Photoexcitation of Tryptophan Groups Induces Reduction of Two Disulfide Bonds in Goat α-Lactalbumin†

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ABSTRACT: Illumination of goat α-lactalbumin (GLA) with 280 or 295 nm light results in tryptophan-mediated photolysis of disulfide bonds within the protein. The photolysis is not dependent on the absence or presence of Ca^{2+} and is observed as well on illumination of native and of partially unfolded GLA. However, photolysis of native GLA results in a partial unfolding of the protein. The latter phenomenon is most clearly observed on fluorescence measurements at low temperatures (near 3 °C). The photolysis induces some dimerization and oligomerization, but most GLA molecules remain monomeric. To obtain more information about the reaction products, the illuminated protein is treated with iodoacetamide to label the free thiol groups, it is fragmented with trypsin, and the fragments are analyzed by mass spectrometry. Via this approach, we observe that the cleavage of disulfide bonds is restricted to Cys6−Cys120 and Cys73−Cys91 bonds. The photolytic cleavage of either of these disulfide bonds results in the formation of a single free thiol, a phenomenon restricted to Cys120 and Cys91, respectively. We also found indications that a thioether linkage is formed between Cys73 and Trp60. The alkylsulfenylation of Trp60 presumably results from a combination of primary thiyl and tryptyl radicals.

Exposure of enzymes to near-UV radiation induces structural changes and alters their biocatalytic function (*1*). The damage is initiated through photon absorption of the chromophoric amino acids. The indole nucleus is the most strongly near-UV absorbing group in proteins. Therefore, tryptophan residues are primary components in the activation of protein photodegradation. The nature of these degradation reactions depends on the microenvironment within the protein (*2*). Some decades ago, it was suggested that absorption of near-UV light by the aromatic amino acid residues contributes to reductive splitting of disulfides in proteins (*3*). Despite the important impact that this phenomenon may have on the inactivation of enzymes and hormones, only recently has a clearly certified example of tryptophan-mediated photoreduction of a disulfide bond within a protein (cutinase) been registered (*4*). For a better understanding of the phenomenon, it is important that more examples of this kind of photolysis be described and that the concomitant structural changes becharacterized. Indeed, at present, very little is known about the resulting photoproducts.

In the past years, we have studied the conformational changes of α-lactalbumins (LAs).[1] LAs are small globular proteins. The variant from goat milk (GLA) consists of 123 amino acids. Its three-dimensional X-ray structure determined by Pike et al. (*5*) is presented in Figure 1. A deep cleft divides the molecule into two lobes. One lobe comprises residues 1−39 and 85−123. That lobe contains four helices. The second, consisting of residues 40−84, is characterized by the presence of a three-stranded β-sheet. The unfolding of LAs has been studied intensively because the molecule adopts a "molten globule" state under mild denaturing conditions. In this situation, the protein is still compact but lacks well-defined tertiary interactions, and the hydrophobic interior is readily accessible for solvent molecules (*6*, *7*). Near-UV circular dichroism and fluorescence spectroscopy have been widely used for the characterization of the molten globule state (*8*−*14*). Both spectroscopic properties mainly reflect the nature of the interactions of the tryptophan residues with their surroundings. GLA contains four Trp residues, located at positions 26, 60, 108, and 118. The protein also contains four disulfide bridges between residues 6 and 120, 28 and 111, 61 and 77, and 73 and 91. In the native state, the fluorescence signal of Trp60 and Trp118 is significantly quenched by the disulfide bridges in their vicinity (*15*). These structural characteristics make LA a suitable protein for a more thorough investigation of disulfide disruption under the influence of photoexcited Trp residues.

