Selectivity of Tryptophan Residues in Mediating Photolysis of Disulfide Bridges in Goat α-Lactalbumin†

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ABSTRACT: Goat α-lactalbumin (GLA) contains four tryptophan (Trp) residues and four disulfide bonds. Illumination with near-UV light results in the cleavage of disulfide bridges and in the formation of free thiols. To obtain information about the reaction products, the illuminated protein was carbamidomethylated and digested with trypsin and the peptides were analyzed by mass spectrometry. Peptides containing Cys120Cam, Cys61Cam, or Cys91Cam were detected, as well as two peptides containing a new Cys−Lys cross-link. In one, Cys6 was cross-linked to Lys122, while the cross-link in the second was either a Cys91−Lys79 or Cys73−Lys93 cross-link; however, the exact linkage could not be defined. The results demonstrate photolytic cleavage of the Cys6−Cys120, Cys61−Cys77, and Cys73−Cys91 disulfide bonds. While photolysis of Cys6−Cys120 and Cys73−Cys91 disulfide bonds in GLA has been reported, cleavage of the Cys61−Cys77 disulfide bonds has not been previously detected. To examine the contribution of the individual Trp residues, we constructed the GLA mutants, W26F, W60F, W104F, and W118F, by replacing single Trp residues with phenylalanine (Phe). The substitution of each Trp residue led to less thiol production compared to that for wild-type GLA, showing that each Trp residue in GLA contributed to the photolytic cleavage of disulfide bridges. The specificity was expressed by the nature of the reaction products. No cleavage of the Cys6−Cys120 disulfide bridge was detected when the W26F mutant was illuminated, and no cleavage of the Cys73−Cys91 disulfide bridge was seen following illumination of W26F or W104F. In contrast, Cys61Cam, resulting from the cleavage of the Cys61−Cys77 disulfide bridge, was found following illumination of any of the mutants.

The presence of endogenous chromophores makes proteins sensitive to photoinduced degradation. The major chromophoric amino acids are tryptophan (Trp), tyrosine (Tyr), phenylanaline (Phe), histidine (His), cysteine (Cys), and cystine. The absorption of UV light can produce excited state species and radicals, which both initiate various degradation processes (*1*−*3*).

In the near-UV region, the indole side chain of Trp has a significantly greater molar absorption coefficient than the other amino acid side chains, making it the prime candidate for mediating protein photodegradation. The triplet state of Trp (^{3}Trp) can undergo electron transfer with suitable acceptors. Disulfides, such as cystine, readily take up an electron, leading to the formation of a disulfide radical anion, which can easily dissociate (*4*). Electron transfer from an excited Trp to cystine and the subsequent reductive splitting of disulfide bonds in proteins were postulated several decades ago (*5*), but it is only recently that the first unambiguous example of such Trp-mediated photoreduction was demonstrated in *Fusarium solani pisi* cutinase (*6*). A strong indication for Trp-mediated degradation of cystine bonds has been observed in goat and human α-lactalbumin (*7*, *8*) and in bovine somatotropin (*9*).

F. solani pisi cutinase contains a single Trp residue, allowing the link between the excitation of Trp and the disruption of the disulfide bond to be easily made. α-Lactalbumins (LAs) contain four disulfide bridges and, depending on the species, also contain three or four Trp residues, making examination of the photolytic process more complicated. Goat α-lactalbumin (GLA),[1] used in this study, contains four Trp residues. The structure of GLA is homologous to that of other LAs. The protein consists of two lobes separated by a cleft (Figure 1A). The larger lobe, formed by residues 1−34 and 86−123, consists mainly of helical structures, namely, α-helices A−C (Figure 1B), the flexible loop/helix D, and a 3_{10}-helix. The smaller lobe,

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[1] Abbreviations: GLA, goat α-lactalbumin; rGLA, recombinant goat α-lactalbumin; HLA, human α-lactalbumin; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MS, mass spectrometry; MS/MS, tandem mass spectrometry; MALDI-TOF, matrix-assisted desorption ionization time-of-flight mass spectrometry; ESI-MS, electrospray ionization mass spectrometry; Q-TOF, quadrupole time-of-flight.

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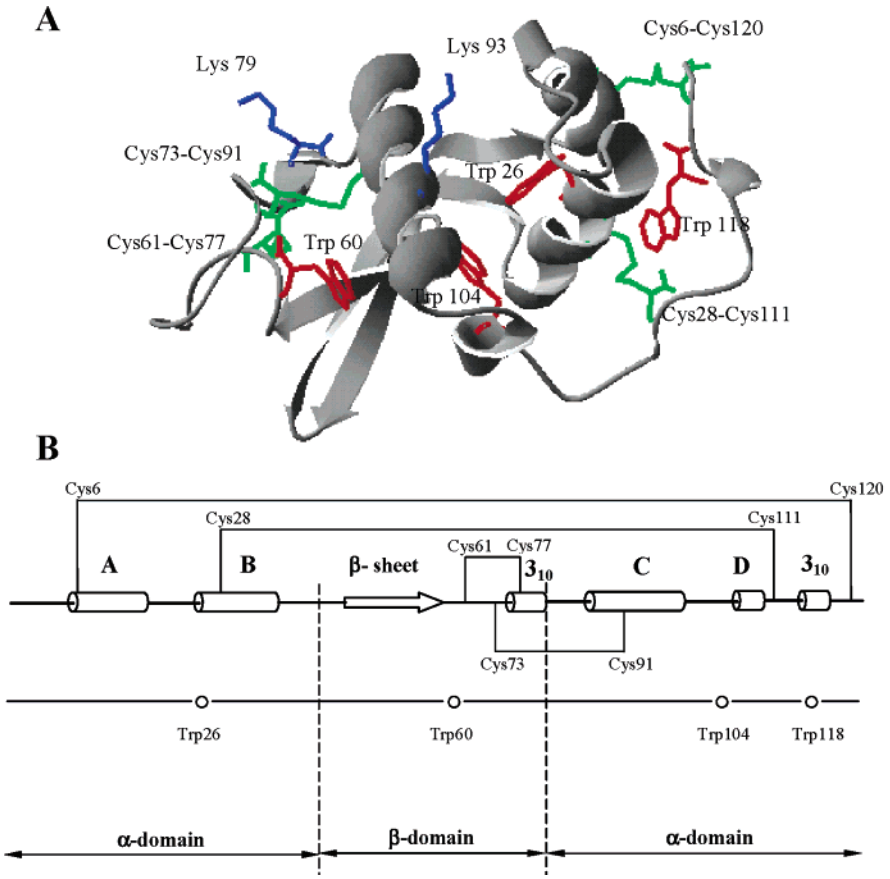


