targets due to a combination of their UV absorption characteristics and their abundance in cells. UV radiation can mediate damage *via* two different mechanisms: (a) direct absorption of the incident light by the cellular components, resulting in excited state formation and subsequent chemical reaction, and (b) photosensitization mechanisms, where the light is absorbed by endogenous (or exogenous) sensitizers that are excited to their triplet states. The excited photosensitizers can induce cellular damage by two mechanisms: (a) electron transfer and hydrogen abstraction processes to yield free radicals (Type I); or (b) energy transfer with O_2 to yield the reactive excited state, singlet oxygen (Type II). Direct UV absorption by DNA leads to dimers of nucleic acid bases including cyclobutane pyrimidine species and pyrimidine (6-4) pyrimidone compounds, together with their Dewar isomers. These three classes of dimers are implicated in the mutagenicity of UV radiation, which is typified by a high level of CC→TT and C→T transversions. Single base modifications can also occur *via* sensitized reactions including Type 1 and Type II processes. The main DNA product generated by $^1O_2$ is 8-oxo-Gua; this is a common lesion in DNA and is formed by a range of other oxidants in addition to UV. The majority of UV-induced protein damage appears to be mediated by $^1O_2$, which reacts preferentially with Trp, His, Tyr, Met, Cys and cystine side chains. Direct photo-oxidation reactions (particularly with short-wavelength UV) and radicals can also be formed *via* triplet excited states of some of these side chains. The initial products of $^1O_2$-mediated reactions are endoperoxides with the aromatic residues, and zwitterions with the sulfur-containing residues. These intermediates undergo a variety of further reactions, which can result in radical formation and ring-opening reactions; these result in significant yields of protein cross-links and aggregates, but little protein fragmentation. This review discusses the formation of these UV-induced modifications and their downstream consequences with particular reference to mutagenesis and alterations in protein structure and function.

Key words: DNA, free radicals, photoproducts, protein, singlet oxygen, ultraviolet.

Introduction

Nature of UV and solar radiation

UV light is defined as the region of the electromagnetic spectrum with wavelengths from 200 to 400 nm. This light is broken down into three distinct wavelength bands, known as UVC (*ca.* 200–280 nm), UVB (*ca.* 280–320 nm) and UVA (*ca.* 320–400 nm). As with all electromagnetic radiation, the shortest wavelength radiation (UVC) is the most energetic, and has the greatest potential for biological damage.

The major source of human exposure to UV light is *via* the sun. Solar radiation contains all three forms of UV radiation, but UV radiation with wavelengths below 295 nm (i.e., the entire UVC region) is absorbed by the Earth's upper atmosphere, and does not reach the Earth's surface. The UV light that does reach the Earth's surface comprises primarily (*ca.* 95%) UVA wavelengths, with the remainder (*ca.* 5%) comprising the shorter wavelength (295–320 nm) UVB radiation. This chapter will focus on the biological effects of UVB and UVA radiation, as these are the most biologically relevant [1, 2].

Mechanisms of UV-induced biological damage

UV radiation is capable of inducing biological damage *via* two discreet mechanisms [1–4]. Firstly, there is direct absorption of UV photons by the cellular material (particularly DNA or proteins) that can lead to photo-induced reactions. Secondly, there is the possibility of photosensitized processes, where UV light is absorbed by an endogenous or exogenous (in the case of therapeutic methods such as photodynamic therapy) sensitizer. The electronically excited sensitizer (typically in the triplet state) can harmlessly revert back to its ground state *via* intramolecular decay processes, or can damage other cellular material. Cellular damage can occur by two major pathways often called Type I and Type II mechanisms. Type I damage involves one electron oxidation or hydrogen atom abstraction from cellular targets, resulting in free radical formation. The Type II mechanism involves energy transfer from the molecule that originally absorbed the UV light (the sensitizer) to molecular oxygen, with the consequent formation of an excited state of oxygen – singlet oxygen. The latter species is a powerful oxidant that can undergo further reactions with cellular material.

Singlet oxygen is the first excited singlet state ($^1\Delta_g$ $^1O_2$) of molecular oxygen. This state, which has both electrons in the same molecular orbital with paired spins, is formed readily, being only *ca.* 94 kJ mol^{-1} above the ground triplet state ($^3\Sigma$), and has a relatively long lifetime of a few microseconds (reviewed in [2, 5, 6]). Other excited states can also be formed (e.g., the $^1\Sigma_g$ state, which has the two highest energy electrons in different orbitals with paired spins), but these are of higher energy and much shorter lived, and usually undergo rapid decay rather than chemical reaction [5]. The $^1\Delta_g$ state (henceforth denoted simply as $^1O_2$) is therefore the most important excited state of oxygen in biological systems. The transfer of energy from the original chromophore to molecular oxygen to give $^1O_2$ is rarely 100% efficient, and hence energy transfer to oxygen (Type II) and electron/hydrogen transfer (Type I) reactions usually occur simultaneously, and competitively. Thus, many photosensitizers give both $^1O_2$ and radicals such as $O_2^{\cdot-}$ [5]; the yields of species generated by these two processes are known to be dependent on the sensitizer, the excitation wavelength and the reaction conditions [5]. A number of reviews of the yields of $^1O_2$ and the rate constants for the reactions of these species are available [7, 8].

UVB radiation is responsible for the majority of damage resulting from direct absorption of UV light by cellular structures. The primary macromolecular structures that absorb UVB light are DNA and proteins, but a number of other low-molecular-weight materials (e.g. heme groups, carotenoids and vitamin A, eumelanin and phaeomelanin, pyridoxamine, urocanic acid; reviewed in [2]) can also be major chromophores for UVB radiation. The significance, and extent, of absorption by these other compounds is obviously tissue and situation dependent, with some cells and organs containing higher levels of these chromophores (either endogenously or as a result of exogenous addition). Thus, melanocytes in the skin contain particularly high levels of melanins and the retina particularly high levels of carotenoids and related species. The levels of particular chromophores within tissues can also be artificially raised by, for example, exposure to heme compounds or their precursors (e.g. 5-aminolaevulinic acid) in photodynamic therapy [2]. Other macromolecular structures such as lipids and polysaccharides do not have any major absorption bands in the UVB region, and thus do not undergo direct damage by UVB radiation.

It is well established (reviewed in [9–11]) that UVB radiation is both genotoxic and mutagenic, and plays a key role in DNA damage and skin cancer. Thus, there have been considerable efforts to develop sunscreens that protect the skin from UVB radiation. However, in the past many sunscreens have not offered protection against UVA radiation, and this has led to increased exposure of the population to UVA radiation, as the dose-limiting side effects of UVB exposure (e.g. sunburn) are attenuated [10]. The extent of human exposure to UVA radiation has also increased due to the use of sun beds and tanning salons as an alternative to sun exposure. These artificial tanning methods use light that is almost exclusively in the UVA region to induce skin tanning.

UVA radiation is typically less damaging than UVB light, but is directly absorbed to some extent by DNA and proteins. However, the majority of damage induced by UVA radiation is *via* photosensitization and Type I- and II-mediated mechanisms (reviewed in [4, 10–12]), resulting in free radical and $^1O_2$ formation, and ultimately oxidative damage to DNA, proteins and lipids. The processes that give rise to such damage, and its consequences, are reviewed below.