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[1] Abbreviations: LA, α-lactalbumin; GLA, goat α-lactalbumin; 3SS-GLA, goat α-lactalbumin in which the Cys6−Cys120 disulfide bond is selectively reduced; 3SS-CAM-GLA, goat α-lactalbumin in which the Cys6−Cys120 disulfide bond is selectively reduced and Cys6 and -120 are carbamidomethylated; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB^-, 2-nitro-5-thiobenzoate ion; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; ESI-MS, electrospray ionization mass spectrometry; Q-TOF, quadrupole time-of-flight; MS/MS, tandem mass spectrometry; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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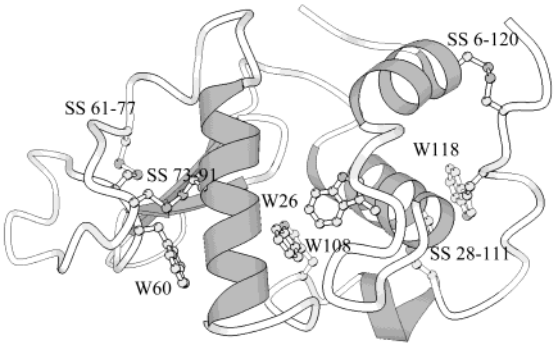

FIGURE 1: Molscript diagram (*31*) of the X-ray structure of GLA (PDB entry 1HFY) showing the four disulfide bridges and the four Trp side chains.

In this work, we describe an unusual increase and red shift of the fluorescence of GLA by irradiation of the protein at 280 or 295 nm. We provide evidence that these fluorescence changes are accompanied by cleavage of disulfide bonds. Mass spectrometry of the tryptic fragments of the carbamidomethylated protein has been used as an effective method for the analysis of the free thiol groups within partly reduced LA (*16*). On applying this method to illuminated GLA, we find that out of the four S−S bonds in intact GLA only the Cys6−Cys120 and Cys73−Cys91 bonds become disrupted. This observation makes it clear that not all the disulfide bonds in GLA are in a favorable environment for Trp-mediated photolysis. The ability of an excited Trp to initiate cleavage of a disulfide bond seems to be enhanced as well by direct van der Waals contact (Trp60 with the Cys73−Cys91 bond) as by the weakness of the bond (the Cys6−Cys120 bond is more distant from Trp118 than the Cys28−Cys111 bond, but the former bond is weaker than the latter). With regard to the resulting products, our results indicate that Trp-mediated photolysis only results in a partial reduction of the involved disulfide bond and that only a single free Cys is created in each case. We find indications that the second Cys can form a thioether linkage with the indole group of the Trp residue that is involved.

MATERIALS AND METHODS

Materials. Goat α-lactalbumin (GLA) was prepared from fresh milk whey. After addition of Tris and EDTA to final concentrations of 50 and 1 mM, respectively, and adjustment of the pH to 7.5 with HCl, the whey was applied to a Streamline-Phenyl column (Pharmacia, Uppsala, Sweden). Apo-GLA was bound hydrophobically to the column, while the other whey proteins were eluted with the Tris-EDTA buffer (pH 7.5) (*17*). GLA was eluted by changing the eluting buffer to 50 mM Tris and 25 mM Ca^{2+} (pH 7.5), and Ca^{2+}-bound GLA was then demetallized as described previously (*18*). All experiments were performed in 10 mM Tris-HCl buffer (pH 7.5) containing either 2 mM Ca^{2+} or 2 mM EGTA. The GLA concentration was determined by spectrophotometry using an ϵ_{280} of 28 500 M^{-1} cm^{-1}. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent), used for the analysis of free thiol groups, was from Eastman (Rochester, NY).

Preparation of Illuminated GLA. A degassed solution (2.5 mL) containing 27 μM GLA or 3SS-CAM-GLA (see below) at pH 7.5 was illuminated in a 10 mm × 10 mm cuvette within an Aminco-Bowman (Rochester, NY) Series 2 spectrofluorimeter. The excitation wavelength was centered at 280 nm. To ensure an important photon flux, a broad band-pass of 16 nm was applied. Ferrioxalate actinometry showed that under these conditions, the incident flux was 5×10^{14} photons/s. During illumination, the solutions were stirred at 120 rpm. Spectroscopic studies were carried out immediately after illumination. The concentration of free thiol groups was determined with DNTB using, at 412 nm, a molar extinction coefficient of 13 600 M^{-1} cm^{-1} for TNB^- (*19*, *20*).

