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Direct Radiation Effects on the Structure and Stability of Collagen and Other Proteins

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In this review, recent progress in understanding the direct effects of radiation on the structure and stability of collagen, the most abundant protein in the human body, and other proteins is surveyed. Special emphasis is placed on the triple-helical structure of collagen, as studied by means of collagen mimetic peptides. The emerging patterns are the dose dependence of radiation processes and their abundance, the crucial role of radicals in covalent-bond formation (crosslinking) or cleavage, and the influence of the radiation energy and nature. Future research should allow fundamental questions, such as charge transfer and fragmentation dynamics triggered by ionization, to be answered, as well as developing applications such as protein-based biomaterials, notably with properties controlled by irradiation.

1. Introduction

Proteins are crucial for cellular functions of living organisms, in which proteins, for instance, catalyze chemical reactions, perform signal transduction, transport ligands, allow motility, and replicate DNA. Moreover, mechanical properties of the extracellular matrix (ECM), such as stiffness and elasticity, and ECM functions (intercellular communication, cell adhesion, tissue protection, etc.) are mediated by proteins. The ECM is mainly composed of glycoproteins, proteoglycans, polysaccharides, elastin, and collagen. The last of these is the major protein in the ECM, and provides most of its frame and stiffness, through collagen supramolecular structures made of assemblies of fibers. Collagen is also the most abundant protein in the human body, present in a wide range of different (mostly connective) tissues, and the main component of cartilage, tendons, ligaments, and skin. Characteristic properties of collagen, such as primary to quaternary structures, stability, and mechanical properties, are tissue specific and, to date, 27 unique collagen types have been identified in vertebrates. Common to all of these members of the collagen family is the presence of long sequences (from 100 to 1000 residues) made of repeated XX′G triplets (G is glycine; X and X′ are any other amino acid). These sequences form the triple-helical secondary structure that is typical of collagen, but which can also be found in other proteins, such as complement protein C1.[1] The structure–function relationship is particularly important for collagen, and it appears at different scales from molecular to macroscopic. The length of single collagen proteins can vary from 14 to 2400 nm,[2] fibril lengths are on the micron scale, and fibers can extend to the millimeter range.[3] This fibrous structure is even visible in tissues such as tendons, and drove very early interest in deciphering the molecular basis of their mechanical properties. Indeed, in 1938, the first structural study on collagen reported on X-ray diffraction (XRD) analysis of a rat tendon.[4] Later, a structure with better resolution was reported and is shown in Figure 1. The triple helix is composed of three protein strands tightly wound around each other, with all peptide bonds in *trans* configuration; thus allowing for a strong

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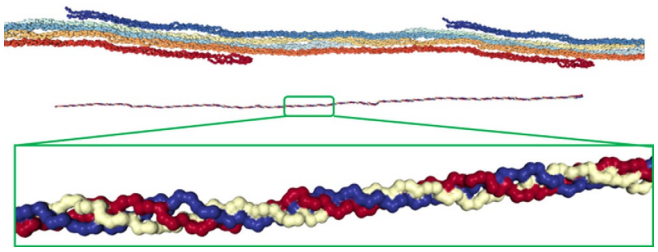

Figure 1. Crystal structure of collagen from an XRD experiment on a tendon of *Rattus norvegicus* (PDB ID: 3HQV).[8] Top: Each triple helix in the fibril is depicted in rainbow colors, from one end to another. Middle: a single triple helix is shown; a magnified image is shown at the bottom in which each protein is colored differently.

interstrand hydrogen-bonding network between backbone amide groups that are commonly referred to as Rich and Crick II.[5] Each of the strands has a polyproline II secondary structure, which is consistent with the very high (ca. 20%) proline content in collagen triple-helical domains. An unusually high fraction of these proline residues located at the X′ position are post-translationally modified by substituting one side-chain H atom by one OH group. The resulting hydroxyprolines (denoted O in the amino acid letter code) are known to stabilize the triple helix.[6] Mandatory for the existence of the triple-helical structure is a one-residue staggering of the three strands, as well as the presence of glycine every three residues, because it has the smallest side chain of all amino acids, and therefore, sterically allows the three backbones to be closely bound. This requirement is so important that mutations of this glycine (called collagen interruptions) can lead to destabilization of the helix; collagen misfolding; and the emergence of diseases, such as osteogenesis imperfecta.[7]

Mutations in proteins can be induced by external factors; therefore, probing the response of biological matter under their influence is crucial for understanding the way living organisms survive and adapt to their environment. In particular, biological systems have always been interacting with radiation, especially light from the sun. Advanced medical techniques use nonionizing and ionizing radiation, such as X-rays in radiography, computed tomography (CT), mammography, angiography, and fluoroscopy. Higher energy (MeV) X-rays are also employed for the treatment of certain types of cancer by radiotherapy because these photons are able to penetrate the body and kill tumor cells. Ion beams (mainly protons, but also carbon ions) at MeV energies are also used in hadron therapy. This technique has been receiving growing interest over the last decades because of its advantages over radiotherapy, in terms of precise targeting of the tumor, superior ballistic properties, and biological efficiency to kill cells.[9] Another way of targeting cancer cells is to attach radioisotopes to ligands that specifically bind to membrane receptors: this is called radioligand therapy. Radiopharmaceuticals can also be used for molecular imaging, in positron emission tomography or single-photon emission CT. UV light, X-rays, and gamma rays, as well as electron beams, are also routinely used to sterilize food or medical products.[10]

