Direct Ultraviolet Laser-Induced Reduction of Disulfide Bonds in Insulin and Vasopressin

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ABSTRACT: Ultraviolet (UV) light has been shown to induce reduction of disulfide bonds in proteins. The photoreduction is proposed to be a result of electron donation from excited Tyr or Trp residues. In this work, a powerful UV femtosecond laser was used to generate photoreduced products, while the hypothesis of Tyr/Trp mediation was studied with spectroscopy and mass spectrometry. With limited irradiation times of 3 min or less at 280 nm, the laser-induced reduction in arginine vasopressin and human insulin led to significant yields of ~3% stable reduced product. The photogenerated thiols required acidic pH for stabilization, while neutral pH primarily caused scrambling and trisulfide formation. Interestingly, there was no direct evidence that Tyr/Trp mediation was a required criterion for the photoreduction of disulfide bonds. Intermolecular electron transfer remained a possibility for insulin but was ruled out for vasopressin. We propose that an additional mechanism should be increasingly considered in UV light-induced reduction of disulfide bonds in solution, in which a single UV photon is directly absorbed by the disulfide bond.

INTRODUCTION

Disulfide bonds are important for the structure and function of peptides and proteins. UV light-induced reduction of the disulfide bond in solution and the molecular consequences has been reported in a diverse range of proteins. The predominant hypothesis to describe the UV photoreduction of disulfide bonds in peptides and proteins is based on a Tyr/Trp-mediated model. Upon absorption of UV light by the chromophoric Tyr or Trp side chain, the excitation energy can eject an electron that may reduce a local disulfide bond. The excitation energy can reduce a disulfide bond in a Tyr chromophore. The hypothesis has been used more recently to explain disulfide bond scrambling (formation of non-native disulfide bonds) by reoxidation of photogenerated thiols in human growth hormone and IgG1 antibody.

UV light has been used to excite solution mixtures of acetone and alcohol to activate radical chemistry that cleaves disulfide bonds into reduced and alkylated sulfur atoms in specific ratios depending on the alcohol. In the gas phase, ultraviolet photodissociation (UVPD) of the covalent bonds in peptides and proteins can be used as a fragmentation method in tandem mass spectrometry. The current commercially available setup utilizes a 213 nm laser for the absorption of UV photons by the peptide bonds, leading to backbone fragmentation. However, ions fragmented only at the S–S and C–S bonds by UVPD may also be observed in triplet sulfur patterns of zero, one, or two sulfur atoms because the disulfide bond is also a UV chromophore. While the above-referenced in-solution studies discuss UV light-induced disruption of disulfide bonds as a result of a partner chromophore absorbing the UV light, some in-solution studies as well as a more recent series of gas-phase studies present a different model. Here, the disulfide bond itself absorbs the UV light and homolytically cleaves into thyl radicals. Examples have also been reported of UV light-induced cleavage of disulfide bonds in nonaromatic peptides and small compounds. Thus, the in-solution photochemistry field that has been in favor of an electron-donating Tyr/Trp-mediated model for proteins may have underappreciated the model widely recognized in the gas-phase photochemistry field. At 280 nm, the disulfide bond is a significant chromophore, which is why it has an assigned extinction coefficient (125 M⁻¹ cm⁻¹) in protein concentration calculations.

We have previously demonstrated that femtosecond laser technology is useful for accelerating the 280 nm light stress of peptides and for generating large quantities of photoproducts in limited irradiation time for characterization of photo-modifications by liquid chromatography–mass spectrometry. We have previously demonstrated that femtosecond laser technology is useful for accelerating the 280 nm light stress of peptides and for generating large quantities of photoproducts in limited irradiation time for characterization of photo-modifications by liquid chromatography–mass spectrometry.
An electron from Trp in somatostatin-14 was proposed to be donated to the adjacent Phe to form a novel Phe-Trp cross-link. The disulfide bond of vasopressin was photounstable and involved in multiple dimeric species with scrambled intermolecular disulfide bonds. It was hypothesized that the scrambling was due to the initial photoreduction in which the free thiols reoxidized at neutral pH.

So far, data has been limited regarding the state of the proposed electron-donating residues in the photoreduced products. The aim of this work was to study the proposed Tyr/Trp-mediated photoreduction in simple model systems. Femtosecond laser technology was used to accelerate the intrinsic UV light-based mechanisms of the molecules for the reduction of the disulfide bonds in solution. The photoreduced products were separated with liquid chromatography and online-characterized with absorption spectroscopy and tandem mass spectrometry. Gas-phase UVPD was also used to study UV light-induced fragmentation of the disulfide bonds.