FIGURE 1: (A) Crystal structure of goat α-lactalbumin generated from coordinates deposited in the Brookhaven Protein Data Bank (entry 1HFY) using SWISS-MODEL (*26*). The side chains of the four Trp residues (red), the disulfide bonds (green), and two lysine residues (Lys79 and Lys93, blue) are represented by sticks. Lys122 is not represented, as it is not possible to define the conformation of residues 121−123 due to the poor definition of the electron density maps in this region (*23*). (B) Schematic representation of GLA. Secondary structure elements and the disulfide bonds in native GLA are indicated at the top. The domain boundaries are shown as dashed lines. Each of the tryptophan residues (○) is indicated.

formed by residues 35−85, contains a small three-stranded antiparallel β-sheet, a small 3_{10}-helix, and a large part of nonstructured loops. Two disulfide bridges (Cys6−Cys120 and Cys28−Cys111) are located in the α-helical lobe. The Cys61−Cys77 disulfide bridge, located in the smaller lobe, connects nonstructured loops, while the Cys73−Cys91 disulfide bridge forms a bridge between the two lobes. Upon analysis of the reaction products of illuminated GLA, we observed photolytic cleavage of Cys6−Cys120 and Cys73−Cys91 (*7*). As Trp118 is close to the former disulfide bridge and Trp60 is close to the latter, a direct relationship between the excitation of these two Trp residues and the reduction of their nearby disulfide bridge was postulated. Nevertheless, a number of questions remained. Trp118 is closer to the sulfur atoms of Cys28−Cys111 (3.4 Å) than to those of Cys6−Cys120 (9.8 Å), so we expected to see photoreduction of Cys28−Cys111, rather than Cys6−Cys120. Furthermore, following illumination of human α-lactalbumin (HLA), which lacks Trp26, no reduction of Cys6−Cys120 was observed (*8*), unexpectedly suggesting that the more distant Trp26 (at 13.6 Å) might mediate the photolysis of Cys6−Cys120, rather than Trp118. Last, Permyakov et al. (*8*) observed photolytic cleavage of Cys61−Cys77 in HLA, a cleavage not seen in our work with GLA. In contrast, in the tryptic digest of irradiated GLA, we observed a 1553.0 Da fragment, which, on the basis of the available MS analysis, was interpreted as being a peptide in which the Cys61−Cys77 bridge was conserved (*7*). Another point of interest in our previous study is that the photoreduction of disulfide bonds resulted in the formation of a single free thiol, suggesting that intermediately formed thiyl radicals may be involved in the creation of new bonds. However, we could not unambiguously identify these newly formed cross-links.

The first goals of this study were to obtain more information about possible bonds created upon photolysis and to clear up the differences between the earlier studies on goat and human LAs. Illuminated wild-type rGLA was therefore carbamidomethylated and digested with trypsin, and the peptides that formed were extensively analyzed using two new devices: a MALDI-TOF-TOF-MS instrument and a chip-based nano-ESI MS instrument, which allowed better identification of the peptide fragments than was possible in our earlier work (*7*).

To examine the relationships between the photoexcitation of a particular Trp and the associated reduction of disulfide bridges, we constructed four GLA mutants by replacing single Trp residues with Phe. The recombinant LAs (W26F, W60F, W104F, and W118F) were expressed in *Pichia pastoris*. We compared the photolytic degradation products of each mutant with that of wild-type rGLA and also followed the effects of photolysis on the conformation and enzymatic activity of the proteins.

MATERIALS AND METHODS

Protein Expression and Purification. The Trp mutants of GLA, constructed as described by Vanhooren et al. (*10*), were expressed in *P. pastoris* and purified by hydrophobic interaction and affinity chromatography (*10*, *11*). The integrity of all proteins was confirmed by electrospray ionization mass spectrometry (ESI-MS). Protein concentrations were determined from the optical density at 280 nm (*12*). The calculated extinction coefficients were 28 840 M^{-1} cm^{-1} for wild-type GLA and 23 150 M^{-1} cm^{-1} for the single mutants (W26F, W60F, W104F, and W118F). All experiments were performed in 10 mM Tris-HCl buffer (pH 7.5) containing 2 mM Ca^{2+}.

Lactose Synthase Assay. The lactose synthesis regulatory activities of the recombinant LAs were compared to that of authentic GLA using bovine β-galactosyltransferase (GTase) and UDP-galactose according to the method of Brew et al. (*13*). The assays were performed in 200 μL of 50 mM Tris-HCl buffer (pH 7.5), 20 mM $MnCl_2$, 40 mM glucose, 0.3 mM UDP-galactose, 0.4 μCi of UDP-[6-^{3}H]galactose, 6 milliunits of Gtase, and 4−40 μg of recombinant protein (wild-type rGLA, W26F, W60F, W104F, and W118F). The amount of synthesized lactose was measured using a liquid scintillation counter (Packard Tri-Carb 1900TR).

Illumination of GLA. Wild-type GLA and its Trp mutants were illuminated in an Aminco-Bowman (Rochester, NY) Series 2 spectrofluorimeter. The excitation wavelength was centered at 290 nm, with a band-pass of 16 nm, in contrast to the illumination at 280 nm used in our previous work (*7*). We choose the wavelength of 290 nm to reduce the likelihood of interference by Tyr excitation. A 2 mL sample of a protein solution (30 μM in the above Tris/Ca^{2+} buffer) was illuminated at 4 °C in the cuvette holder of the spectrofluorimeter. During illumination, the solution was stirred with a magnetic bar at 120 rpm. To avoid oxidation, all solutions were thoroughly degassed prior to use and kept under a N_2 atmosphere during illumination. The number of free thiol groups was determined using DTNB (*14*, *15*). The lactose synthesis regulatory activities (*7*) of the illuminated and nonilluminated recombinant proteins were compared to that of authentic LA.