To understand the effects of ionizing radiation on proteins, it is crucial to control the experimental conditions, especially in terms of temperature and phase. Irradiation of a solution at room temperature leads to the formation of free radicals from the solvent (e.g., hydroxyl from water) as the main primary products. These species then chemically react with proteins, leading to secondary processes, such as backbone cleavage and, in some cases, aggregation of the fragments formed;[11] crosslinking between proteins to form larger systems; or even nanoparticles with diameters in the 10 nm range,[12] and generally quenching or enhancement of biological activity.[13] The effect on activity is thought to be due to a radioinduced conformational change, in accordance with other reports that provide evidence for the unfolding of proteins after irradiation.[14,15] All of these indirect effects require diffusion of free radicals from the solvent to the protein, which occurs at rates that decrease by several orders of magnitude from room to cryogenic temperatures.[16] This free-radical-mediated mechanism is the reason why the radioinduced loss of protein activity with rising temperature does not depend on the protein in solution. Therefore, freezing proteins allows the direct effects of radiation to be probed. Studies aimed at understanding these effects were reviewed more than 10 years ago.[16,17] At that time, no gas-phase studies on proteins had been performed; thus lyophilized, crystallized, and dried or frozen samples were irradiated and direct effects analyzed thanks to experimental techniques such as radiation target analysis and capillary electrophoresis; IR, UV, and electron paramagnetic resonance (EPR) spectroscopies; or gas chromatography. For instance, the last two have been employed to investigate homopolymers of amino acids, and it was established that the loss of side chains occurred regardless of the amino acid, whereas backbone cleavage was quenched in the case of tyrosine and phenylalanine, presumably due to radical trapping at their aromatic rings.[18] In proteins, cleavages also occur, and their locations on the backbone have been found to be random for some, but specific for others, mostly nonmembrane proteins. For instance, fragmentation is more likely at loops and turns between α-helices and β-sheets of aspartate transcarbamylase.[19] Interestingly, covalent-bond cleavage does not necessarily induce loss of the secondary, tertiary, or quaternary structure of a protein.[16] However, in some cases, irradiation leads to separation of noncovalently bound protein subunits.[20] This has been attributed to energy transfer between subunits after irradiation, as in β-galactosidase, which is active as a tetramer, but inactivated after fragmentation in monomers.[21]

Although some aspects of the structure and stability of collagen, as well as direct effects of radiation on proteins, were described ten years ago, other points remained poorly understood, as, for instance, Kempner[16] wrote, "the nature of molecular damage as a function of energy deposited in the primary ionization deserve[d] further investigation". In this review, we survey the efforts undertaken during the last decade towards a description and understanding of direct effects on the primary, secondary, and tertiary structure, as well as the stability of proteins, especially collagen, upon irradiation by photons, ions, and electrons.

2. Structure and Stability of Collagen Mimetic Peptide (CMP) Assemblies

A large amount of information on the structural properties and stability of collagen has been gained by studying systems smaller than entire collagen proteins or even collagen fragments. CMPs are, for instance, easier to crystallize for XRD studies. As a consequence, better resolution, and thus, more precise structural details, can be achieved. CMPs can be synthesized to include any mutation in a desired amino acid sequence. It was demonstrated that the $(PPG)_{10}$ sequence formed stable triple helices in solution[22] and in a crystal.[23] The latter study clearly revealed hydrogen bonds linking a given peptide backbone to the two others (Figure 2).

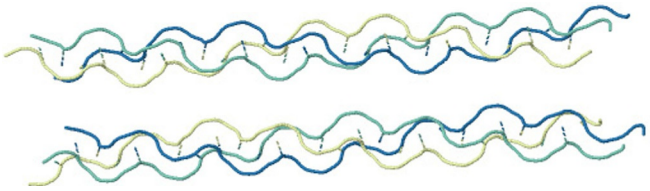

Figure 2. Crystal structure of the two triple helices of the $(PPG)_{10}$ CMP (PDB ID: 1K6F).[24] Each peptide of a given triple helix is depicted in a different color, and intermolecular hydrogen bonds are drawn in dashes.