Preparation of 3SS-GLA. GLA in which the Cys6−Cys120 disulfide bridge is selectively reduced (3SS-GLA) was prepared according to the method of Kuwajima et al. (*21*) with the following modifications. After the experiment was started with 5 mg/mL GLA and after reduction of the disulfide bridge, 3SS-GLA was immediately separated from dithiothreitol by passing the reaction mixture through a Sephadex G-25 column equilibrated with 10 mM Tris-HCl buffer (pH 7.5) containing 2 mM EDTA. The number of free thiol groups was checked with DNTB as described above.

Labeling of the Free Thiol Groups. The thiol groups in 3SS-GLA or in illuminated GLA were carbamidomethylated by adding an equal volume of 10 mM iodoacetamide in 0.9 M Tris-HCl buffer (pH 8) containing 1 mM $CaCl_2$, allowing the reaction to proceed for 30 min in the dark at room temperature (*16*). The proteins were separated from excess reagent by gel filtration through a Sephadex G-25 column equilibrated with 10 mM ammonium bicarbonate (pH 7.5) and lyophilized. 3SS-GLA that has been carbamidomethylated and purified in this way is called 3SS-CAM-GLA.

Peptide Mapping. The proteins were resuspended in 25 mM ammonium bicarbonate (pH 8) at a concentration of approximately 100 pmol/μL. Trypsin (Promega) was added in a 1:40 ratio (w:w), and the mixture was incubated for 4 h at 37 °C. One microliter of the resulting peptide mixture was subjected to matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF) on a Micromass (Manchester, U.K.) M@ldi instrument using α-cyanohydroxycinnamic acid as a matrix. Alternatively, electrospray ionization mass spectrometry (ESI-MS) on a Q-TOF mass spectrometer (Micromass) was used, especially for MS/MS experiments. Also, the intact proteins were analyzed on the Q-TOF mass spectrometer.

Fluorescence Measurements. Steady-state fluorescence was measured with an Aminco-Bowman Series 2 spectrofluorimeter provided with a 150 W xenon lamp for continuous radiation. The GLA concentration was approximately 5 and 27 μM in the irradiation experiments without and with simultaneous stirring, respectively. Amounts of 2.5 mL of the degassed protein solutions were transferred into a 10 mm × 10 mm cuvette for fluorescence spectroscopy. Except when explicitly mentioned, the excitation wavelength was 280 nm. The band-passes for the excitation and emission slits were 4 and 1 nm, respectively. The cuvette holder was thermostated by circulating water from an external water bath. The fluorimeter was equipped with a magnetic stirrer mounted under the cuvette holder. In order to stir, a small magnetic stirring bar (5 mm in length and 2 mm in diameter) at the bottom of the cuvette was rotated at the speed of an external field (120 rpm).

Circular Dichroism Spectroscopy. The CD measurements were performed on a Jasco (Tokyo, Japan) J-600 spectropo-

larimeter. Cuvettes of 10 and 1 mm were used for the near-UV and far-UV regions, respectively. During the 5 min equilibration at 4 °C, the illuminated samples were shielded from the light beam. In the near-UV region (250−350 nm), the ellipticity monitored at 270 nm is mainly due to aromatic residues and reflects the tertiary conformation of the protein.

High-Performance Gel Filtration. Analysis of LA oligomers was carried out using an HPLC apparatus equipped with a gel filtration column (Superose 12 HR 10/30, Pharmacia). Equilibration, calibration, and elution were carried out at room temperature with a 0.5 mL/min flow rate of 50 mM $NaH_2PO_4 \cdot H_2O$ and 0.15 M NaCl (pH 7). The eluting protein was monitored by its absorbance at 230 nm. Calibration with standard proteins (IgG, BSA, β-lactoglobulin, cytochrome *c*, and vitamin B_{12}) allowed an estimation of the molecular masses of the LA components.

Absorption Meaurements. The absorption measurements were performed on a Uvikon 933 double-beam UV−vis spectrophotometer (Kontron Instruments, Milano, Italy) at room temperature.