Spectroscopic Measurements. Fluorescence, circular dichroism, and absorption measurements were carried out immediately after illumination, as described previously (*7*). In short, steady state fluorescence spectra were acquired at 4 °C using an Aminco-Bowman Series 2 spectrofluorimeter. CD measurements were performed at 25 °C on a Jasco (Tokyo, Japan) J-600 spectropolarimeter. Cuvettes of 10 or 1 mm were used for the near-UV or far-UV region, respectively. The absorption measurements were performed on an Uvikon 933 double-beam UV−vis spectrophotometer (Kontron Instruments, Milan, Italy) at room temperature.

Peptide Characterization. Iodoacetamide-treated samples of the illuminated proteins were prepared as described in our previous study (*7*). However, compared to this earlier study, novel mass spectrometric approaches were used. The tryptic peptides were analyzed using MALDI-TOF-TOF-MS (4700 Proteomics Analyzer, Applied Biosystems). Previously, we used a single MALDI-TOF instrument, which did not allow us to perform MS/MS on MALDI-generated ions. In the current setup, a total of 2000 shots were collected for MS data acquisition (40 subspectra, each accumulated from 50 laser shots). All MS/MS data were acquired in 1 keV MS/MS mode, using air as the collision gas (1.2×10^{-7} Torr). A total of 3000 shots (40 subspectra, each accumulated from 75 laser shots) were acquired with the timed-ion-selector window set to 250 resolution (fwhm). Electrospray MS and MS/MS were performed on a Q-TOF mass spectrometer (Micromass) following the manufacturer's instructions, except that the original nano-electrospray source was replaced by a chip-based nano-ESI device (NanoMate 100, Advion Biosciences).

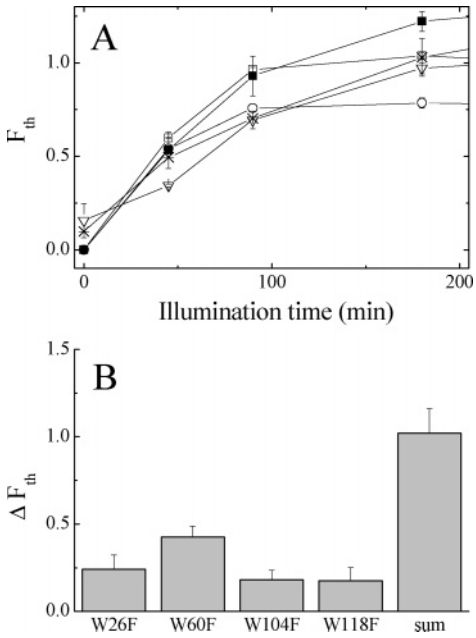


FIGURE 2: (A) Number of free thiol groups per recombinant GLA (F_{th}) generated as a function of illumination time measured using Ellman's reagent. Wild-type GLA (■), W118F (□), W60F (○), W26F (▽), and W104F (∗). The samples were illuminated as described in the Materials and Methods. The error bars represent the SEM for 3−5 different experiments. (B) Difference between the amount of thiols per LA unit formed by wild type and mutant GLA (W26F, W60F, W104F, or W118F) (ΔF_{th}) after 3 h of illumination. The sum of the differences for all Trp mutants is shown in the last column.

RESULTS

Disulfide Bond Cleavage in the Trp Mutants

Wild-type rGLA and the four single-Trp mutants (W26F, W60F, W104F, and W118F) were excited with light at 282−298 nm to determine whether the replacement of the various Trp residues with Phe affected the photoinduced cleavage of disulfide bonds. The number of free thiols generated per protein molecule (F_{th}) was measured using Ellman's reagent and plotted as a function of irradiation time (Figure 2A). The change with time in the F_{th} for wild-type rGLA was in good agreement with that for authentic GLA from milk under the same conditions (*7*), indicating that both proteins behaved similarly upon irradiation. For all the recombinant proteins (wild type and mutants), the rate at which free thiols were formed during illumination gradually decreased, as expected for a reaction with decreasing availability of disulfide bonds. Interestingly, the maximum F_{th} value was reached more rapidly with mutants W60F and W118F than with mutants W26F and W104F or wild-type rGLA. This difference in

time needed to reach the maximum F_{th} was the first clear-cut indication that the different Trp residues direct the photolytic cleavage of disulfide bonds in different ways, showing that the disulfide groups which are potential targets for photolytic cleavage mediated by Trp26 and Trp104 became exhausted more rapidly than those for which photolysis was mediated by Trp60 and Trp118.

To obtain more quantitative information about the contributions of the different Trp groups to disulfide cleavage, we calculated the difference between the amount of thiols formed in wild-type and mutant rGLAs after illumination for 3 h (ΔF_{th}; a larger value means fewer free thiols were formed in the mutant). The data, shown in Figure 2B, show the extent by which the progress of photolytic degradation was decreased by each Trp replacement. Irradiation of W60F and W26F resulted in 0.43 ± 0.06 or 0.24 ± 0.06 fewer thiol/LA molecule, respectively, than irradiation of wild-type rGLA (1.22 ± 0.05 thiols/LA molecule), while irradiation of W104F or W118F resulted in 0.18 ± 0.08 fewer thiol/LA molecule than wild-type irradiation. The sum of the individual decreases caused by each of the four Trp mutants was 1.03 ± 0.14, close to the value for wild-type rGLA. This suggests that each Trp residue has an independent impact on the photolytic degradation process. These data also showed that potential photolytic cleavage of disulfide bonds not mediated by Trp residues was negligible compared to Trp-mediated cleavage.