RESULTS AND DISCUSSION

UV Photoreduction of Disulfide Bonds in Solution. Somatostatin-14, arginine vasopressin, and human insulin were selected for the attempt to UV photoreduce the disulfide bonds (Chart 1). The 280 nm irradiation of insulin cleaved the two native interchain disulfide bonds to produce free B-chain into its two redox forms depending on the pH (Figure 1). The acidic conditions stabilized the photoreduction, while neutral pH stabilized the reoxidized B-chain with a scrambled intrachain disulfide bond. The remaining photoproducts eluting after the precursor were primarily high-molecular-weight species. Peptide mapping under nonreducing conditions revealed a total of three disulfide-scrambled bonds that were enriched after the UV irradiation (Figure 2). Cys7 of the B-chain was involved in the scrambling to either Cys19 (forming the intrachain bond), to a homo Cys7 disulfide bond, or to Cys20 of the A-chain. For each scrambled disulfide bond, a trisulfide variant was also observed. A trisulfide bond was also observed in vasopressin at neutral pH.28 Overall, these data suggest that UV light-induced disulfide scrambling may lead to trisulfide bonds in proteins. Interestingly, trisulfide bond impurities have previously been reported in recombinant monoclonal antibodies.29,30

The hypothesized photoreduction of vasopressin (M + 2H) was also confirmed at acidic pH (Figure 3A). Like the B-chain of insulin, reoxidation of the photogenerated thiols was observed but as intermolecular disulfide bonds that formed the dimeric 2M photoproduc. An additional photoproduc containing one thiol was observed as a shoulder to the precursor elution peak. This product was identified as dithiohemiacetal-vasopressin, previously also identified as scrambled dimeric species at neutral pH as well as observed in the vasopressin homolog oxytocin.23,31

The disulfide bond in somatostatin-14 was stable toward photoconversion despite the presence of a Trp residue. No photoproduc involving disulfide bond reduction was identified
The photoreduction yield of insulin B-chain and vasopressin was 2−3%. The quantities were enough for LC-MS/MS (-MS^2) analysis after 60 s of laser irradiation, and the fragmentation spectra of the photoreduced products provided improved sequencing due to the removal of the disulfide bonds, supporting that the products were reduced (Figure S1). The 280 nm photoreduction was also achievable using a continuous source xenon lamp of 45-fold less power at prolonged exposure (data not shown). This indicated that the photocleavage of the disulfide bond was a one-photon reaction. In one-photon reactions, a linear correlation is expected between the product quantity and irradiation power, which was observed for both insulin B-chain and vasopressin (Figure 4).

**Evaluation of Tyr-Mediated Photoreduction.** The data did not support Tyr-mediated electron transfer to the photoreduction of the disulfide bonds in insulin and vasopressin. In electron donation from Tyr, it would be expected that Tyr is simultaneously converted to a covalent product in the photoreduced molecules. For both photoreduced products, Tyr immonium ion was present in the MS^2 fragmentation, while UPLC absorbance spectroscopy revealed negligible excitation of Phe is expected to have occurred in the irradiation experiments. Phe has a local absorption maximum at 258 nm with an extinction coefficient of 188 M^-1 cm^-1 and at 275 nm with a coefficient of 4 M^-1 cm^-1, while for concentration measurements at 280 nm, Phe has no assigned extinction coefficient due to very low absorption above 275 nm. The observed UV light-induced reduction is unlikely to be significantly contributed by Phe-mediated electron donation because of the negligible absorption of Phe at the irradiated wavelengths and because the Phe immonium ion was present in the MS^2 fragmentation of photoreduced vasopressin (data not shown). A recent photostudy varied the distance between the Trp and the disulfide bond. A photostudy of insulin used electron scavengers under Ar saturation to deplete any hydrated electrons from the Tyr de bond. A photostudy of insulin used electron scavengers under Ar saturation to deplete any hydrated electrons from the Tyr de bond. A photostudy of insulin used electron scavengers under Ar saturation to deplete any hydrated electrons from the Tyr de bond. A photostudy of insulin used electron scavengers under Ar saturation to deplete any hydrated electrons from the Tyr de bond.