RESULTS

Unusual Fluorescence Behavior of Native GLA. The first indication that irradiation at 280 nm affects GLA came from the changes in the emission spectra at 3 °C. Figure 2 represents spectra of nonirradiated and irradiated GLA under different conditions of pH and Ca^{2+} content. The solid line spectra were obtained when the GLA solutions were carefully kept in the dark until scanning. The dotted spectra were obtained when the same, nonstirred solutions were irradiated, for a 28 min period, by the excitation beam within the fluorimeter (excitation wavelength of 280 nm, band-pass of 4 nm). While the solutions were being smoothly stirred, the fluorescence spectra rapidly regained the initial value, but started to evolve to the dotted scans after stirring was stopped. The most plausible explanation for the above observations is obtained by accepting that the measured changes in fluorescence result from the combined effects of a radiation-dependent structural transformation of GLA and a diffusion of these molecules. Under conditions of limited diffusion and the absence of perturbation, an excess of transformed GLA molecules remains accumulated in the light path of the excitation beam. The rapid return to the original fluorescence upon smooth stirring results from the homogenization of the solution.

To study the effect of dissolved oxygen on the fluorescence change, the GLA solution was saturated with pure nitrogen. As the photoinduced effects did not change, oxygen from the dissolved air did not affect the results. It is also worth noting that, upon excitation at 295 nm, where absorption by any other chromophore except Trp is excluded, observations identical to those as at 280 nm are made. However, due to the decreased absorption at 295 nm, the intensity of the fluorescence change is less pronounced than upon excitation at 280 nm.

The fluorescence spectra in Figure 2 provide information about the nature of the irradiation-induced fluorescence changes. The solid lines in panels A and B of Figure 2 represent spectra of nonirradiated apo and Ca^{2+}-bound GLA solutions, respectively, at 3 °C and pH 7.5. These spectra are characteristic of nontransformed GLA, in the quasi native state (Figure 2A) and in the native state (Figure 2B). The

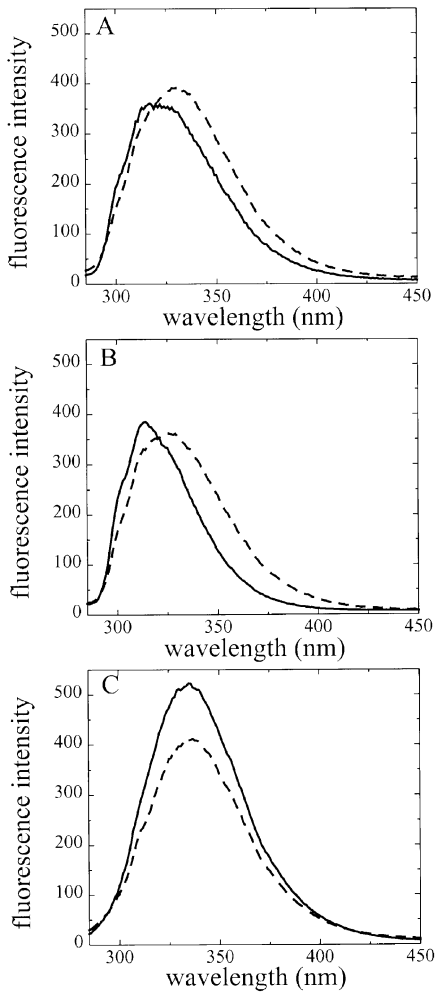

FIGURE 2: Fluorescence spectra at 3 °C and pH 7.5 of 5 μM apo-GLA (A), Ca^{2+}-loaded GLA (B), and GLA at pH 2 in 10 mM HCl (C). Degassed solutions of 2.5 mL, containing 5 μM apo- or Ca^{2+}-loaded GLA, were illuminated in a 10 mm × 10 mm cuvette. The fluorescence of the center of the cuvette was observed at a right angle. Excitation was carried out at 280 nm with a band-pass of 4 nm; emission was measured with a band-pass of 1 nm. The solid lines represent the spectra of GLA at the start of irradiation. To ensure homogeneity, the solutions were smoothly stirred during the registration of the spectra. The dashed lines are the spectra of nonstirred GLA after irradiation for 28 min at 280 nm.