Spectroscopic Studies

Emission fluorescence and near-UV CD spectroscopy were used to monitor conformational changes caused by disulfide bond cleavage in the illuminated proteins. In general, the partial unfolding of LA can be monitored by two different features of the fluorescence spectrum (*16*). An increase in fluorescence intensity is seen, as the number of contacts of Trp with quenching residues is reduced in the expanded state, while a red shift of the maximum wavelength is caused by enhanced solvent accessibility of the Trp residues in the partially unfolded state.

Figure 3A shows the area under the fluorescence curve as a function of irradiation time. In general, the fluorescence increased during the first 100 min of illumination and then decreased. The first phenomenon corresponds to the loosening of the tertiary structure of wild-type GLA and its mutants upon illumination, while the subsequent loss of fluorescence probably results from photodestruction of Trp residues. As shown in Figure 3B, all the recombinant proteins exhibited a continuous red shift of the fluorescence upon irradiation, reflecting a continuous process of unfolding induced by irradiation. The value for the emission maximum (λ_{max}) after illumination for 6 h tended to a plateau at 340 nm. However, Trp in water has an emission maximum (λ_{max}) of 348 nm (*17*), and the λ_{max} for wild-type GLA in 6 M guanidinium hydrochloride is 345 nm (*18*, *19*). None of the recombinant proteins reached this fluorescence wavelength, which may indicate that parts of the native structure were conserved in the illuminated GLA and/or that a fraction of the protein was still native.

Lactose Synthesis

The influence of irradiation on the ability of LA to act as a stimulator of lactose synthase activity was investigated.

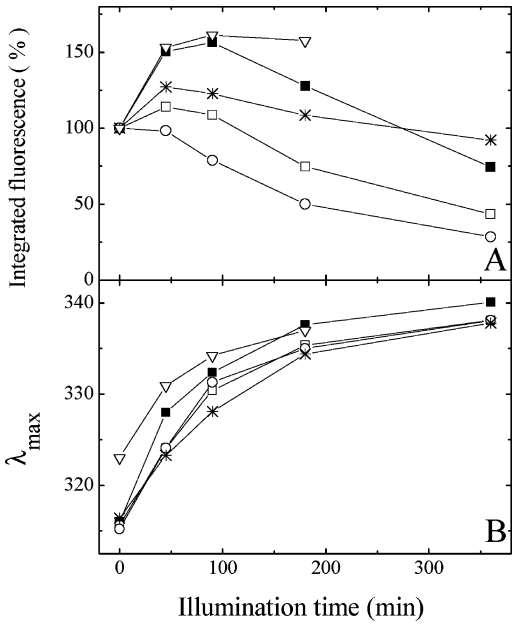


FIGURE 3: (A) Area under the fluorescence curve expressed as a percentage of the zero time value and (B) λ_{max} as a function of illumination time for wild-type GLA (■) and mutants W118F (□), W60F (○), W26F (▽), and W104F (∗). The magnitude of the variation within separate experiments is within the dimensions of the depicted symbols.

For wild-type rGLA, ~35% of the original lactose synthetase regulatory activity was conserved after illumination for 3 h. The lactose synthase regulatory activity of the two mutants, W26F and W60F, was similar to that of wild-type rGLA and responded similarly to irradiation. Replacement of Trp118 with Phe caused almost complete loss of lactose synthesis, as this Trp is part of the catalytic cluster in lactose synthetase (*20*), while replacement of Trp104 reduced lactose synthetase regulatory activity to 20% of that of wild-type rGLA. Because of the low activity of W118F and of W104F, no data were obtained for the influence of irradiation on the rate of lactose synthesis.

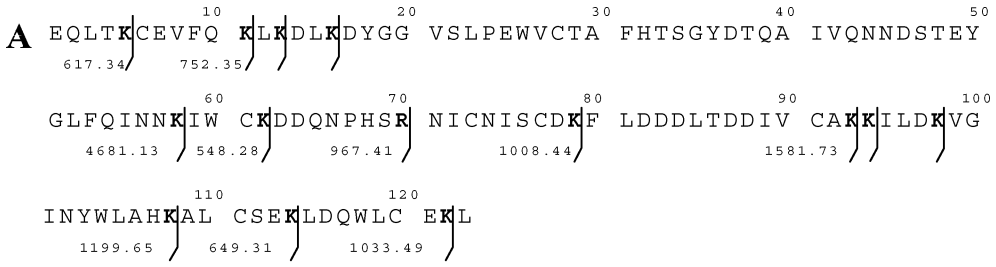
Mass Spectrometry

Wild-type rGLA and the mutants were exposed to UV irradiation at 282−298 nm for 180 min; then the free thiol groups were carbamidomethylated with iodoacetamide and the proteins digested with trypsin, and the resulting peptides were analyzed using MALDI-TOF-TOF-MS and nano-ESI-MS. The amino acid sequence of GLA and potential tryptic digestion sites are presented in Table 1A. A summary of the tryptic peptides obtained from intact wild-type GLA is shown in Table 1B. The differences in the spectra of the digests from carbamidomethylated nonirradiated and irradiated GLA were then analyzed by a combination of MALDI-MS/MS and nano-ESI MS/MS.

Mass Spectra of Wild-Type GLA. Comparison of the MS spectrum of the digest of carbamidomethylated nonirradiated wild-type GLA with that of the carbamidomethylated irradiated protein revealed significant differences. The spectrum of the digest of the illuminated protein showed a decrease in the magnitude of the two peaks at 1784.80 and 3135.38 Da (data not shown), masses corresponding to two peptides with intact disulfide bridges (Table 1B), and the disappearance of these peaks suggests that these disulfide bridges were

Table 1: (A) Amino Acid Sequence of Wild-Type rGLA[a] and (B) Peptides Containing Intact Disulfide Bridges Derived from Wild-Type GLA by Tryptic Digestion