Follow-up irradiation of a vasopressin-Tyr2Phe analogue revealed it was also photoreducible since the M + 2H and 2M photoproducts were observed (Figure 3B), further indicating that another mechanism than Tyr mediation accounted for the photoreduction. Here, hydrogen for the reduction may derive from the solution. With a ±7 nm full width at half-maximum (fwhm) bandwidth of the laser pulses at 280 nm maximum, negligible excitation of Phe is expected to have occurred in the irradiation experiments. Phe has a local absorption maximum at 258 nm with an extinction coefficient of 188 M^-1 cm^-1 and at 275 nm with a coefficient of 4 M^-1 cm^-1, while for concentration measurements at 280 nm, Phe has no assigned extinction coefficient due to very low absorption above 275 nm. The observed UV light-induced reduction is unlikely to be significantly contributed by Phe-mediated electron donation because of the negligible absorption of Phe at the irradiated wavelengths and because the Phe immonium ion was present in the MS^2 fragmentation of photoreduced vasopressin (data not shown). A recent photostudy varied the distance between the Trp and the disulfide bond in a simple peptide model system and reported no correlation between the distances and quantities of photogenerated thiols. The study assumes that the intramolecular distance between Trp and the disulfide bond is the only critical feature in the observed disulfide bond cleavage. However, intermolecular electron transfer may also contribute to the observed reduction, thus masking any intramolecular effects. In agreement with our observations, the authors proposed that other mechanisms than Trp mediation could account for the photocleavage of the disulfide bond. A photostudy of insulin used electron scavengers under Ar saturation to deplete any hydrated electrons from the Tyr residues concluded that 33% of the total photogenerated thiols was due to Tyr mediation. One of the two photoreduced disulfide bonds in goat α-lactalbumin was unexpected due to the distance to the nearest Trp. Protein dynamics were therefore proposed to provide a shortened distance that allowed the Trp-mediated photoreduction. The independence of Tyr and Trp observed here can also explain the unexpected photoreduced disulfide bond.

The fluorescence of Tyr was degraded in the UV laser-irradiated samples of insulin and vasopressin (Figure S2). Loss of Tyr fluorescence due to UV light stress may derive from covalent photomodifications of the Tyr side chain or misfolding that quench the emission or cause blue/red shifting. Photodegradation of Tyr fluorescence could not be located to the photoreduced insulin B-chain and vasopressin because the studies of peptides have shown that energy transfer can lead to fragmentation of the covalent bond.

![Figure 3](https://dx.doi.org/10.1021/acsomega.9b04375)

**Figure 3.** UHPLC–MS analysis of the 280 nm photoreaction and photoreduction of the intrachain disulfide bond at acidic pH of (A) arginine vasopressin and (B) arginine vasopressin-Tyr2Phe. The power of the laser irradiation was 50 mW.

![Figure 4](https://dx.doi.org/10.1021/acsomega.9b04375)

**Figure 4.** Quantity of the disulfide-cleaved photoproducts (oxidized insulin B-chain at pH 7.4 and reduced vasopressin at pH 3.0) as a function of laser power at 280 nm. Irradiation time was 3 min. Data was fitted to linear regression.
Tyr residues were intact in these products. This was despite the case of insulin where the degradation of Tyr fluorescence was roughly equivalent to the total precursor degradation (Figure S2A). The loss of Tyr fluorescence could therefore primarily be assigned to the high-molecular-weight species (Figure 1), which may include di-Tyr cross-linking as a result of UV irradiation as suggested in a spectroscopic study of insulin. It is a possibility that the fluorescence-modified Tyr residues were significant donors for intermolecular electron transfers in the reduction of the interchain disulfide bonds. For vasopressin, which is a simpler molecular system, the photoconversion of the disulfide bond into reduction and photodegradation of the Tyr residue could be clearly observed as two independent processes, not only by identification of the photoproducts with LC–MS but also because the degradation of all residues in the precursor exceeded the degradation of the Tyr emission (Figure S2B). Roughly, the photodegradation of Tyr fluorescence corresponded to half of the total degradation, which included Tyr and the disulfide bond.