Consequences of UV irradiation on DNA

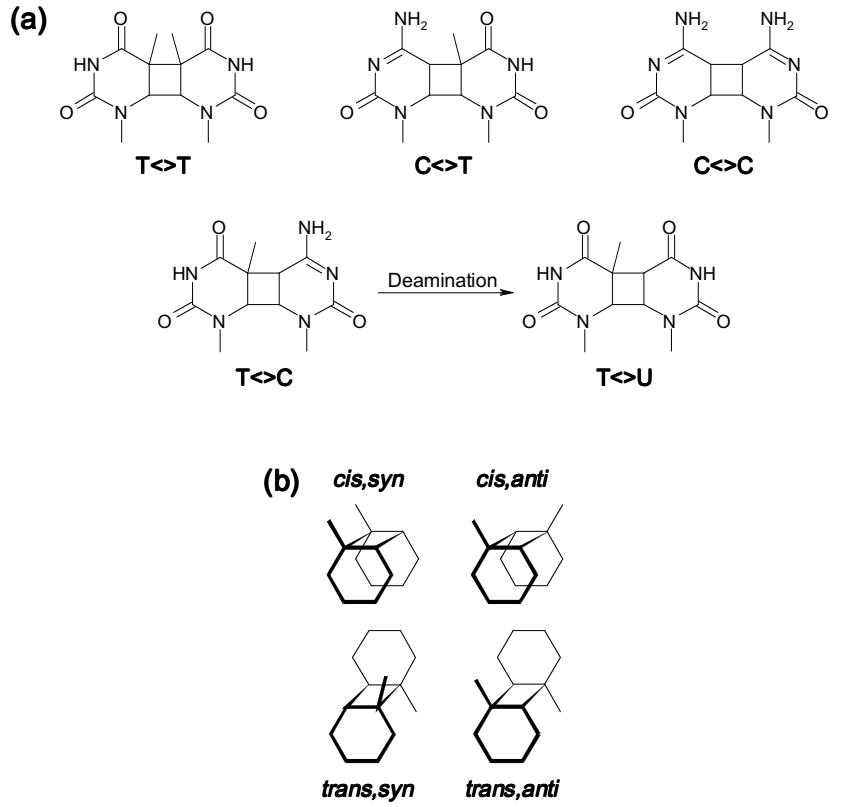
The UV absorption spectrum for DNA comprises a rising band in the far UV (<220 nm) with a further peak at 260 nm, which tails into the near-UVB wavelengths at around 300 nm [13]. The purine and pyrimidine bases are responsible for the absorption maximum at 260 nm [2], and it is these bases that dominate the UV photochemistry of DNA.

DNA photoproducts induced by direct UV absorption

The majority of products detected in DNA following UVB irradiation are pyrimidine products, as these bases absorb further into the near-UV region than the purine bases. Most of these products are photoadducts between adjacent bases to yield dimers (reviewed in [9, 12, 14, 15]). Typically these occur on the same DNA strand, but there is also evidence for inter-strand dimer formation in double-stranded DNA under certain conditions [16]. These lesions are described in detail below.

Cyclobutane pyrimidine dimers

Cyclobutane pyrimidine dimers (P<>P; Scheme 1a) are formed when a pyrimidine base in the triplet excited state undergoes a [2 + 2] addition to the C5–C6 double bond of a second pyrimidine base (reviewed in [12]). In free solution a



Scheme 1. Structures (a) of the cyclobutane pyrimidine dimers (P<>P) and the possible diastereoisomers (b) of T<>T.

mixture of diastereoisomers (Scheme 1b) is generated that differ in the orientation of the two pyrimidine rings relative to the cyclobutane ring, and on the relative orientations of the C5–C6 bonds in each pyrimidine base. In double-stranded DNA in its natural configuration (i.e. in the B form), where the dimer involves two adjacent pyrimidine bases on the same strand, only the *syn* isomers can be generated, and the *cis* isomer is greatly preferred over the *trans* isomer [12]. In single-stranded or denatured DNA, the *trans,syn* isomer becomes more prevalent due to the increased flexibility of the DNA backbone. Dimer formation (typically the *cis,syn* or *trans,anti* isomers) between the two strands of double-stranded DNA can also be detected in trace amounts in aqueous solutions with UVC irradiation [16]. However, in situations where a different DNA conformation is adopted (e.g., in 80% ethanol or in the dry state) the incidence of inter-strand dimers dramatically increases, as evidenced by the increased proportion of *anti* isomers [16].

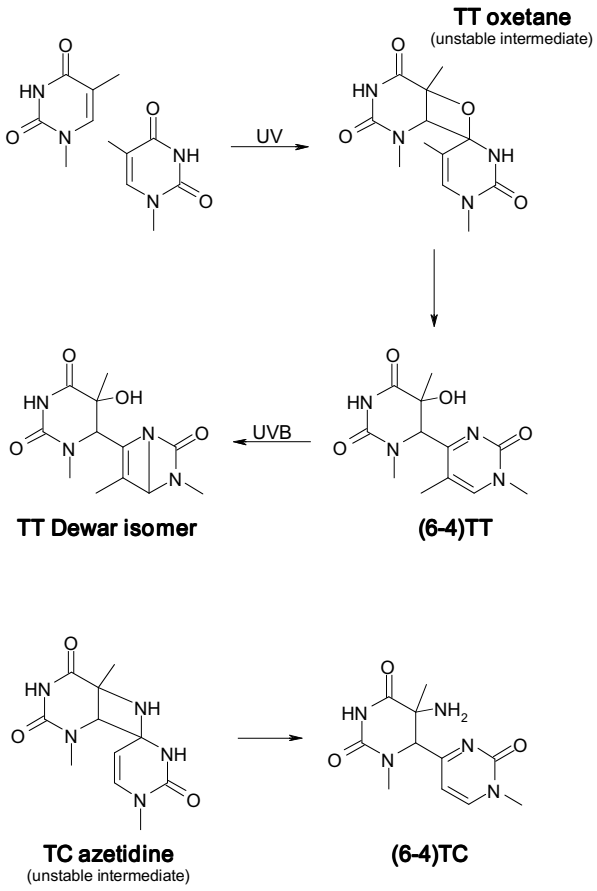
The P<>P dimers themselves are only mildly photoactive but under irradiation with UVC light the dimerization can be reversed by photo-induced splitting of the cyclobutane ring, to yield the original monomer bases [17, 18]. There are also a number of DNA repair processes invoked *in vivo*; in many organisms these include photolyase enzymes that are activated upon exposure to UV light (reviewed in [9, 13]).

For P<>P dimers that contain cytosine a further reaction can occur; due to the saturation of the C5–C6 bond in these products, they undergo deamination *via* hydrolysis of the C4 amino group to yield a carbonyl function (Scheme 1) [19, 20]. This results in the formation of uracil-containing products, for example, T<>C becomes a T<>U dimer. This has implications in their mutagenic properties, as discussed in the section 'Mutagenicity of the DNA lesions' [21].