A



B

Disulfide bridge	Peptide sequence	Mass $[M+H]^+$
6-120	$L^{115}DQWLC^{120}EK^{122}$ $C^{6}EVFQK^{11}$	1784.84
61-77 73-91	$F^{80}LDDDLTDDIVC^{91}AK^{93}$ $N^{71}IC^{73}NISC^{77}DK^{79}$ $I^{59}WC^{61}K^{62}$	3135.38
28-111	$D^{17}YGGVSLPEWVC^{28}TAFH(\ldots)NK^{58}$ $A^{109}LC^{111}SEK^{114}$	5329.44

[a] Potential trypsin digestion sites and the masses of the peptides in a tryptic digest that would not contain disulfide bridges are given.

ruptured. In addition to the decrease in the magnitudes of these two peaks, four new peaks (1553.70, 1639.0, 1841.80, and 3192.49 Da) appeared in the spectrum of the digest of the illuminated protein. The sequences of all peptides were investigated using MS/MS analysis, which selects a precursor ion for low-energy gas-phase collisional activation and subsequently determines the *m*/*z* value of the resulting product. Under these conditions, peptide ions fragment primarily at the amide bond, yielding a ladder of sequence ions. Retention of charge on the N-terminal portion of the peptide ion yields a b-type ion; retention on the C-terminal portion of the peptide ion yields a y-type ion. Subtracting the masses of adjacent ions reveals the amino acid sequence. MALDI-TOF MS/MS of disulfide-linked peptides also yields specific fragments due to disruption of the C−S−S−C linkage at any of the three bonds (*21*). We designated these as F-fragments, as shown in panels A and B of Figure 4.

MS/MS analysis of the peptides at *m*/*z* 1784.80 and 1841.80 is illustrated in Figure 4. For nonilluminated GLA, the series of b-type ions, y-type ions, and F-type ions generated in the analysis of the *m*/*z* 1784.80 ion ([peptide + H]$^+$) (Figure 4A) was consistent with the sequence of a peptide containing an intact Cys6−Cys120 bond. After UV exposure, the peak for the peptide at 1784.80 Da was reduced in height (data not shown), suggesting that the Cys6−Cys120 bond was broken, and an additional peak was detected at *m*/*z* 1841.80. MALDI-MS/MS (Figure 4B), as well as ESI-MS/MS (data not shown), suggested that this peak corresponded to a complex peptide formed by a novel cross-link between Cys6 and Lys122 and containing a carbamidomethyl group (Cam) at Cys120.

MS/MS analysis of the peptide at 1553.70 ([M + H]$^+$), which appeared after UV irradiation, revealed that it was the Ile59−Arg70 peptide, with a carbamidomethyl group on Cys61 (Figure 5). Clearly, this carbamidomethylated peptide resulted from the rupture of the Cys61−Cys77 disulfide bridge. However, the appearance of the Ile59−Arg70 peptide was unusual, as digestion with trypsin would be expected to lead to the Ile59−Lys62 peptide due to cleavage after the Lys residue (Table 1A). Moreover, the theoretical mass of the Ile59−Arg70 peptide is 1555.67 Da, rather than the observed value of 1553.70 Da, suggesting a loss of two protons after UV exposure. The mass difference between the y-ions in the MALDI-MS/MS and ESI-MS/MS spectra confirmed that the Cys61Cam had a mass of 158 Da, instead of the expected mass of 160 Da (Figure 5). This suggests the formation of a double bond between Cα and Cβ of the cysteine residue, probably as a result of a radical reaction following UV-induced disulfide bridge cleavage.

The MS/MS data for the new peaks at 3192.49 and 1639.0 Da provided evidence for the cleavage of the Cys73−Cys91 disulfide bridge. The latter peak has been previously shown to correlate with the Phe80−Lys93 peptide containing carbamidomethylated Cys91 (*7*). The appearance of the novel peak at 3192.49 Da revealed an interesting effect seen after reduction of this disulfide bond. In the nonilluminated protein, a peptide containing two disulfide bridges (Cys61−Cys77 and Cys73−Cys91) was found at 3135.38 Da (Table 1B). After UV exposure, ESI-MS revealed that this fragment has gained 57 Da due to a carbamidomethylated thiol. The fact that the peptide was still present, although one of its cysteines was carbamidomethylated, suggested that a second