solid line in Figure 2C represents the spectrum of nonirradiated GLA at 3 °C and pH 2. This acid medium is commonly accepted as providing the mild denaturing conditions necessary to obtain the molten globule state of LA (*7*). The solid line spectrum of Figure 2C is characteristic of LA in such a partially unfolded conformation. The relatively high emission maximum wavelength is indicative of the good solvent accessibility to the hydrophobic clusters that contain the fluorescent Trp groups within LA. The relatively high fluorescence intensity is indicative of the reduction of fluorescence quenching that has been observed in this slightly expanded conformation. Indeed, according to Sommers and Kronman (*8*), fluorescence quenching of native LA (solid lines in panels A and B of of Figure 2) is promoted by transfer of excitation energy from Trp26 and Trp104 to Trp60 and further onto two vicinal disulfide bridges. This postulated model of energy transfer and contact quenching is supported experimentally by the phosphorescence decay observed at low temperatures (*22*). In a recent study, Chakraborty et al.

(*15*) observed that the adjacent disulfide bonds also significantly quench Trp118.

The dashed lines in panels A and B of Figure 2 represent the spectra of the nonstirred GLA solutions irradiated at 280 nm for 28 min at neutral pH and 3 °C. At this pH, either in the absence or in the presence of Ca^{2+}, the irradiation of the native protein provokes a red shift and an increase in the fluorescence yield. The latter phenomenon is evidenced by the surfaces under the spectra. The red shift refers to an increased polarity due to an improved access of solvent to the fluorophores. The increase in yield results from a loss of quenching due to loosening of the protein structure (*8*). The dashed line in Figure 2C is the spectrum for the nonstirred, irradiated GLA solution at pH 2 and 3 °C. At this pH, the fluorescence intensity of GLA decreases by prolonged irradiation without a perceptible shift in wavelength. The emission maximum of 340 nm is a characteristic property of molten globule LA. The loss of yield could be due either to the photodestruction of the fluorophore or to an increase in the level of quenching by new neighboring residues in the altered conformation. The lack of a wavelength shift supports the idea that the potential irradiation products do not fluoresce.

Photoinduced Cleavage of Disulfide Bridges. The fluorescence changes upon irradiation of native GLA at 280 or 295 nm are thought to refer to a photoinduced loss of tertiary structure. Indeed, it has been shown recently that illumination of a particular Trp residue mediates photoreduction of an adjacent disulfide bond in the enzyme "cutinase" (*4*). To investigate whether the above-mentioned fluorescence changes can be linked to photoreduction of disulfide bonds in LA, we have set up a series of illumination experiments at higher protein concentrations (27.3 μM GLA). The solutions were illuminated using a broad band-pass of wavelengths ranging from 272 to 288 nm to increase the incident photon flux. In this way, the concentration of generated free thiol groups becomes accessible for chemical analysis. During irradiation, the homogeneity in the sample solution was maintained by smooth magnetic stirring. The number of free thiol groups and the fluorescence changes were followed at different temperatures, in the presence and absence of Ca^{2+}. The creation of free thiol groups upon illumination was followed by their reaction with DTNB. To ascertain that the reduction of DTNB results from free thiol groups and not from other potential reducing agents, a sample of illuminated GLA was treated with 2-iodoacetamide prior to addition of DTNB, a treatment which selectively carbamidomethylates the free thiol groups. Since no TNB^- was formed in this case, only free thiol groups were responsible for the reduction of DTNB.

In Figure 3, the number of free thiol groups generated per protein molecule (F_{th}) is presented as a function of the irradiation time at pH 7.5 and at 3 °C in 2 mM EGTA. F_{th} increases according to the equation

$$F_{th} = F_{th,max}(1 - e^{-t/\tau}) \quad (1)$$

Under the experimental conditions, the relaxation time (τ) resulting from the curve fit was 152 ± 20 min. (Undoubtedly, τ depends on a number of factors related to the instrument settings such as the intensity of the incident light, the illuminated surface, the total volume, and the concentration of the protein.) The limiting number of free thiol groups ($F_{th,max}$) was 1.83 ± 0.02 in this case. From this analysis, we calculated that within the first minute of illumination 2.7 × 10^{14} free thiol groups were formed while 290 × 10^{14} photons were absorbed. Therefore, under the conditions described above, the quantum yield of free thiol groups in intact native apo-GLA was 0.009. Illumination in the presence or absence of Ca^{2+} was carried out at different temperatures. The $F_{th,max}$ values obtained under these different experimental conditions were always less than 2.