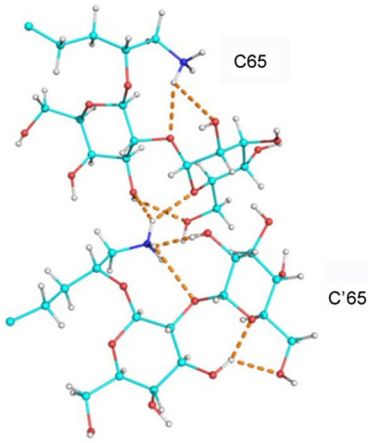

Figure 3. Proposed structure of a region of a peptide trimer mimicking the collagenous domain of adiponectin, showing hydrogen bonds (in dashes) between glycosylated hydroxylysine residues C65 and C′65. Carbon, nitrogen, oxygen, and hydrogen atoms are depicted in cyan, blue, red, and white, respectively. Adapted from ref. [37] with permission. Copyright: Wiley, 2016.

The melting temperature, T_m, and increase of enthalpy, ΔH°, for denaturation of $(PPG)_{10}$ are reported to be $T_m = 24.5\,^\circ C$ and $\Delta H^\circ = 150–180\ kJ\,mol^{-1}$.[25] Hydroxylation of all prolines in the Y position leads to the $(POG)_{10}$ peptide, which forms an even more stable triple helix, with $T_m = 56.9\,^\circ C$ and $\Delta H^\circ = 370–390\ kJ\,mol^{-1}$. This is consistent with stabilization observed for triple-helical domains of collagen proteins, even if their hydroxyproline content is lower than that of $(POG)_{10}$. Contradictory claims have been reported about the role of hydroxyproline in the X position.[26,27] The mechanism of triple-helix stabilization by hydroxyproline is not based on direct intrahelix hydrogen bonds involving the OH group of its side chain because the crystal structures show no such hydrogen bonds. Instead, a water-mediated hydroxyproline backbone hydrogen-bond network is observed, similar to the case of hydration networks in collagen fibrils.[28] However, a series of further studies (see reviews by Bella[29] and Shoulders and Raines[6] for details) provided evidence that intrapeptidic stereoelectronic effects involving the hydroxyproline OH were mainly responsible for triple-helix stabilization.[30,31] A totally different interaction is responsible for stabilization through cystine knots: the intermolecular covalent bond between two sulfur atoms of cysteine residues.[32] Another crucial stabilizing interaction in collagen was discovered thanks to CMP: interstrand salt bridges between lysine and aspartic or glutamic acid residues.[33] Their strength is due to Coulombic attraction between deprotonated acids and protonated lysine side chains. The requirement for this interaction to exist is the presence of KGE or KGD sequences.[34] Although local effects have been successfully applied to predict the stability of synthetic CMP,[33] extending this to collagen proteins is more delicate. Indeed, suppression of a single salt bridge can have long-range impacts, such as micro-unfolding of a proline-poor region of the triple helix, as demonstrated by Xu et al.[35] Experiments on CMPs have shown that these regions lacking hydroxyproline or proline can form a stable triple helix, if they contain another modified residue: O-glycosylated threonine.[27] This modification is covalent binding of a polysaccharide group to the side-chain oxygen of a threonine residue. It explains the stability of some cuticle collagens.[36] Recently, it has been proposed that intermolecular hydrogen bonds between glycosylated hydroxylysine residues stabilize triple-helical domains of adiponectin (Figure 3).[37] Interestingly, Huang et al.[38] showed that glycosylated hydroxyproline slightly destabilized triple-helical CMPs, but increased their assembly rate; this might be due to these intermolecular interactions. Therefore, the way glycosylation influences the triple helix seems to be different from that of hydroxyproline. We recently shed light on the role of the latter, by performing experiments on the structure and stability of CMPs in the gas phase.

The intrinsic structural properties of collagen-related systems were only scarcely investigated: IR[39] and rotational[40] spectroscopy experimental studies, focusing on stereoisomers of hydroxyproline, found that stereoelectronic effects stabilized the collagen-relevant isomer. Collision-induced dissociation coupled to mass spectrometry has been used to determine the glycosylation site of collagen-related glycopeptides.[41] Assemblies of whole type I, III, and V collagen proteins have been put in the gas phase by means of matrix-assisted laser desorption ionization (MALDI) and detected by mass spectrometry.[42] The polyproline II structure of small collagen-related isolated peptides was investigated by means of DFT,[43] but, before our recent work,[44] there was no report on triple-helix models in the gas phase. Therefore, we applied tandem ion mobility coupled to mass spectrometry to unravel the intrinsic structure and stability of these systems. Mass spectrometry allows perfect control of the stoichiometry of the molecular assembly. Ion mobility spectrometry consists of measuring the arrival time of molecular ions after a drift in a tube filled with rare gas: at a given charge state, a more extended conformation results in a higher number of collisions with gas, and thus, the arrival time. CMP assemblies were put in the gas phase thanks to electrospray ionization; a technique that does not damage thermally fragile molecular systems. First, we have shown that protonated $(PPG)_{10}$ and $(POG)_{10}$ peptide trimers are triple helical in the gas phase, if they contain more than seven protons. Thus, water is not required for the triple helix to exist. Furthermore, activation of these systems by low-energy collisions with helium demonstrated that hydroxyproline increased the dissociation energy of the triple helix; thus intrinsic effects play a crucial role (Figure 4). Second, the $(PPG)_{10}$ dimer with nine protons retains its crystal structure without water, consisting of two antiparallel triple helices (Figure 2), presumably bound by