**UV photomodifications of Tyr and Trp.** The major Tyr product of vasopressin at acidic pH was identified (Figure 3A). Consistently, while Tyr was intact in the photoreduced product of vasopressin, oppositely the Tyr product in the photoreaction had an intact disulfide bond and instead suffered a total mass loss corresponding to one ammonia (NH₃). The M−NH₃ Tyr photo-modification was previously identified for vasopressin at neutral pH and contained a new local maximum of 294 nm instead of the classical Tyr profile. Characterization of the chemically reduced deaminated product revealed that the mass loss was localized in the a₂/b₂ (Cys1−Tyr2−) pair fragments (Figure S3). Also, the Tyr immonium ion was absent in the MS² fragmentation. It is therefore likely that the N-terminus is the source of deamination in a photoreaction involving Tyr. Interestingly, electron transfer to a protonated N-terminus is the source of deamination in a photoreaction involving Tyr. Electron transfer to a protonated N-terminus is the source of deamination in a photoreaction involving Tyr. Even though the disulfide bond of the deaminated Tyr photoproduct of vasopressin was intact it could be hypothesized that this modified Tyr donated electrons in an intermolecular transfer to the observed photoreduction. This can however be rejected because the vasopressin-Tyr2Phe analogue was also photoreducible and because no M−NH₃ photoproduct was identified in the photoreaction.

The oxidation products of Tyr and Trp are well established. Besides the novel Phe−Trp cross-link in somatostatin-14, the conversion of Trp to the common photodegradation product N-formylkynurenine was also observed, consistent with UV laser stress at neutral pH (data not shown). NFK-somatostatin-14 was for the first time reported as a product from fluorescent light. Established Tyr oxidations were searched for in arginine vasopressin and human insulin. Under the conditions employed here, we did not observe any abundant conventional oxidation of Tyr in vasopressin. A minor M + H₂O product was observed (Figure 3A). LC−MS² localized the hydration to the side chain of Tyr by the presence of hydrated a₂/b₂ pair and hydrated Tyr immonium ion (data not shown). The under-representation of conventional Tyr oxidation in vasopressin and identification of the novel deamination product can be due to the N-terminal position of Tyr2. Thus, photoinduced reaction between the aromatic side chain and a primary amine may outcompete modifications involving the addition of oxygen derived from the matrix. Interestingly, under the acidic conditions, conventional oxidation (+16 Da) was observed in the case of human insulin, which has its four Tyr residues positioned further apart from the N-termini (Chart 1). The M + O product required a flat gradient for separation and UV detection (Figure S4A). Top-down tandem mass spectrometry was applied to identify the oxidation site. LC−MS² analysis localized the oxidation to A-chain (data not shown). The M + O photoproduct was also detectable by infusion (Figure S4B). The A-chain + 16 Da MS² fragment was further dissociated by adding a third MS event (MS³). The oxidation site of the human insulin M + O photoproduct was localized to Tyr14 through both the b and y series of fragments (Figure S4C,D). Thus, UV light irradiation of human insulin can convert Tyr14 on A-chain to dihydroxyphenylalanine.

**Thiol Radical Formation by UV Photodissociation of the Disulfide Bond.** The cleavage of the interchain disulfide bonds in human insulin was also observed at 213 nm irradiation in the gas phase by the identification of dissociated A- and B-chain species (Figure 5). The excitation from the absorbed 213 nm photons induced cleavage at the S−S and C−S bonds in the A-chain C−S=S−C₈-B-chain group, forming the triplet sulfur patterns. For both chains in the 213 nm photodissociation, the loss or gain of one sulfur atom went to or derived from the partner chain, respectively. The triplet sulfur pattern was also observed with vasopressin through the y-ions (data not shown). These A- and B-chain fragments dissociated only at the interchain disulfide bonds and the fragmentation into sulfur triplets in both molecules suggested direct absorption of the UV photons by the disulfide bonds.

The patterns of the isotope clusters of the chain fragments reflected a heterogeneous ion population varying in the number of hydrogen atoms (Figure 5). The intensities of the m/z values corresponding to intact oxidized chain with two sulfur atoms may derive from thyl radicals that additionally could reform as an intrachain disulfide bond. The formation of thyl radicals is supported by the observed loss of hydrogen atoms through intermolecular hydrogen transfer to thyl radicals from the partner chain. Also, the photoexcitation of Tyr followed by ejection of an electron, forming the unstable thyl radical that rapidly deprotonates, could also contribute to hydrogen loss.22
solubilized in 20 mM phosphate buffer at pH 3.0 or 7.4 to the final concentration of 100 μM and final volume of 3 mL for the following irradiation in macro fluorescence cuvettes (101-QS, Hellma Analytics, Germany). The 280 nm UV irradiation was performed with a femtosecond laser setup or a xenon lamp-grating system in a spectrophotometer. The femtosecond laser setup constituted of a Millennia eV laser that pumped a Tsunami XP laser to an output of 840 nm frequency tripled to 280 nm by a UHG module (all from Spectra-Physics, CA). The full width at half-maximum (fwhm) was ±7 nm. The remaining instrumentation, procedures of the UV irradiation, and fluorescence spectroscopy are described elsewhere. The control and UV-irradiated samples were stored at −18 °C. Human insulin laser-irradiated under pH 7.4 was digested with Endoproteiase Glu-C Sequencing Grade (Roche Diagnostics GmbH, Germany) in the microgram ratio of 1:20 protease/insulin for 3 h at 37 °C under nonreducing conditions. Chemical reduction of laser-irradiated vasopressin was prepared by incubation with 1 mM TCEP for 30 min at room temperature. For static nanospray infusion, 50 μL of samples of 100 μM was desalted using ZipTip C18 (Millipore) and eluted in 10 μL of 50% acetonitrile and 1% formic acid.