Pyrimidine (6-4) pyrimidone dimers and their Dewar valence isomers

The formation of pyrimidine (6-4) pyrimidone dimers [(6-4)PP; Scheme 2] occurs when a pyrimidine base in its singlet excited state reacts *via* a [2 + 2] cycloaddition with a second pyrimidine base (reviewed in [12]). This process is similar to the formation of P<>P dimers, but the (6-4)PP adducts are generated by cyclization between the C5–C6 bond of a pyrimidine and the C4 carbonyl or imino groups (for thymine or cytosine, respectively) of its 3' neighbor. The resulting oxetane and azetidine products are unstable and rapidly rearrange to yield the (6-4)PP adducts, in which the carbonyl or imino group of the 3' base is transferred to the C5 position of the 5' base.

The UV spectra of the (6-4)PP dimers exhibit an absorption band that is shifted by *ca.* 50 nm from the native bases into the near UV region of the spectrum. Thus, upon exposure to UVB or UVA light, the (6-4)PP adducts are readily converted into their Dewar valence isomers (Scheme 2) [22, 23]. These Dewar isomers are only moderately photoactive, but can undergo reversion to the (6-4)PP adducts upon exposure to short-wavelength UV radiation [13].

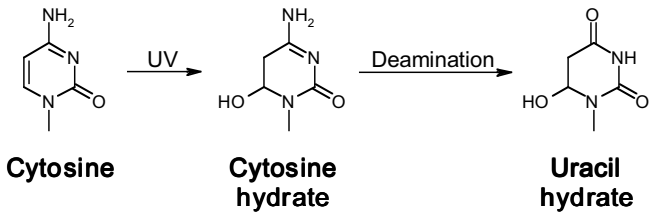


Scheme 2. Mechanism of formation of (6-4)TT and (6-4)TC dimers, and the isomerization of (6-4)TT to its Dewar isomer.

As with the P<>P dimers, (6-4)PP adducts containing cytosine (and the corresponding Dewar isomers) can undergo deamination reactions to yield uracil-containing adducts [24]. However, deamination can only occur when the cytosine residue is on the 5' side of the dimer, as the transfer of the amine group to the 5' base during adduct formation when cytosine is in the 3' position prevents deamination from occurring.

Monomeric pyrimidine photoproducts

Exposure of monomeric cytosine compounds to UV radiation has been shown to efficiently yield the hydrated product, 6-hydroxy-5,6-dihydrocytosine, commonly known as the “cytosine photohydrate” (Scheme 3) [25]. The formation



Scheme 3. The formation of "cytosine photohydrate" and its deamination to "uracil hydrate".

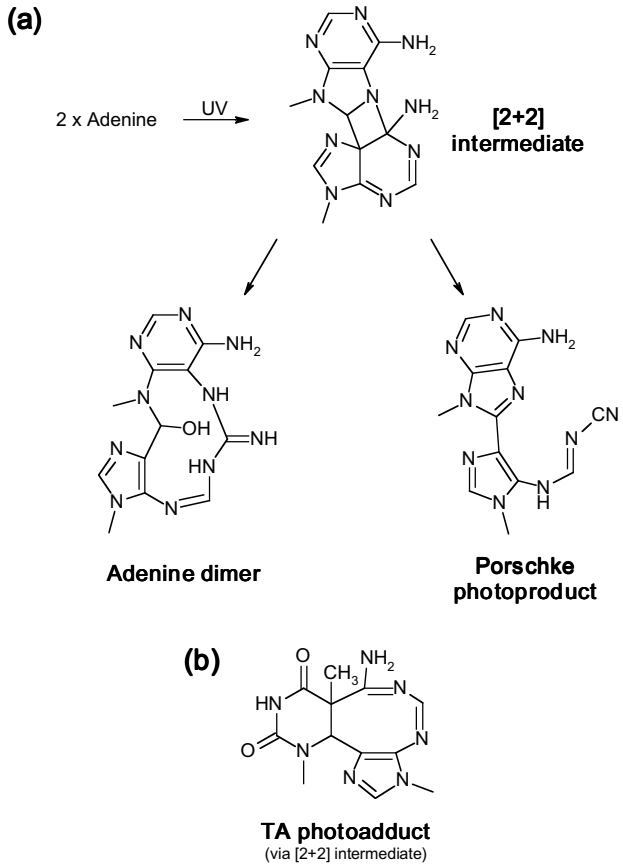
of this species has been proposed to involve the nucleophilic addition of H_2O to a low-lying vibrational level of the first excited singlet state [25]. This product is, however, unstable [25] and escaped detection in UV-exposed DNA for many years [26]. As with the dimeric compounds where the C5–C6 bond of cytosine is saturated, this material undergoes deamination to yield the uracil analogue (Scheme 3) [25]. The increased stability of the latter product has allowed the quantification of this material in isolated and cellular DNA [27]. These materials are, however, only minor products with their yields *ca.* 100 and 1000 times lower, in isolated and cellular DNA, respectively, than the P<>P adducts.

Photoproducts of purine bases

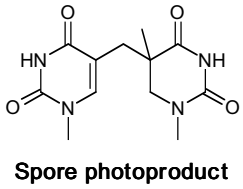
As described above, the primary targets for UV-induced DNA damage are the pyrimidine bases, but photodamage involving the purine bases also occurs. As observed with pyrimidine bases, dimeric products can be formed between two adjacent adenine residues [28, 29], or between adenine and a vicinal thymine base [30]. In both cases the primary intermediate is a [2 + 2] cycloadduct, which undergoes rearrangement reactions (Scheme 4). Exposure to UVC radiation forms the AA cycloadduct, which can undergo one of two rearrangement processes [28, 29, 31]. These lead to the formation of either an adduct containing a large ring structure (Scheme 4a), or to a structure where ring opening of one adenine residue has occurred (the Porshke photoproduct; Scheme 4a). For the TA photoproduct (generated by UVB radiation), rearrangement of the cyclobutane intermediate results in a product with an 8-membered ring structure (Scheme 4b) [30]. Thus, photochemistry of the purine bases can occur in DNA, but the quantum yields of these are very low [32], such that the yields of these products are almost negligible when compared to the various pyrimidine dimers.

Spore photoproduct

The spore photoproduct (Scheme 5) has been detected on exposure of bacterial spores to UV light [33, 34], but this material is only generated in high



Scheme 4. Mechanism of formation of the photoinduced adenine dimers (a) and the structure of the thymine adenine photoadduct (b).



Scheme 5. Structure of the spore photoproduct generated from two thymine bases.

yields *in vitro* by irradiating dry, isolated DNA with UVC light [35]. This dimeric material arises from addition of the methyl group of one thymine residue to the C5 position of a neighboring thymine. As this product requires

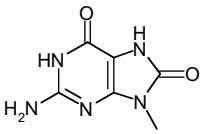
anhydrous conditions for its formation, it is of little relevance in most cellular environments.

Oxidized DNA photoproducts

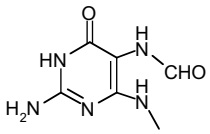
In addition to the direct absorption of UV radiation to yield the products outlined above, a series of oxidized DNA lesions can be generated by UV light. These are typically induced by the longer wavelength UVA radiation, and are mediated by photosensitized Type I (one electron oxidation or hydrogen atom abstraction) and Type II ($^1O_2$ oxidation) mechanisms (reviewed in [12]). For Type I oxidation mechanisms the base moieties of DNA are the favored sites of attack, with guanine the most susceptible due to its low ionization potential. After guanine, adenine is the second most susceptible base, followed by approximately equal reaction for thymine and cytosine. The primary intermediates generated by Type I mechanisms are the radical cations, which undergo rapid hydration or deprotonation. These processes have been reviewed extensively [12] and are outlined briefly below. Due to its low ionization potential, guanine is also the most susceptible base to oxidation by Type II, $^1O_2$-mediated, reactions.