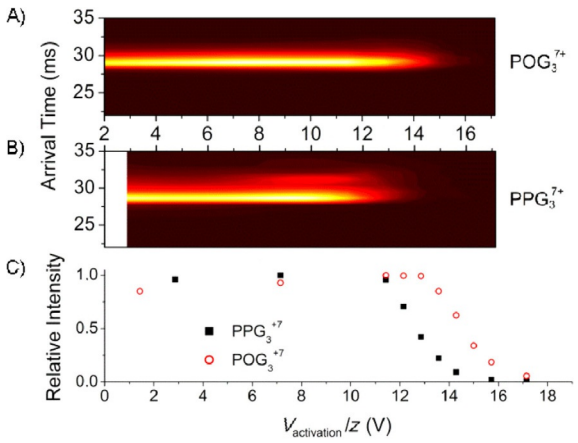

Figure 4. The 2D maps of the arrival time distributions of A) $[((POG)_{10})_3+7H]^{7+}$ and B) $[((PPG)_{10})_3+7H]^{7+}$ (noted as $POG_3{}^{+7}$ and $PPG_3{}^{+7}$) as a function of collision voltage. C) Evolution of the relative intensity of $POG_3{}^{+7}$ and $PPG_3{}^{+7}$ as a function of collision voltage. Reproduced from ref. [44] with permission. Copyright: Wiley-VCH, 2018.

the interaction between their strong dipole moments. This is challenging the claim of the absence of these small assemblies of triple helices in solution without hydroxyproline.[45] We are currently investigating these systems more deeply, to gain an insight into the first steps of collagen growth into fibrils. We also plan to study how a controlled number of water molecules change the structure and stability of CMP assemblies.

3. Irradiation by Nonionizing Radiation

UV light is a major factor of skin ageing and cancer induction. Because the dermis of skin is mainly composed of a dense ECM containing numerous collagen fibrils, UV irradiation of collagen has been studied by a range of experimental techniques. Particular attention has been given to effects on the characteristic triple-helical 3D structure of collagen because of its crucial role in the mechanical properties of connective tissues. Investigations into collagen proteins in solution by using experimental techniques such as differential scanning calorimetry (DSC) or circular dichroism (CD), as well as various chemical analyses, demonstrated that UV irradiation had an effect on their geometrical and chemical structures. A transition from the triple helix to random coil occurs through an intermediate state characterized by Miles et al.[46] UV absorption is thought to occur mainly at tyrosine and phenylalanine (tryptophan is almost absent in collagen) aromatic side chains, creating crosslinks between collagen strands and backbone cleavages.[47,48] These crosslinks and cleavages are due to free radicals created by tyrosine and phenylalanine photoproducts. The intermediate state is more flexible, and thus, has a higher entropy than that of the native triple helix, resulting in a decrease of the entropy change for denaturation, but a similar enthalpy change.[46] Interestingly, it has been reported that low doses of UV light increase the denaturation temperature of collagen in tendons,[49,50] which would be due to crosslinking between triple helices. At higher doses, atomic force microscopy (AFM) and DSC showed that the high number of backbone cleavages leads to destruction of collagen fibers, loss of the triple-helix structure, and protein disintegration into small peptides.[51,52] It has been proposed that the radicals created by UV absorption in aromatic residues migrate to proline and then glycine residues, which might finally lead to backbone cleavage.[53] Consistent with this picture, a higher probability of damage for proline-containing CMPs and preferential glycine–proline backbone cleavage have been observed by means of mass spectrometry analysis of UV degradation products of different CMPs.[54]

Proteins other than collagen have also been studied by means of UV and IR spectroscopy, notably taking advantage of the high flux of synchrotron radiation to reach good signal-to-noise ratios for far-UV–CD studies. Heat-induced denaturation of matrix proteins[55] and antifreeze protein III[56] has been observed, but this process also occurs upon sufficient exposure to UV light. By controlling synchrotron radiation, CD–UV denaturation can even provide information about protein stability and receptor–ligand binding interactions, particularly if thermal studies are inconclusive. For instance, it has been shown that gold nanoparticles or ligands stabilize human serum albumin upon irradiation over the wavelength range of 185–250 nm.[57]

4. Irradiation by Ionizing Radiation

Proteins are exposed to radiation other than UV light, such as gamma rays and electrons with a kinetic energy of about 10 MeV, which are routinely used for the sterilization of biomaterials. From studies reported over the last two decades, several main conclusions can be drawn: first, the magnitude and nature of direct effects are often highly dose dependent. Second, radicals created by ionization of atoms or groups within individual proteins are involved in side-chain loss and backbone cleavage, as well as crosslinking. Third, the nature and energy of the ionizing radiation often play a big role. We detail all of these points for collagen and other proteins in the following section.