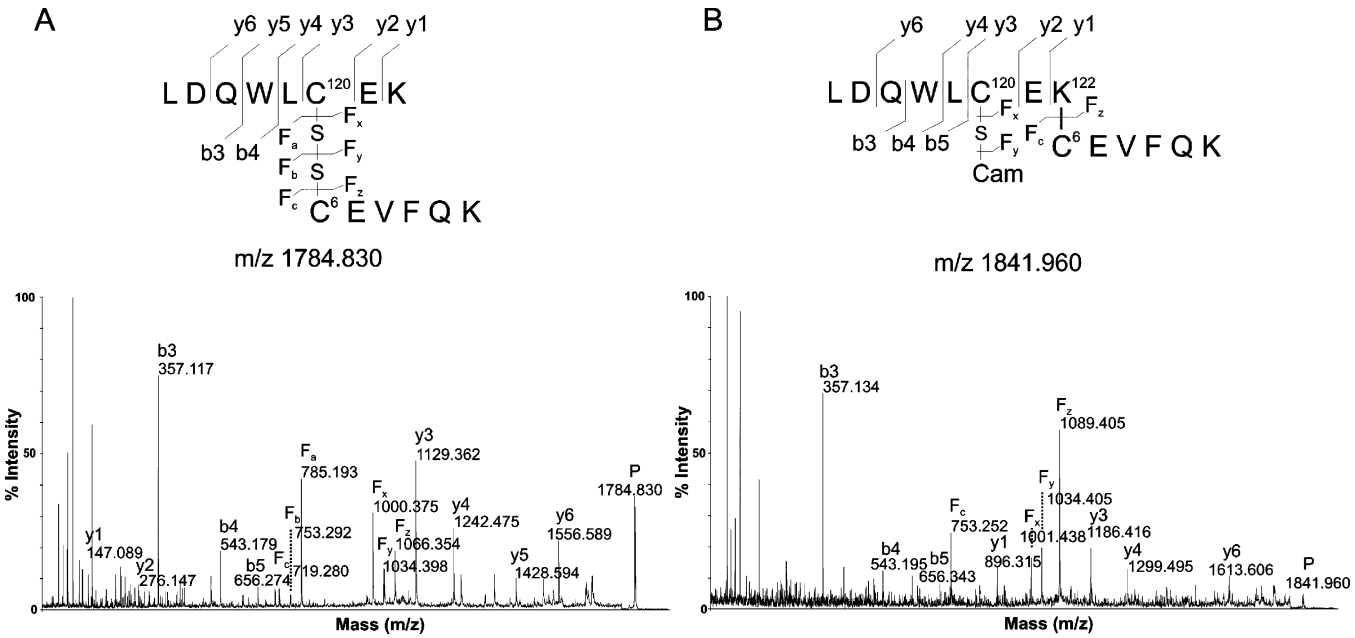


FIGURE 4: MALDI-MS/MS spectra of (A) the peptide (*m*/*z* 1784.80) derived from carbamidomethylated nonilluminated wild-type GLA containing the intact Cys6−Cys120 disulfide bridge and (B) the peptide (*m*/*z* 1841.80) linked by a Cys6−Lys122 bond derived from carbamidomethylated irradiated wild-type GLA.

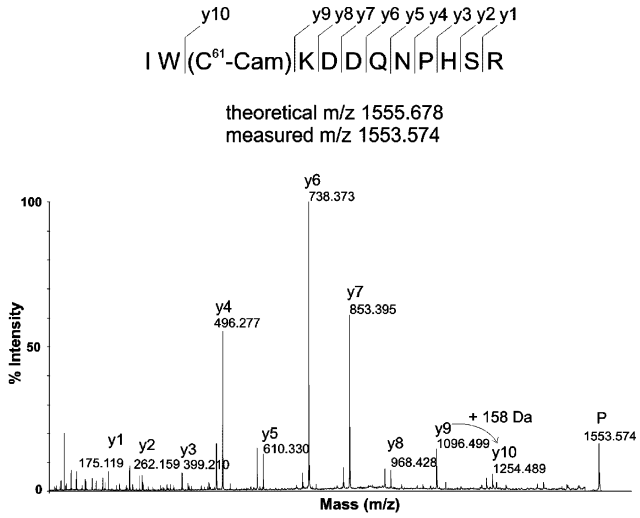


FIGURE 5: MALDI-MS/MS spectrum of a peptide (*m*/*z* 1553.7) derived from the tryptic digest of carbamidomethylated irradiated wild-type GLA. A fragmentation ion with a 2 Da difference between the theoretical and measured mass is indicated with an arrow.

Cys−Lys bond, similar to the Cys6−Lys122 bond, was formed. To test this hypothesis, the fraction containing the 3192.49 Da peptide was treated with DTT, resulting in a new peptide at *m*/*z* 882.60 ($[M + 3H]^{3+}$). This mass corresponds to a fragment composed of a Phe80−Lys93 peptide linked to an Asn71−Lys79 peptide, with either a Cys91−Lys79 bond and carbamidomethylation at Cys73 or a Cys73−Lys93 bond and carbamidomethylation at Cys91. Both possibilities are shown in Figure 6. ESI-MS/MS confirmed the nature of this cross-linked peptide but could not determine the exact linkage.

In conclusion, MS analysis of illuminated wild-type GLA revealed the reduction of three disulfide bonds (Cys6−Cys120, Cys73−Cys91, and Cys61−Cys77) accompanied by two unusual phenomena, the formation of at least one Cys−Lys bond and the remarkable 2 Da loss at the carbamidomethylated Cys61.

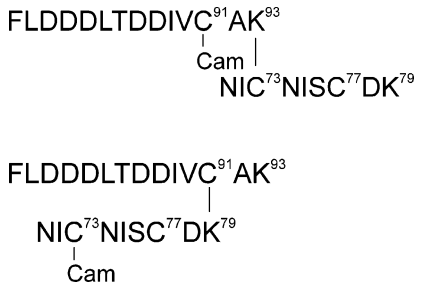


FIGURE 6: Possible structures of the peptide at *m*/*z* 882.60 ($[M + 3H^+]$). After UV exposure, iodoacetamide treatment, and tryptic digestion, wild-type GLA gave a 3192.49 Da carbamidomethylated peptide. Following treatment with DTT, this complex peptide was reduced to an 2644.8 Da peptide.

Mass Spectra of the Tryptophan Mutants. Analogous experiments were performed on the Trp mutants to define which of the four Trp residues were essential for inducing the photoreduction of the disulfide bonds. Table 2 summarizes whether a particular disulfide bridge (6−120, 28−111, 61−77, or 73−91) in wild-type GLA and its mutants was cleaved by irradiation. For each disulfide bond ruptured, carbamidomethylated thiols (Cys-Cam) identified by MS/MS are indicated. Table 2 also indicates whether a new link between a cysteine and a lysine was found. We here list the most striking results, which will be discussed in the next section. For each photolyzed disulfide bridge, only one carbamidomethylated Cys could be detected. The Cys28−Cys111 disulfide bridge was not affected by UV radiation in any of the recombinant proteins. In contrast, in all proteins, UV illumination resulted in disruption of the Cys61−Cys77 disulfide bridge. Evidence for the cleavage of the Cys6−Cys120 disulfide bridge was found in all proteins except mutant W26F. Furthermore, in mutants W26F and W104F, no evidence was found for disruption of the Cys73−Cys91 disulfide bridge.

Table 2: Rearrangement of Disulfide Bridges in Illuminated Recombinant GLAs[a]

	Cys6−Cys120	Cys28−Cys111	Cys61−Cys77	Cys73−Cys91	Cys6−Lys122	Cys91−Lys79 or Cys73−Lys93[b]
wild type	Cys120Cam	ND	Cys61Cam	Cys91Cam	yes	yes
W26F	ND	ND	Cys61Cam	ND	no	no
W60F	Cys120Cam	ND	Cys61Cam	Cys91Cam	yes	yes
W104F	Cys120Cam	ND	Cys61Cam	ND	no	no
W118F	Cys120Cam	ND	Cys61Cam	Cys91Cam	yes	yes

[a] The formation of carbamidomethylated cysteines demonstrates cleavage of the disulfide bond. ND (not detected) indicates that no evidence of disulfide bond cleavage was found. [b] The exact nature of the Cys−Lys cross-link could not be determined.