Type I oxidation products of purine bases

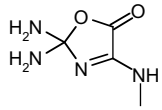
Hydration of the radical cation of guanine gives a reducing radical intermediate [36]. Under reducing conditions this radical is converted to 2,6-diamino-4-hydroxy-5-formamidoguanine (FapyGua; Scheme 6), but in oxidizing conditions (e.g., in the presence of O_2) it converts to 8-oxo-7,8-dihydroguanine (8-oxo-Gua; Scheme 6). The guanine radical cation can also deprotonate



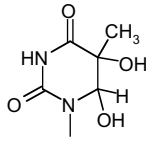
8-oxo-Gua



FapyGua



Oxazolone



Thymine glycol

Scheme 6. Chemical structures of the oxidized products of guanine and thymine.

(reviewed in [12]) and undergoes a complex series of reactions, ultimately generating a stable oxazolone product (Scheme 6).

The products generated on one electron oxidation of adenine are similar to those for guanine, with FapyAde and 8-oxo-Ade arising from hydration of the radical cation [36]. This is, however, only a minor pathway, with the major product, 2'-deoxyinosine, arising from deprotonation of the radical cation at the exocyclic amine function.

Type I oxidation products of pyrimidine bases

The pyrimidine bases are not major targets for Type I oxidation. However, when the radical cations of pyrimidine bases are generated, the most common reaction is hydration at the C6 atom, to yield a carbon-centered radical at C5 (reviewed in [12]). In the presence of O_2 this radical forms hydroperoxyl radicals and hydroperoxides that undergo a series of subsequent reactions to yield products including pyrimidine glycols (Scheme 6) and fragmented materials such as formamide. Deprotonation of the pyrimidine radical cations is a minor pathway, with this resulting in myriad products, including modified bases and dimers, *via* peroxyl radical intermediates (reviewed in [12]).

Type II oxidation of guanine

Guanine is the only DNA base that reacts rapidly with $^1O_2$ [7, 37]. The primary intermediates are endoperoxide species generated by cycloaddition reactions of the imidazole ring with $^1O_2$ [38]. The major decomposition product of these endoperoxides is 8-oxo-Gua (Scheme 6). This product can also be generated by a plethora of other oxidants, and thus is a poor marker for $^1O_2$ involvement in DNA damage.

Spectrum of DNA damage induced by different UV sources

Irradiation of isolated or cellular DNA with UVB light typically yields the same pattern of lesion formation. Many studies have shown by a variety of biochemical assays, such as immunoblotting or the use of repair enzymes and DNA sequencing, that the major class of UVB-induced damage to DNA is formation of P<>P, followed by (6-4)PP generation, and low levels of Dewar isomers (reviewed in [12]). The formation of oxidized bases does not occur to a major extent. Although the extent of formation of different classes of lesion can be assessed by gel-electrophoresis methods, this methodology does not yield information on the levels of specific dimers. Cadet et al. have developed HPLC-MS/MS assays that allow the quantification of individual dimer products [e.g., T<>T, C<>T, C<>C, (6-4)TT, (6-4)TC, and Dewar isomers]; this

approach has been expanded to allow the yield of inter-strand dimers, spore photoproducts, and pyrimidine photohydrates to be determined in isolated or cellular DNA [16, 27, 35, 39, 40]. These studies consistently show that T<>T is the most common dimeric product formed by UVB radiation, followed by similar yields of T<>C and (6-4)TC (with *ca.* half the frequency of T<>T formation) [40]. Interestingly, the overall yield of TT and TC dimers are very similar, but the proportion of P<>P:(6-4)PP differs for the two classes of dimers with *ca.* 10:1 for TT dimers, and 1:1 for TC dimers, respectively. The dimeric adducts [P<>P or (6-4)PP] at CT and CC sites were detected in much lower yields (from 5 to 10 times in cellular DNA) than the TT and TC lesions. In cellular studies, the total ratio of P<>P:(6-4)PP lesions was 3:1 [40], which is similar to that detected by other methods [41]. In isolated DNA, Dewar adducts were detected at low levels, but these were not detected in cellular DNA [40]. Similarly, oxidized bases such as 8-oxo-Gua are relatively minor products of UVB radiation, with yields that are two orders of magnitude lower than the P<>P dimers [42, 43].

In contrast to UVB exposure, exposure of cellular DNA to UVA radiation results in much lower levels of direct damage, with the observed lesions appearing to be predominantly mediated *via* sensitized reactions (reviewed in [12]). Thus, the major products generated by UVA would be expected to be oxidized photoproducts of purine bases such as 8-oxo-Gua. These materials are indeed present at higher levels in DNA exposed to UVA than UVB [43], but recent studies have shown that P<>P dimers are the major products of UVA damage, with these present at threefold greater levels than 8-oxo-Gua [43]. Despite being the most prevalent lesion, the P<>P dimers are formed at lower levels by UVA than UVB. Interestingly, the pattern of damage induced by UVA is different to that given by UVB, with T<>T lesions predominating, together with *ca.* 10% T<>C lesions [43, 44]. It has been shown that UVA radiation and aromatic ketone sensitizers (e.g., benzophenone) give a similar spectrum of damage *in vitro* [43], suggesting that unknown sensitizers within cells are responsible for the formation of these lesions *via* sensitized reactions, rather than direct UV absorption by DNA. The prevalence of T<>T lesions is probably a consequence of the more facile triplet energy transfer from endogenous sensitizers to T than C residues. In addition to these products, UVA radiation can also induce strand breaks [43]. These observations are consistent with a significant role for $^1O_2$-mediated reactions in UVA-induced DNA damage.

Exposure of cells to simulated sunlight, gives a damage spectrum that is similar, but not identical, to that observed with UVB [43]. Thus, the majority of DNA damage by sunlight is probably induced by direct UVB absorption. However, UVA-induced photosensitized reactions also play a role, with the levels of T<>T dimers and oxidized purine bases present at higher levels than with UVB alone. A further consequence of simulated sunlight is that the yield of (6-4)PP Dewar isomer lesions are increased relative to UVB irradiation, as UVA light readily promotes the isomerization reaction [22, 23, 43].

Mutagenicity of the DNA lesions

A series of repair mechanisms are available *in vivo* to minimize the impact of UV-induced DNA damage including, for example, excision repair and photoreactivation pathways (reviewed in [9–11, 13]). The most abundant mutations observed in DNA following exposure to UV radiation are C→T and CC→TT transversions, which are considered as fingerprints of solar-induced DNA damage [45, 46]. However, this mutation pattern does not match the product profile detected, where TT dimers are the most abundant. It has been suggested that the TT dimers are relatively non-mutagenic as DNA polymerase incorporates adenine residues by default opposite non-readable bases (reviewed in [10]). In the case of a TT lesion this does not alter the DNA sequence, but for a CC dimer this results in a mutation to a TT site, and for a CT or TC lesion, a C→T mutation occurs.

The increased mutagenicity at C sites has also been attributed to the ready deamination of cytosine residues that have their C5–C6 bond saturated in UV-induced dimers [21]. If DNA repair occurs following deamination of a cytosine site, this leads to the replacement of a C residue by a U site. This in turn leads to the incorporation of a T residue in place of a C residue once the DNA is replicated.