Direct effects are usually reported to start being detectable for doses of around 10 kGy, which is higher than that of indirect effects due to the presence of free radicals after irradiation of a solution at room temperature.[58] However, it should be noted that EPR spectroscopy of bovine hemoglobin (Hb) powder after irradiation by gamma rays at doses as low as 5 Gy allowed a signal assigned to peroxyl and tyrosyl radical formation to be observed.[59] The authors attribute this to "the high sensitivity of Hb protein to irradiation". The signal then rises linearly with increasing dose up to 300 kGy. Interestingly, gamma rays at the same very low dose of 5 Gy have also been found to increase the diameter of collagen fibrils without significant crosslinking.[60] In contrast, Hu et al. studied the modification of collagen thin films by space radiation, which is composed of electromagnetic radiation, such as X-rays, gamma rays, protons, and other ions, and observed extensive crosslinking and a decrease in thermal stability of collagen proteins.[61] This thermal behavior might be dependent on the protein because the opposite has been observed for proteins from sunflower meal irradiated with gamma rays between 10 and

50 kGy.[62] It was attributed to crosslinking, but might also be due to conformational change. Indeed, a progressive transition from an α-helix to β-sheet and random-coil secondary structures was observed thanks to FTIR spectroscopy. This process has been reported for different proteins and radiation types, for instance, bovine serum albumin (BSA) irradiated by N^+ ions[63] or protons,[64] although the underlying mechanism remains unclear.

In the high-dose regime, from 1 to 100 MGy, other processes, mainly involving covalent-bond cleavage, can be observed at the molecular or atomic levels by techniques such as near-edge X-ray absorption fine structure (NEXAFS) spectroscopy or XRD. The latter requires protein crystals and low temperatures, usually below 150 K. In proteins containing cystine, disulfide bond cleavage is usually observed, for instance, in acetylcholinesterase and hen egg white lysozyme irradiated by X-rays at 10 MGy.[65] Other bonds are also broken, such as C–H, C–N, and C=O from the amide backbone of surface proteins,[66] cytochrome *c*, or BSA.[67] A mechanism common to several polymers containing amide groups has been proposed after C–O cleavage: H transfer from N to the carbonyl C or C_α, forming imine or nitrile groups, respectively (Figure 5).[67] In the last case, it leads to fragmentation of the protein backbone.

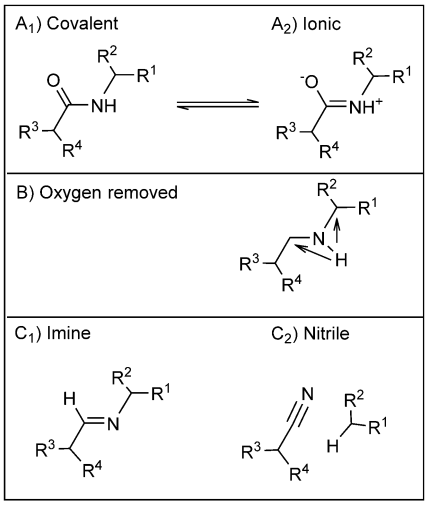

Figure 5. A) Covalent and ionic structures of the amide group. B) Amide group after O removal and illustration of H transfers. C) Imine and nitrile groups formed. Adapted from ref. [67] with permission. Copyright: AIP Publishing, 2011.

C–H and C=O cleavages lead to loss of H and O atoms, which can give rise to H_2 and O_2 if these radicals self-recombine.[68,69] C–O cleavage within serine and threonine side chains leads to loss of the hydroxyl group.[70] OH loss after C–O cleavage of the tyrosine side chain is controversial because it has been reported several times, but challenged by recent work.[71] Contradictory results have also been reported for C–C bonds: formation in surface proteins, but cleavage in other proteins, including hen egg white lysozyme, acetylcholinesterase, and chymotrypsin inhibitor.[72] This cleavage explains CO_2 loss from glutamic and aspartic acid side chains. Interestingly, all of these specific atomic displacements have been shown to trigger cooperative movements of protein structural subdomains.[68] Another characteristic of these cleavages is the involvement of radicals created by ionization, as outlined below.

The mechanisms proposed to account for direct effects of radiation on proteins are often radical mediated. For instance, crosslinking has been attributed to the formation of dityrosine after binding of two tyrosyl radicals (Figure 6).[73] Different radicals might play a role in collagen, such as CH_2–$CH_2^{\lambda\cdot}$, which has been detected by EPR spectroscopy of collagen samples, irradiated by 2 MeV protons.[74] This is consistent with a recent study by Kornacka et al.,[75] who used the same technique and concluded that gamma-ray irradiation at 5 kGy created radicals at proline side chains. CO_2 loss from glutamic and aspartic acid side chains, as well as H_2 and O_2 formation and backbone cleavage, are suggested to be due to radicals (see previous paragraph). Loss of neutral molecules from side chains has also been observed from isolated peptides or proteins after ionization. In the next paragraph, we discuss how the advent of gas-phase studies brought complementary information to condensed-phase investigations, notably by varying the nature and energy of radiation.