DISCUSSION

To study the effect of individual Trp residues on the photoinduced degradation of disulfide bonds in GLA, we constructed single mutants in which each of the four Trp residues were substituted with Phe (W26F, W60F, W104F, and W118F). In a first step, the number of free thiol groups created following photolysis was followed as a function of the time of illumination (Figure 2A). Substitution of any Trp led to less thiol production. Interestingly, at a given time (90 min or 3 or 6 h), the sum of the decreases seen for the four different Trp mutants approximated the amount of thiols created in the wild-type protein, as shown by the 3 h results in Figure 2B. This provides evidence that Trp-mediated cleavage accounts for almost all of the photolytically cleaved disulfide bridges in GLA. After 3 h, the number of thiol groups generated by Trp60 was clearly higher than that generated by any of the other Trp residues. This was expected, as the fluorescence emission of Trp60 is strongly quenched (*10*), and nearby disulfide bridges, responsible for the quenching effect, should be primary candidates for undergoing cleavage. However, since Trp118 is also strongly quenched in GLA (*10*), we were surprised to observe a clearly smaller effect of Trp118 on thiol formation (Figure 2B). At least in the crystal structure, the indole group of Trp118 is in direct contact with the Cys28−Cys111 disulfide bridge (at 3.4 Å), so considerable transfer of energy or of electrons from photoexcited Trp118 to the Cys28−Cys111 disulfide bridge seems inevitable. It is possible that the poor contribution of Trp118 to disulfide cleavage is due to the Cys28−Cys111 disulfide bridge being situated in a very stable region of the protein. Indeed, Peng et al. (*22*) found that the region surrounding the Cys28−Cys111 disulfide bond has a very high preference for adopting a nativelike structure. Thus, this activated disulfide bond tends not to cleave, and even if it is cleaved, the resulting thiyl radicals would tend not to migrate away from each other; therefore, the disulfide bond might re-form before cross reactions can occur. This idea is in agreement with the fact that the Cys28−Cys111 disulfide bridge was the only disulfide bridge in GLA which was not cleaved (Table 2). Finally, Figure 2B shows that Trp26 and Trp104, which are quenched considerably less than Trp118, also contributed to the photolytic reduction of disulfide bridges. No contribution of Trp104 was expected in previous papers dealing with the photolytic cleavage of disulfide bridges in LA (*7*, *8*).

For a more thorough investigation of the effects of individual Trp residues on different disulfide bods, irradiated and nonirradiated samples of the various recombinant GLAs were treated with iodoacetamide and digested with trypsin, and then MALDI-MS/MS and nano-ESI-MS/MS analysis were used to unambiguously identify the newly created peptides.

For wild-type GLA, photoinduced cleavage of the following three disulfide bridges was seen: Cys6−Cys120, Cys61−Cys77, and Cys73−Cys91. We have previously demonstrated cleavage of the Cys6−Cys120 and Cys73−Cys91 bonds (*7*). In this earlier study, we also pointed to the existence of a peptide fragment of 1553.0 Da with Ile-Trp at the N-terminus. On the basis of its mass and the terminal amino acids, this was interpreted as the linked GLA fragments 59−62 and 71−79 (Table 1B), and the Cys61−Cys77 bond was therefore considered to be resistant to photolysis. However, the MALDI-MS/MS spectrum presented here allowed a far more thorough analysis of this peptide. The extended data showed that it consisted of GLA sequence 59−70 with a carbamidomethyl group on Cys61 and, consequently, pointed to photolysis of the Cys61−Cys77 bond (Figure 5). In the previous study, this possibility was not considered, as we expected that peptide 59−70 would be cleaved at Lys62 during tryptic digestion, yielding a peptide with the same mass. Furthermore, the ESI MS/MS spectrum obtained previously provided rather poor information about the lower-molecular mass part of the spectrum, leaving some uncertainty about the C-terminal part of the peptide. In addition, the carbamidomethylated peptide containing residues 59−70 of GLA was 2 Da smaller than expected, due to the removal of two hydrogens in the side chain of Cys61, a previously unexpected phenomenon.

The data summarized in Table 2 provide insight into the specificity with which the Trp residues mediate the photoactivation of a specific disulfide bond. First, mutant W26F was the only mutant which did not generate a peptide with a carbamidomethylated Cys120 or a peptide with a cross-link between Cys6 and Lys122, indicating that photolysis of the Cys6−Cys120 bond was exclusively mediated by photoexcitation of Trp26. In our earlier study (*7*), we assumed that photolysis of this bond would be mediated by Trp118. Indeed, the shortest distance between the indole ring of Trp118 and the Cys6−Cys120 disulfides is ∼9.8 Å, while that between the disulfides and Trp26 is 14.5 Å (*23*). However, Permyakov et al. (*8*) studied photoreduction of disulfide bonds in HLA, which has the same Trp residues as the W26F mutant of GLA, and observed photolysis of Cys61−Cys77 and Cys73−Cys91 bonds, but not of the Cys6−Cys120 bond, and postulated that Trp26 mediates photolysis of the Cys6−Cys120 bond. In an attempt to explain this preferential impact, Permyakov et al. (*8*) suggested that Trp26 may exist in different rotamer forms by which the inter-residue distance with the Cys6−Cys120 bond could be shortened to as little as 8.0 Å. Our study offers an alternative explanation for why Trp26, rather than Trp118,

mediates photolysis of the Cys6−Cys120 disulfide bond. As mentioned above, photoexcited Trp118 readily transfers energy or electrons to the Cys28−Cys111 bond, which does not tend to undergo net cleavage. Furthermore, the fact that a new Cys−Lys cross-link was detected following photolysis of the Cys6−Cys120 bond, as well as following photolysis of the Cys73−Cys91 bond (Table 2, column 7), is an indication that the mediation of a Lys group can also be important in the cleavage of disulfide bonds. Hawkins and Davies (*24*) demonstrated that one-electron oxidations of a free amine group give rise to nitrogen-centered radicals, namely, aminium cation radicals ($RNH_2^{+\bullet}$) or neutral aminyl radicals ($RNH^{\bullet}$). These authors also mentioned that the side chain aminyl radicals formed from the ϵ-amino group of Lys side chains generate radicals at either C-3 or at the α-carbon atom (*24*). Conversely, Fu et al. (*25*) observed that Cys residues in peptides form intramolecular and intermolecular −S−NH− bridges with lysine and arginine residues when radicals are induced by hypochloric acid. Although our analysis did not identify which atoms of the Cys6 and Lys122 side chains were involved in the cross-links, the generation of such sulfenamide bonds appears to be likely when dealing with radical reactions initiated by photoexcitation of Trp.