**Sample Analysis.** For LC−MS and −MS analyses, a Vanquish Horizon UHPLC system with a VF-D40 detector was coupled to an Orbitrap Fusion Lumos mass spectrometer (both from Thermo Fisher Scientific, CA). For online absorption spectroscopy, an Acquity UPLC Classic system with an Acquity photodiode array was used, which was coupled to a Synapt G2Si mass spectrometer (both from Waters, U.K.). The reverse-phase columns were Acquity UPLC CSH C18, 1.0 μm × 150 mm², 1.7 μm (Waters, U.K.), with the mobile phase of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B).

Top-down characterization of the human insulin photodegradation product (M + O) and 213 nm UVPD of human insulin was conducted on an Orbitrap Fusion Lumos to increase spectra quality, the inlet was changed from LC to infusion to avoid the limited acquisition time available during elution of the analyte. The desalted samples were infused by static nanospray using metal-coated borosilicate emitters (Thermo Scientific, ES381) at 1.2 kV. The infusion-MS spectra were combined by 5 scans each of 100 microscans at 120k resolution. Electron-transfer/higher-energy collision dissociation (ETD)-MS analysis of insulin and its M + O photoprocessed was performed by an electron-transfer dissociation (ETD) reaction time of 40 ms and 30% energy for the higher-energy collision-induced dissociation (HCD). MS analysis of EThCD-derived A-chain and A-chain+16 Da fragments, that were cleaved at the interchain disulfide bonds, used HCD at 20% energy. The MS spectra were combined by 8 scans each of 100 microscans at 120k resolution. The UVPD-MS spectrum of insulin was combined by 5 scans each of 100 microscans at 120k resolution. The irradiation time for insulin was 100 ms and for vasopressin was 300 ms.

The remaining settings and software for UV detection, mass spectrometry, and data analysis were default. GraphPad Prism 7.04 was used for the plotting. GPMAW 12.0 (Lighthouse Data, Denmark) was used for the simulated isotopic patterns of human insulin A and B chains. The yield of the photoreduced products, defined as the degraded quantity of precursor converted to a stable photoreduced product, was quantified using 215 nm UHPLC by dividing the area of the photoreduced product with the area of the degraded precursor.

**EXPERIMENTAL SECTION**

**Sample Preparation and Fluorescence Spectroscopy.** Zinc-free human insulin was expressed in *S. cerevisiae* and purified with high-pressure LC following standard procedures. Somatostatin-14, arginine vasopressin, and arginine vasopressin-Tyr2Phe analogue were solid-phase synthesized in-house following standard procedures. The molecules were synthesized in solution, to consider an additional model without Tyr/Trp mediation where the disulfide bond intensity should be given to the possibility that intermolecular electron transfer can occur. The photoreduction can be stabilized at acidic pH, confirming that UV light-induced scrambling of disulfide bonds can be secondary products from photogenerated thiols. The results presented here are consistent with gas-phase studies of disulfide bonds, where it is accepted that the bond is directly and homolytically cleaved by UV light.

**Scheme 1. Proposed Model of Direct Photoinduced Reduction of the Disulfide Bond in Solution**

![Scheme 1](https://dx.doi.org/10.1021/acsomega.9b04375)
Ultradвозветь illumination-induced reduction of alpha-lactalbumin disulfide bridges. *Proteins 2003*, 51, 498–503.

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Notes
The authors declare no competing financial interest.

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ABBREVIATIONS
UV, ultraviolet; UVPD, ultraviolet photodissociation; LC–MS, liquid chromatography–mass spectrometry; MS/MS, MS2; TCEP, tris(2-carboxyethyl)phosphine; HCD, higher-energy collision-induced dissociation; ETD, electron-transfer dissociation; EThcD, electron-transfer/higher-energy collision dissociation

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