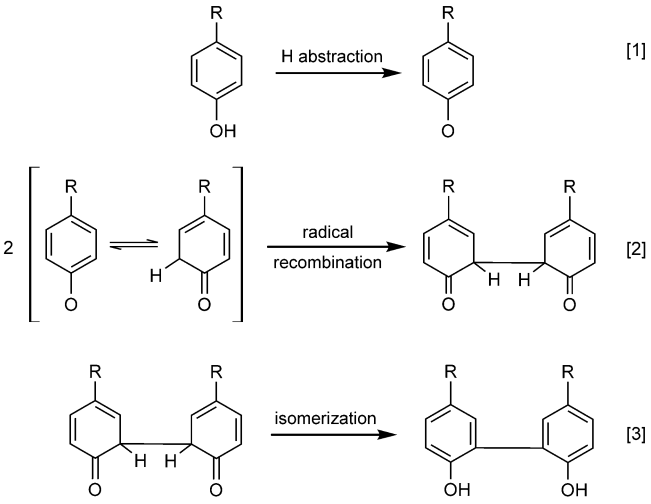

Figure 6. Radical-mediated mechanism of dityrosine formation from two tyrosine side chains. Adapted from ref. [73] with permission. Copyright: Springer, Vienna, 2003.

In recent decades, advances in soft sources of gas-phase molecules, such as ESI or MALDI, allowed larger biologically relevant systems than DNA bases or amino acids to be studied. However, due to the very low current intensity (of the order of 1 pA) delivered by these sources, and even with the highest radiation fluxes available (cyclotrons, lasers, synchrotrons, etc.), ion traps had to be employed to irradiate these biomolecules for 0.1–1 s to obtain decent signal count rates. In these experiments, the number of interactions can be controlled by measuring depletion of the precursor ion. Zubarev,[76] Giuliani et al.,[77] and Schlathölter et al.[78,79] pioneered experiments involving irradiation of mass-over-charge selected peptides and proteins and analysis of the product ions by means of mass

spectrometry. Early studies with electron beams of controlled kinetic energy allowed the first ionization cross-sections and energies of protonated peptides, such as substance P or vasopressin, to be measured.[80] The latter are in the 10–15 eV range, which is consistent with the ejection of valence electrons. Further work established a 1.1 eV increase in ionization energy if the protonated peptide charge state increased, which was assigned to Coulombic attraction undergone by the ejected electron.[81] This behavior has been confirmed by photoionization experiments.[82] Much lower photon energy is required to detach one electron from multiply deprotonated peptides, which supports the electrostatic picture.[83] After ionization or electron detachment, loss of neutral molecules from the radical species was also detected in the mass spectra; these molecules were first proposed to come from side chains and the C-terminal carboxylic acid.[84] Later, these losses from ionized peptides were identified as coming from side chains of specific amino acids, such as tyrosine, aspartic acid, glutamic acid, and serine.[85,86] Photoionization of substance P as a function of photon energy, thanks to synchrotron radiation, allowed appearance energies of a few eV to be measured for these losses, consistent with radical-driven processes.[87] We studied a peptidic sequence of type I collagen and peptidic models of the collagen triple helix by synchrotron radiation in the vacuum ultraviolet (VUV) and soft X-ray ranges,[88,89] and observed the loss of neutral molecules with low appearance energy that we attributed to radical-driven processes at amino acid side chains (Figure 7), akin to the case of irradiation of proteins in the condensed phase (see the previous section). However, in contrast, gas-phase radicals were mainly located at aspartic acid and hydroxyproline side chains. These radicals might be too short-lived to be observed in the condensed phase.

At higher photon or electron energies, peptide backbone fragmentation is observed, with an abundance that decreases with peptide size at a given photon energy.[90,91] This has been interpreted as fragmentation in the ground state after redistribution of part of the initial photon energy in the internal degrees of freedom, the latter increasing with peptide size; thus almost quenching fragmentation for proteins.[92] Nondissociative multiple ionization has even been observed for cytochrome *c* (Figure 8), ubiquitin, and insulin, after absorption of one soft X-ray photon.[90,93] In this energy range, Auger electron emission occurs and has been shown to induce secondary ionization of melittin.[94] Thanks to NEXAFS spectroscopy and mass spectrometry, gas-phase experiments allowed the identification of resonant excitation of 1 s electrons of carbon, nitrogen, and oxygen atoms to unoccupied molecular orbitals, as well as direct ionization for slightly higher photon energies, because Auger decay leads to ejection of one electron for excitation and two electrons for direct ionization (cf. Figure 8). It is important to note that these excitation energies are very similar to those obtained from condensed-phase measurements.[93,95] Nondissociative ejection of several electrons can also be achieved upon irradiation by ions, as illustrated by experiments with multiply charged Xe beams on deprotonated and protonated cytochrome *c*.[96,97] For smaller systems, such as protonated peptides, backbone fragmentation and side-chain loss of neutral molecules occur; thus indicating that the mechanisms are similar to those regarding electron and photon interactions.[98,99] Interestingly, an ion-specific process has been reported: proton detachment, leading to the formation of an intact cation without one charge.[96,100] However, it might also come from a reduced species formed by electron capture, as suggested recently.[101] More investigation is needed to clarify this point.