A further possibility for the discrepancy between the UV-induced damage and mutation spectra may be the relative rates of repair of the various lesions. The rate of excision/repair of (6-4)PP adducts is more efficient than for the P<>P dimers, but the corresponding Dewar isomers are much more resistant to repair [47]. Considerable evidence suggests that the P<>P dimers are responsible for the majority of the UV-induced mutagenicity (e.g. [43, 44, 48]).

8-Oxo-Gua is unlikely to be a major mutagenic lesion following UV irradiation, as it is expected to induce GC→TA transversions [49], and these are only minor mutations in UV exposed cells [45, 46]. These data are consistent with the hypothesis that the mutagenic events initiated by solar radiation arise predominately from pyrimidine dimers.

Consequences of UV irradiation on proteins

Proteins are major cellular targets for photo-oxidation due to their high abundance and the presence of endogenous chromophores within the protein structure (primarily amino acid side chains). Direct photo-oxidation arises from the absorption of UV radiation by the protein structure (primarily side chains), or bound chromophores (sensitizers). Direct absorption by the protein, or energy transfer from sensitizers to the protein (Type 1 processes) generate excited states (singlet or triplet) or radicals as a result of photo-ionization. Type II oxidation mechanisms involving $^1O_2$ also occur for proteins, with the formation of this oxidant sensitized by either protein-bound, or other endogenous chromophores.

Direct oxidation of amino acids, peptides and proteins by UV light is only a significant process if the incident light is absorbed by the protein. For most proteins without bound (covalent or non-covalent) co-factors or prosthetic groups, this only occurs with light with λ *ca.* $\leq$320 nm. The major chromophoric amino acids present in proteins are tryptophan (Trp), tyrosine (Tyr), phenylalanine (Phe), histidine (His), cysteine (Cys) and cystine; the UV spectra of these amino acids are given in [2]. All the other major amino acids do not absorb significantly at λ *ca.*>230 nm. Peptide bonds [-C(O)-NH-] exhibit a weak absorption band at 210–220 nm, which is usually observed as a shoulder on the long wavelength tail of the more intense band at *ca.* 190 nm. Thus, direct absorption of solar UV light ($\lambda > 290$ nm) by the protein backbone is negligible, and the direct photochemistry of proteins is dominated by the above amino acid side chains. The contribution of each side chain depends, amongst other factors, on their abundance within the target protein and the presence of other chromophores.

The indolic side chain of Trp has the longest wavelength ground state absorption spectrum, and also has a significantly greater molar absorption coefficient for each of its major absorption bands than the other species. Thus, Trp is the strongest chromophore in proteins, but the overall significance of the reactions that occur at this residue is reduced by the low abundance of Trp in many proteins. For example, in human serum albumin the Trp:Tyr ratio is only 1:18 [50], thus Tyr, Phe, His, Cys side chains and disulfide bonds become significant targets of UV-induced damage.

Protein photoproducts induced by direct UV absorption

The absorption of UV light by Trp, Tyr, His, Phe, Cys and cystine can give both excited state species and radicals *via* photo-ionization (reviewed in [2]). The major initial species is usually the first excited singlet state. Such excited singlet states are short lived, and readily lose energy *via* direct energy transfer to other groups, by collisional deactivation and vibrations, and *via* inter-system crossing to the triplet state. Most singlet states formed on proteins react *via* such energy transfer rather than *via* chemical (electron or atom transfer) reactions.

The triplet states of Trp, Tyr and Phe have been studied extensively as these are longer lived than the singlets and show chemical reactivity (i.e., can undergo electron, as well as energy, transfer reactions). The first triplet states of Trp (^{3}Trp), Tyr (^{3}Tyr) and Phe (^{3}Phe) have lifetimes on the microsecond timescale, and the (photo)physical properties of these triplets are well established (reviewed in [2, 4]).

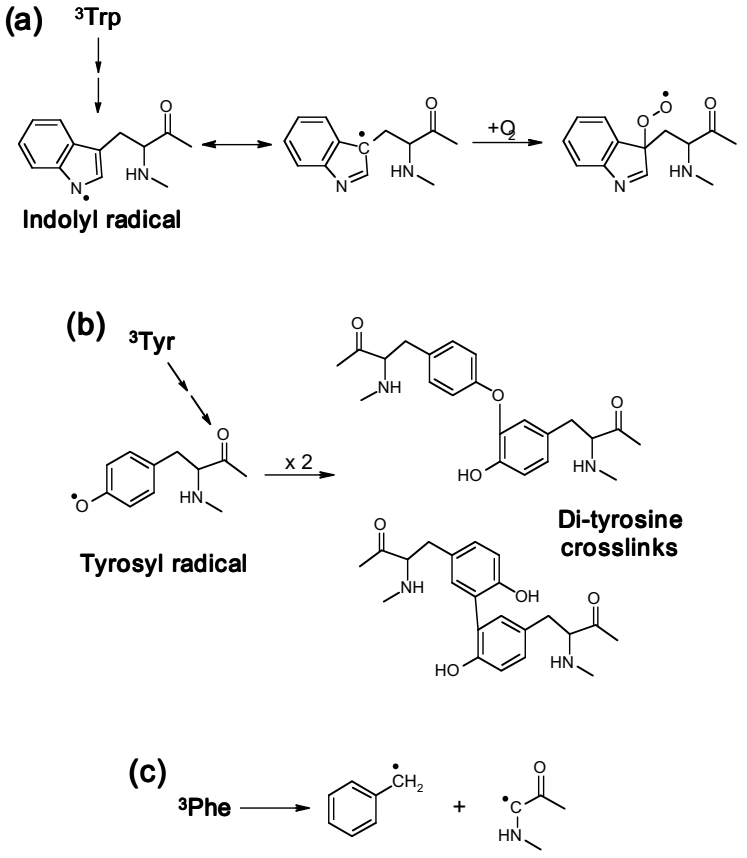
Reactions of Trp, Tyr and Phe triplet states

The first triplet states of Trp and Tyr undergo electron transfer reactions with suitable acceptors [51, 52], including disulfides (RSSR) such as lipoate and

cystine. This results in reduction to give the disulfide radical anion ($RSSR^{-\bullet}$) and the corresponding Trp and Tyr radical-cations ($Trp^{+\bullet}$ and $Tyr^{+\bullet}$; reviewed in [2]). These radical-cations rapidly deprotonate to give the neutral indolyl radical and phenoxyl radical, respectively (Scheme 7a and 7b) [53]. In contrast, the triplet of Phe undergoes direct photo-dissociation to yield a benzyl radical (Scheme 7c) [54].

The indolyl and phenoxyl radicals from Trp and Tyr, respectively, undergo further reactions [53, 55]. In the case of the indolyl radical, these include reaction with O_2 to give a peroxyl radical at position C-3 on the indolyl ring, which can undergo further hydrogen atom abstraction reactions (Scheme 7a) [56]. The phenoxyl radical of Tyr can undergo dimerization (*via* C-O and C-C linkages) to yield di-tyrosine products and hydrogen atom abstraction reactions (see Scheme 7b; reviewed in [55]).