The results, summarized in the third column of Table 2, show that a peptide with a mass corresponding to the peptide expected after cleavage of the Cys28−Cys111 bond was not found in digests of any of the recombinant GLAs, In contrast, similar amounts of a peptide with a mass of 5329.4 Da (*m*/*z* 1333.4, $[M + 4H^+]$), corresponding to the linked GLA fragments 17−58 and 109−114, were observed before and after illumination. These findings suggest that the Cys28−Cys111 bond resists Trp-mediated photoexcitation. As mentioned before, a plausible reason for the high resistance of this disulfide bond is that the Cys28−Cys111 bond is situated in a very stable region of the protein.

In contrast to the stability of the Cys28−Cys111 bond, the Cys61−Cys77 bond was photolyzed in all recombinant proteins (Table 2). This indicates that none of the substituted Trp residues mediated the photoreduction of the Cys61−Cys77 bond in an exclusive way; i.e., at least two Trp residues mediate the photolysis of the Cys61−Cys77 bond. The distances to the disulfide bridge are 15.9 Å for Trp26, 6.4 Å for Trp60, 11.7 Å for Trp104, and 24.5 Å for Trp118. The distance from Trp60 to the Cys61−Cys77 bond is clearly shorter than the distances from the three other Trp residues, suggesting it is most likely involved in the mediation of Cys61−Cys77 photolysis. Evidence supporting this hypothesis is that Trp60 strongly enhanced the production of free thiols upon illumination (Figure 2B), and there is no evidence that it mediates photolysis of any other disulfide bond (Table 2). As Trp104 is the next closest Trp, this Trp residue may also mediate photolysis of the Cys61−Cys77 bond. To unambiguously test this hypothesis, a double Trp60/Trp104 mutant should be constructed.

Another intriguing observation, shown in Table 2, is that the Cys73−Cys91 bond was not subjected to photolytic cleavage when Trp26 or Trp104 was absent. The peptide with a mass corresponding to that expected following rupture of the Cys73−Cys91 disulfide bond was well defined by the analysis of the other GLA mutants and can, therefore, be traced accurately. Consequently, the fact that no 3192.49 or 1639.0 Da peaks were detected in the peptide digest of photolyzed W26F and W104F is a strong indication that photolysis of the Cys73−Cys91 bond did not occur in these mutants. To explain these results, we have to assume that, in one mutant, photolysis has been interrupted in a direct way while, in the other mutant, it was interrupted in an indirect way. The Cys73−Cys91 bond is closer to Trp104 (7.7 Å) than to Trp26 (10.9 Å); therefore, it would seem reasonable that photolysis of this disulfide bridge would most readily be mediated by Trp104, and substitution of this residue may directly prevent photolysis. The indirect interruption of photolysis should then result from displacement of one of the reactive groups upon substitution of Trp26. Pike et al. (*23*) noted that, in HLA, in which residue 26 is leucine, rather than Trp, as in GLA, the 3_{10}-helix at positions 13−15 does not pack as closely as it does in GLA. As Trp104 is located near this helix, the substitution of Trp26 may also reorient Trp104 and, therefore, prevent the transfer of an electron to the Cys73−Cys91 disulfide bond. In favor of this possibility is the fact that the fluorescence maximum of W26F is at 323 nm, rather than 315 nm, as in the other constructs (Figure 3B, λ_{max} at the start of the illumination). As Vanhooren et al. (*10*) showed that Trp60 and Trp118 are strongly quenched, the fluorescence of GLA mainly results from Trp26 and from Trp104, and the fluorescence shift observed upon substitution of Trp26 first of all reflects a change in the polarity around Trp104.

Finally, it is worth remembering that the Cys73−Cys91 bond is the second disulfide bond in GLA for which photolytic disruption may result in the formation of a new Cys−Lys cross-link. Analysis of our ESI-MS/MS spectra did not make it possible to distinguish between a Cys91−Lys79 and a Cys73−Lys93 cross-link. An inspection of the crystal structure (*23*) indicates that, in native GLA, Cys91 does not make direct contact with Lys79, nor does Cys73 with Lys93. As a consequence, the formation of the Cys−Lys bond seems to require some reorganization in GLA following cleavage of the Cys73−Cys91 bond. Furthermore, the crystal structure indicates that the creation of a Cys73−Lys93 cross-link is far less probable than creation of a Cys91−Lys79 linkage. Since Cys73 and Lys93 are located at opposite sides of helix C, any linkage of those residues would require a large displacement of this central helix, which is very unlikely.

In conclusion, the results of this study clarify the relationships between the photoexcitation of particular Trp residues in GLA and the associated reduction of disulfide bridges. The photolytic cleavage of the Cys6−Cys120 bond results exclusively from photoexcitation of Trp26, while that of the Cys73−Cys91 bond is most likely mediated by excited Trp104. At least two Trp residues mediate the photolysis of the Cys61−Cys77 bond; presumably these are Trp60 and Trp104. The Cys28−Cys111 disulfide bridge is not sensitive to photolytic cleavage. In this study, we also show that new Cys−Lys bonds are formed upon photolytic cleavage of disulfide bonds in GLA and that two H atoms are lost in a Cys residue after photolytic cleavage.

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