The role of noncovalent binding on the processes induced by ionizing radiation and the way energy flows through protein subunits was first studied only recently. To do so, we irradiated $(PPG)_{10}$ and $(POG)_{10}$ peptidic models of the collagen triple helix with ionizing photons at the BESSYII synchrotron (Berlin, Germany) and carbon ions at the IRRSUD beamline of the GANIL facility (Caen, France). In the case of VUV photons in the 14–20 eV range, we observed a transition between photoexcitation, leading to fragmentation and nondissociative ionization (NDI) after single-photon absorption (Figure 9).[89] Above 20 eV and up to soft X-ray energies (100–600 eV), internal energy transferred to the system rises with photon energy and leads to increasing fragmentation. As the photon energy increases, intermolecular fragmentation first occurs; thus unfolding the helix and forming isolated peptides, which fragment further if the photon energy is high enough (Figure 9). Inter-

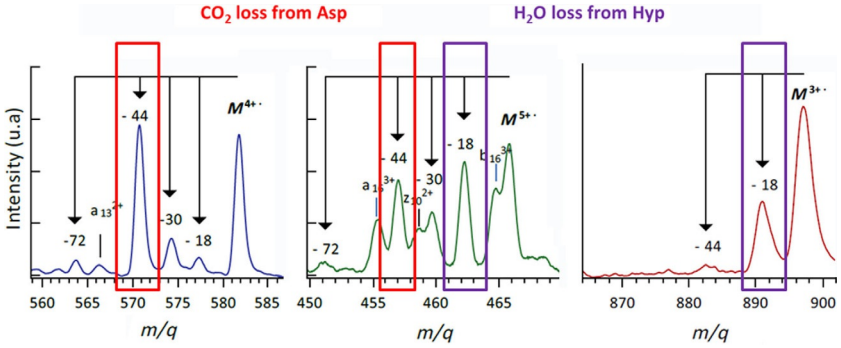

Figure 7. Mass spectra of a protonated type I collagen peptide (left and middle) and of protonated $(POG)_{10}$ (right) after absorption of one 150 eV photon.[88] The mass of neutral molecules lost after ionization is indicated, and losses from aspartic acid as well as hydroxyproline side chains are highlighted. Reproduced from ref. [88] by permission. Copyright: the PCCP Owner Societies, 2017.