The disulfide radical anions ($RSSR^{-\bullet}$) formed *via* reaction of ^{3}Trp or ^{3}Tyr with cystine can readily dissociate, in a reversible reaction, to give the anion



Scheme 7. Reactions of the triplet species formed by UV radiation of (a) Trp, (b) Tyr, and (c) Phe.

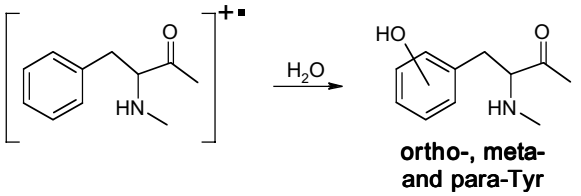
(RS^-) and a radical ($RS^{\cdot}$), or react with O_2 to give the superoxide radical ($O_2^{-\cdot}$) and regenerate the parent disulfide (RSSR) [4, 55]. Both $O_2^{-\cdot}$ and thiyl radicals undergo further reactions. The former primarily undergoes disproportionation to H_2O_2 and O_2 (spontaneously or catalyzed by superoxide dismutase) or one-electron reduction reactions of metal ions. The thiyl radicals typically react with a thiyl anion to regenerate a disulfide radical anion, or with O_2 to give a thiyl peroxyl radical ($RSOO^{\cdot}$) (reviewed in [55]). The resulting thiyl peroxyl radicals revert to thiyl radicals, or can isomerize to a sulfonyl radical [$RS(=O)O^{\cdot}$] and hence give rise to sulfonic (RSO_3H) and sulfinic acids (RSO_2H) (reviewed in [57, 58]).

In addition to the reactions described above, other molecules can also undergo rapid electron transfer reactions with the triplets of Tyr, Phe and Trp. Thus, ^{3}Tyr can be rapidly quenched by electron transfer with O_2, His, and Cys (reviewed in [1]). In each case ^{3}Tyr is converted to the phenoxyl radical, probably *via* the radical-cation and subsequent rapid deprotonation. The partner is converted to the radical anion, which undergoes further reactions (e.g., $O_2^{-\cdot}$) [55]. ^{3}Phe also reacts rapidly with O_2 to yield $O_2^{-\cdot}$ [4].

Formation of ^{3}Trp, ^{3}Tyr and ^{3}Phe also commonly occurs *via* sensitization mechanisms, where light absorption by cellular chromophores followed by energy transfer gives the triplet species that behave as outlined above. Triplet state chromophores can also induce direct electron/hydrogen atom transfer reactions. One-electron oxidation occurs primarily at Trp and Tyr as these side chains are the most readily oxidized, with Tyr the ultimate "sink" for oxidizing equivalents. One-electron reduction can occur at Cys, and also at carbonyl and protonated amine sites, although there is evidence for the rapid transfer of "free" electrons within protein structures with cystine groups being the ultimate sink for reducing equivalents. The transfer of oxidizing and reducing species within proteins and peptides has been the subject of considerable study, and has been recently reviewed [55, 59]. Direct hydrogen atom abstraction reactions mediated by high-energy triplet states of chromophores can occur with most protein side chains. These reactions usually yield carbon-centered radicals (or thiyl radicals from Cys) [55, 59].

Radical formation induced by direct UV absorption

In addition to the reactions of the triplet states of Trp, Tyr and Phe side chains, these residues, together with the His side chain, can also undergo direct photoionization processes. There is, however, some debate on whether these are mono- or bi-photonic processes, particularly when long wavelength UV light is employed ([51, 52, 60], reviewed in [2]). These processes yield the corresponding radical cations that, in the case of Trp and Tyr, undergo the processes described above (Scheme 7). The radical cation from Phe undergoes rapid hydration to yield hydroxylated ring products {*o*-, *m*- and *p*-Tyr (Scheme 8); reviewed in [53]}, though there is also evidence for deprotonation to yield ben-



Scheme 8. Hydration products of the Phe radical cation,

zyl radicals [53]. With Tyr, direct cleavage of the phenolic -O-H bond can occur, yielding the phenoxyl radical, a proton and a hydrated electron [2].

The hydrated electron (e^-_{aq}) produced by direct photo-ionization can add rapidly to O_2, to give $O_2^{-\bullet}$, which can, in turn, induce further protein damage. e^-_{aq}-mediated addition to free carboxyl groups (e.g., the C terminus, or Asp/Glu side chains) and amine groups (e.g., the N terminus, or Lys side chains) results in deamination and $H^\bullet$ elimination. Hydrated electrons also react with cystine to give the disulfide radical anion ($RSSR^{-\bullet}$), and with peptide backbone carbonyl groups yielding a radical anion that can subsequently give rise to backbone cleavage [4, 55, 61, 62].

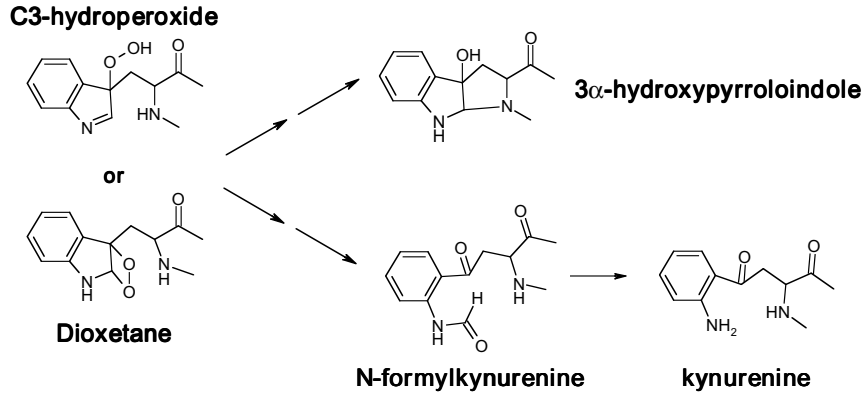
Protein photoproducts induced by $^1O_2$ reactions

As the rate constants for reaction of $^1O_2$ with protein side chains are higher than those with most other cellular targets [7], and proteins are present in most biological systems at particularly high concentrations, these are major targets for $^1O_2$ (reviewed in [3, 4, 6]). The majority of reactions of $^1O_2$ with proteins occur *via* reactions that result in chemical change, rather than quenching pathways that result in relaxation to ground state O_2 without inducing protein damage. Of the common amino acids present in proteins, Trp, His, Tyr, Met and Cys, react with $^1O_2$ at significant rates at physiological pH values [3–7]. At high pH, where Arg and Lys are in their neutral (unprotonated) forms, photo-oxidation also occurs at these residues [63]. Other amino acids can also be consumed as a result of indirect photo-oxidation processes, due to further reactions of $^1O_2$-induced intermediates at the above residues. These reactions have been implicated in cross-linking/aggregation of proteins [5]. The mechanisms and products that arise from $^1O_2$ reaction with these reactive side chains are reviewed below.