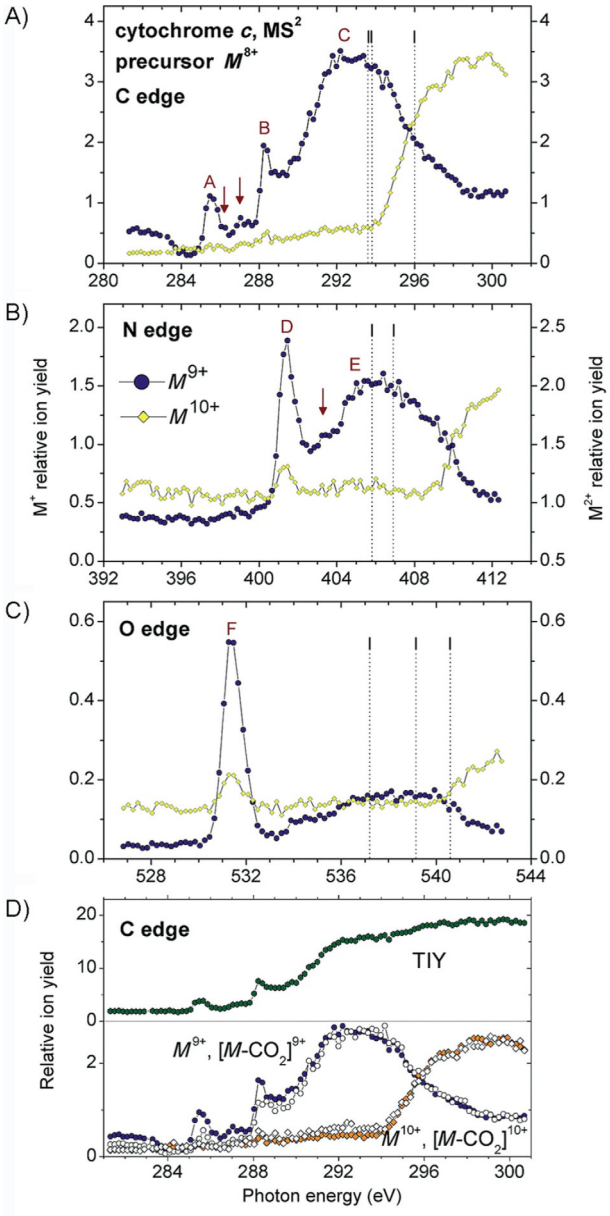

Figure 8. A)–C) Relative yields of nondissociative single (M^+) and double (M^{2+}) ionization after photoabsorption by [cytochrome c+8 H]$^{8+}$ as a function of photon energy. D) Total ion yield (top) and CO_2 loss after single and double ionization. Labels A–F indicate resonant transitions of 1 s electrons to unoccupied molecular orbitals. Reprinted with permission from ref. [93] Copyright: The American Chemical Society, 2012.

estingly, this intramolecular fragmentation is dominated by backbone cleavage between glycine and proline residues, as observed in solution after UV irradiation.[54] It supports the role of intrinsic processes not due to the solvent. We also observed increased stability upon VUV photoabsorption for $(POG)_{10}$ compared with that of $(PPG)_{10}$; this is consistent with the hydroxyproline effect already observed upon collision with helium gas (see Section 2), as well as the well-known effect in crystals and in solution. This definitely shows that at least part of the stabilization by hydroxyproline is not due to water molecules. We recently irradiated these CMPs with carbon ions at the kinetic energy corresponding to irradiation of tumor cells in hadron

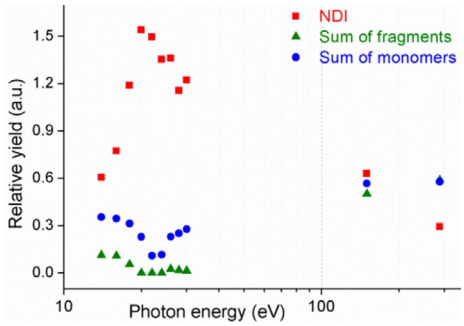

Figure 9. Relative yield of NDI and inter- (sum of monomers) and intramolecular (sum of fragments) fragmentation after single-photon absorption as a function of photon energy for [{$(PPG)_{10}$}$_3$+7 H]$^{7+}$. Reproduced from ref. [89]. Copyright: The PCCP Owner Societies, 2017.

therapy.[100] The same ionization and fragmentation processes as those for X-rays are observed, and the energy transferred to a single molecule has been estimated to be around 20 eV. We are currently extending our research to irradiation of CMP assemblies. Our last results show that proline hydroxylation stabilizes triple-helix dimers (Figure 2), presumably through hydrogen bonds between triple helices.[102]

5. Summary and Outlook

The relationship between structure and stability has been studied in depth for proteins, especially collagen, because of the structure–function relationship. Recent studies on CMPs delivered a wealth of data that elucidated the mechanisms of (de)stabilization of triple-helical domains of collagen. Modeling and designing new collagen-based biomaterials is now an active area of research, mainly based on an understanding of how collagen structure influences its stability.[103,104] These previous findings are also used nowadays to develop collagen-based drug delivery.[105] Open fundamental questions also remain and are to be addressed in a near future about, for instance, the role of glycine interruptions in collagen functions, the influence of supramolecular structure on collagen stability and recognition by other molecules in the ECM, and the dynamics of collagen unfolding. Moreover, it has been shown that interaction with nonionizing (UV) or ionizing (VUV, X-rays, gamma photons, electrons, ions) radiation impacts on the physical and chemical properties of collagen and other peptides and proteins. Upon increasing the dose transferred to the molecules, the observed processes evolve from conformational changes and crosslinking to covalent-bond cleavage in the backbone and side chains. Radical-mediated mechanisms play a big role in all of these phenomena, and much detailed information has been gained by changing the nature and energy of the irradiating particle in a controlled way in gas-phase experiments. However, our understanding of the behavior of peptides and proteins under irradiation is still limited. In particular, little is known about charge transfer and fragmentation dynamics triggered by ionization, but this gap should be filled by pump–probe experiments at the femto- or attosecond timescales, thanks to free-electron lasers operating in the X-ray

energy range.[106] Charge dynamics within amino acids,[107,108] as well as DNA nucleobases and nucleosides,[109] have already recently been measured. Another effect deserving future attention is the fate of a protein after a localized ionization event. Designing experiments at photon energies that target a single atom is notably a direction worth exploring. Further investigation is also needed to precisely measure and potentially control the radioinduced effects on secondary, tertiary and quaternary structures of a protein, which would be reachable by means of ion-mobility spectrometry. Indeed, this powerful technique already allows, for instance, identifying proteins by their unfolding signature,[110] as well as monitoring their conformational changes after collision with a rare gas or absorption of UV photons.[111]

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: collagen · helical structures · structure–stability relationships · proteins · radiochemistry

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