Reaction of $^1O_2$ with tryptophan residues

Reaction of $^1O_2$ with Trp gives both *N*-formylkynurenine (and hence kynurenine *via* hydrolysis) and 3α-hydroxypyrroloindoles, *via* the initial formation of

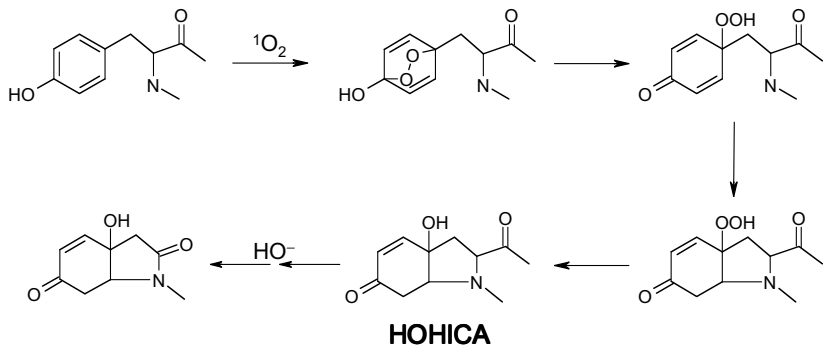
either a dioxetane across the C2–C3 double bond, or a hydroperoxide at C3 (Scheme 9) [64, 65]. Subsequent decomposition of these intermediates gives *N*-formylkynurenine, whereas ring closure yields 3α-hydroxypyrroloindole. Decomposition of these peroxides may involve non-radical reactions, or thermal homolysis of the -O–O- bond to give radicals. Metal ions, heat and UV light have been shown to catalyze peroxide decomposition to radicals; the mechanism and products of such reactions has been reviewed [65]. The overall process appears to be common to both the free amino acid and *N*-blocked Trp, and hence analogous materials are formed on oxidation of Trp oxidation in proteins. Interestingly, *N*-formylkynurenine and kynurenine are more effective photo-sensitizing agents than the parent amino acid [8], thus the formation of these materials on UV-exposed proteins may lead to enhanced photo-oxidation due to further generation of reactive species. This may be of particular significance in the human lens where it is known that free Trp oxidation products (e.g., kynurenine, 3-hydroxykynurenine, 3-hydroxykynurenine *O*-β-D-glucoside and 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid *O*-β-D-glucoside) can become bound to lens proteins (e.g., [66–68]) and subsequently act as photosensitizers of peroxide formation and further damage [69].



Scheme 9. Products of $^1O_2$-mediated oxidation of Trp.

Reaction of $^1O_2$ with tyrosine residues

With free Tyr, the primary products formed are unstable endoperoxides (Scheme 10), which react *via* ring-opening mechanisms to give a C1 hydroperoxide, and cyclized products involving nucleophilic addition of the α-amino group [70–73]. The endoperoxide species are unstable and rapidly decompose. This process can be catalyzed by metal ions and UV light to give radicals; the ultimate products of these reactions are unclear [72, 74]. Thermal decay gives rise to a cyclized indolic product, 3a-hydroxy-6-oxo-2,3,3a,6,7,7a-hexahydro-



Scheme 10. Products of $^1O_2$-mediated oxidation of Tyr.

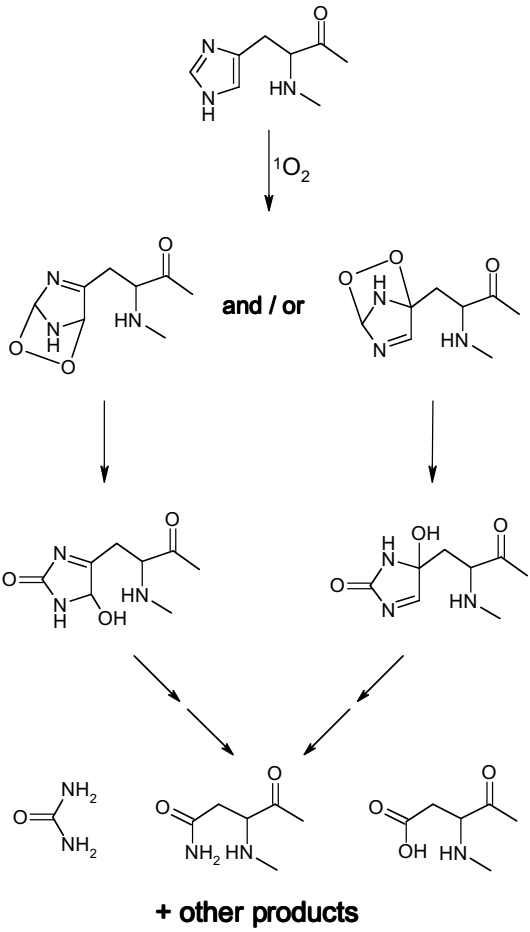
1H-indol-2-carboxylic acid (HOHICA; Scheme 10) [70–73]. This product can be oxidized further in basic conditions, giving rise to a decarboxylated keto compound [70, 71].

In peptides and proteins, the nucleophilic ring closure reactions of the α-amino group are less favorable (due to its incorporation in the peptide bond) allowing other nucleophilic additions to occur (A. Wright, C.L. Hawkins and M.J. Davies, unpublished data); these processes could play an important role in protein cross-linking. Previous studies have reported that 3,4-dihydroxyphenylalanine and di-tyrosine are not major products of $^1O_2$-mediated oxidation of Tyr on proteins [75, 76]; more recent data have suggested the opposite [69].

Studies with Tyr-containing peptides have shown that there is an increase in the yield of free amino groups on reaction with the tri-peptide Tyr-Tyr-Tyr, little change for Tyr-Tyr, and a loss of amine groups for Tyr itself [70]. These observations allude to the occurrence of peptide bond cleavage [70]. This may arise as a result of radical formation on peroxide decomposition, with these species subsequently abstracting a hydrogen atom from the backbone α-carbon site; α-carbon radicals are known intermediates in peptide bond cleavage [55]. Direct evidence for intermolecular hydrogen atom abstraction by similar peroxide-derived radicals has been obtained by EPR spectroscopy (A. Wright, C.L. Hawkins and M.J. Davies, unpublished data).

Reaction of $^1O_2$ with histidine residues

Oxidation of His by $^1O_2$ occurs *via* the initial formation of one or more endoperoxides (Scheme 11) and the consumption of a single molecule of O_2 per mole His [77]. The structure of these endoperoxides has been determined by low temperature NMR in organic solvents [78, 79]. At higher temperatures they undergo a series of ill-defined reactions to give a complex mixture of products which include aspartic acid, asparagines and urea (Scheme 11) [77,



Scheme 11. Products of $^1O_2$-mediated oxidation of His.

80]. The mechanisms probably involve radical processes and ring-opening reactions [74, 81]. The final products of His oxidation can undergo further reactions [82, 83], including the formation of His-His and His-Lys cross-links *via* the reaction of a nucleophilic nitrogen on one His ring, or a Lys side chain, with a keto group on a second oxidized His. Endoperoxide formation appears to occur regardless of whether the α-amino group is free or blocked, so it is likely that similar materials are generated on proteins.

Reaction of $^1O_2$ with methionine residues

Reaction of free Met with $^1O_2$ occurs *via* the formation of a zwitterionic species ($R_2S^+–OO^-$), which undergoes subsequent reaction with a second mole-

cule of the parent to give two moles of the sulfoxide ($R_2S=O$) [84] (reviewed in [5]). With some sensitizers, other intermediates including a stable nitrogen-sulfur cyclic intermediate have been reported (reviewed in [5]). Subsequent hydrolysis of this species yields 1 mol of sulfoxide and 1 mol of H_2O_2 [84]. This type of reaction may only be of significance with free Met, due to the involvement of the free amino group.

With free Met, the stoichiometry of molecular O_2:Met consumption is pH dependent (reviewed in [6]). The stoichiometry and intermediates involved in the corresponding reactions on proteins remain to be fully elucidated, although it is clear that methionine sulfoxide can be a major product.

Reaction of $^1O_2$ with cysteine and cystine residues

Rapid, but non-quantitative generation of the disulfide (RSSR) occurs when free Cys reacts with $^1O_2$ [5, 85]. Other products are formed, probably including cysteic acid (RSO_3H), but these have not been fully elucidated [5].

It has been suggested that reaction of $^1O_2$ with free cystine occurs *via* a zwitterion ($RS^+–OO^-$), similar to that with Met [86]. This species probably reacts with a further molecule of cystine yielding two molecules of the mono-sulfoxide RSS(=O)R. The occurrence of these reactions in proteins, where the 3-D structure is likely to constrain the cystine molecules, remains to be determined.

Physical and chemical consequences of photo-oxidation of proteins

A range of enzymes have long been known to be inactivated on exposure to UV light [87], and a considerable number of studies have reported similar results (e.g., [2, 5, 6]). These studies have shown that much of the photochemistry described above for free amino acids and peptides, also occurs with proteins. Thus, the major amino acids consumed on photo-oxidation are His, Trp, Met, Tyr and Cys, although a few studies have also reported the loss of Phe, Arg and Lys residues. With proteins, which contain all of these residues, His and Trp are usually the most susceptible (e.g., [5]), although there are some exceptions, as would be expected on the basis of the rate constants. It has been shown that photo-oxidation of proteins can give rise to a number of reactive species (reviewed in [3]) including protein peroxides, with these probably localized on His, Trp and Tyr residues. Similar protein-bound peroxides have been detected in a number of cell types exposed to visible light and sensitizers (e.g., Rose Bengal) that generate $^1O_2$ ([88], Policarpio, V. and Davies, M.J., unpublished data). Thus it is likely that exposure of cells to UVA will also generate intracellular protein (and other) peroxides.

The formation of high-molecular-weight aggregates (dimers and higher species) is a common consequence of photo-oxidation of proteins [89]. Some of

these aggregates may arise from radical-radical termination reactions of two Tyr-derived phenoxyl radicals to give di- (or bi-) tyrosine [69, 90], although other reports have suggested that di-tyrosine is not formed [75, 76]. Other reactions probably play a key role in the formation of aggregates, and it has been proposed that many cross-links arise as a result of "dark" reactions that occur after the cessation of light exposure [91]. It has been suggested that cross-links are formed as a result of the oxidation of His residues to products, which then react with Lys, Cys or other His residues [75, 82, 83, 92, 93]. Studies on His derivatives have shown that carbonyl-containing materials are formed [77], and an increase in the yield of (unspecified) protein carbonyls has been reported on photo-oxidation of albumin by porphyrin sensitizers [94, 95]. It is possible that these cross-links arise from reaction of carbonyl compounds, formed by photo-oxidation, with Lys, Arg and Cys side chains. The formation of such cross-links may explain the observed loss of Lys and Arg residues in some photo-oxidized proteins (reviewed in [5]). His residues may be of particular importance in cross-link generation, as such links have been reported to be absent from photo-oxidized proteins that lack this amino acid [75, 76].

In contrast to the prevalence of reports on protein aggregation, there are relatively few reports of backbone cleavage (i.e., fragmentation), at least with short UV exposure times [5, 96]. Photo-oxidation of lysozyme in the presence of a sensitizer has been reported to give peptide fragments as a result of backbone rupture; this has been proposed to occur either *via* oxidation of Trp residues and/or the formation of radicals [97]. Backbone α-carbon radicals are known to be key intermediates in backbone cleavage in the presence of O_2 (reviewed in [4, 59, 62]), and peroxides formed on His and Tyr residues have been shown to yield such radicals in small peptides (Wright, A., Hawkins, C.L. and Davies, M.J., unpublished data) [72, 74].

Photo-oxidized proteins can behave in a markedly different manner to their non-exposed counterparts. Changes include: an increase in susceptibility of the oxidized protein to proteolytic enzymes; alterations in mechanical properties (e.g., of silk and collagen); an increased extent, or susceptibility to, unfolding; changes in conformation; an increase in hydrophobicity; altered light scattering properties and optical rotation; and changes in binding of co-factors and metal ions (e.g., [5, 75, 93, 98, 99]). The physico-chemical bases for these changes are not fully understood, although studies on apo-horseradish peroxidase, which is highly resistant to photo-oxidation, have given useful information. The 3-D protein structure appears to shield most of the susceptible residues from damage [98, 100]; in the holo enzyme the heme iron also appears to play a protective role.

It has been demonstrated that the photo-oxidation of DNA and proteins are not ends in themselves, and that damage to these macromolecules can initiate or exacerbate damage to other molecules. Thus, although DNA and proteins may be the major initial sites of UV-induced damage, the formation of oxidation products on these targets can give rise to secondary damage. It has, for example, been shown that peroxides formed on peptides and proteins can sub-

sequently oxidize susceptible residues on other proteins (and thereby give rise to enzyme inactivation [101, 102]), can deplete low-molecular-mass antioxidants [103], and can also give rise to the formation of oxidized DNA bases, strand breaks and DNA-protein adducts [104–106]. Decomposition of these peroxides to radicals may also initiate lipid oxidation chain reactions and thereby result in significant membrane damage.

Summary and conclusions

The interaction of UV radiation with cells leads primarily to the modification of DNA and proteins, due to a combination of their UV absorption characteristics and abundance in cells. In DNA, several dimeric products between nucleic acid bases are generated by direct UV absorption, together with single base modifications resulting from photo-ionization and radical reactions. A series of products is also generated *via* sensitized reactions, resulting in radical formation or $^1O_2$ generation. The primary product from reaction of $^1O_2$ with DNA is 8-oxo-Gua; this is a common product following DNA oxidation by a variety of oxidants. The dimeric products are strongly implicated in the mutagenicity observed in UV-irradiated DNA, which is typified by a high level of CC→TT and C→T transversions.

In proteins, the majority of UV-induced damage appears to be mediated by $^1O_2$, which reacts preferentially with the side chains of Trp, His, Tyr, Met, Cys and cystine residues, although direct photo-oxidation reactions and radical species may also play a role, particularly with short wavelength UV. The initial products of $^1O_2$-mediated reactions are endoperoxides with the aromatic residues, and zwitterions with the sulfur-containing residues. These species undergo a variety of further reactions, including radical processes and ring-opening reactions that ultimately lead to disruption of protein structure. Cross-linking and aggregation of proteins predominates over fragmentation processes for $^1O_2$-mediated oxidation.

The chemical manifestations of UV radiation on proteins and DNA are now reasonably well characterized, although there is still considerable work to be done in elucidating the mechanisms of these reactions and their relative importance in different scenarios (e.g., different UV wavelengths, cellular *versus* isolated environments, pH). It is also clear that initial damage to one molecule can subsequently give rise to significant secondary damage and have major biological ramifications (e.g., DNA mutations in the early stages of carcinogenesis, disruption of cellular and tissue function by, for example, protein cross-linking and enzyme inactivation); these aspects have yet to be fully elucidated.

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