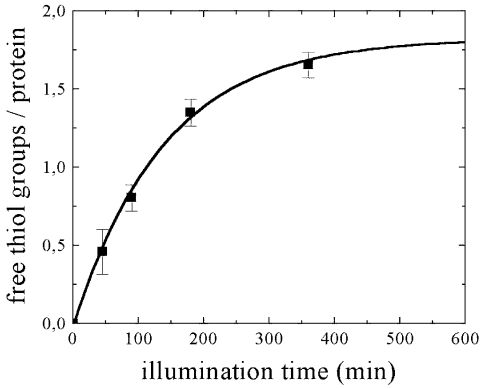

FIGURE 3: Number of free thiol groups per GLA as a function of illumination time measured with Ellman's reagent. A sample of 2 mL of a degassed apo-GLA solution in 2 mM EGTA at pH 7.5 was illuminated in the cuvette holder of an Aminco-Bowman Series 2 spectrofluorimeter. The excitation wavelength was 280 nm with a band-pass of 16 nm, a temperature of 3 °C, and a protein concentration of 28.1 μM. During illumination, the solution was stirred with a magnetic stirring bar at 120 rpm. The error bars represent experimental divergences.

Near 280 nm, the Tyr groups are responsible for approximately one-fifth of the absorption of GLA (*23*). The question of whether these chromophoric groups importantly contribute to the reduction of disulfide bonds arose. In answer to that question, we compared the ratio of created free thiol groups to the absorbed light quanta when an GLA solution was excited by a 272−288 nm light beam with the ratio obtained upon excitation with a 292−308 nm beam. Tyr residues do not absorb at the latter wavelengths. The resulting yield on excitation at 272−288 nm was smaller by 10−13% than on excitation at 292−308 nm, indicating that the yield by which an excited Tyr residue might reduce a disulfide bridge is much smaller than the yield by which an excited Trp residue may do so. From the above results, we conclude that when illuminated near 280 nm the Tyr residues do not effectively participate in the photoreduction of disulfide bounds, first because they absorb fewer photons than the Trp residues and second because their excited state is considerably less apt to reduce disulfide bonds in GLA than the excited state of Trp.

The formation of free thiol groups in the experiments described above of intensive irradiation and combined stirring was always accompanied by changes in the fluorescence emission spectra. As shown in Figure 4, the illumination of apo-GLA at pH 7.5 and 3 °C provoked a gradual red shift in the emission maximum. At first, the fluorescence yield increased, but upon prolonged illumination, it decreased again. These results agree with the observations on nonstirred solutions upon illumination with a narrow excitation slit (Figure 2A). Also, under these common conditions of

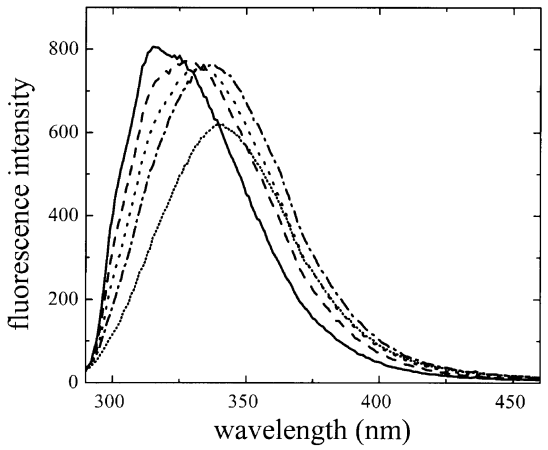

FIGURE 4: Fluorescence spectra of apo-GLA after different periods of illumination at 280 nm: 0 (—), 45 (– – –), 90 (- - -), 180 (—•—), and 360 min (•••). The sample and illumination conditions are as described in the legend of Figure 3. Despite the strong absorption, the fluorescence spectra were not corrected for inner filter effects.

illumination, a decrease in the fluorescence intensity was observed when apo-GLA was already partially unfolded (Figure 2C).

The near-UV ellipticity of native GLA (Figure 5) drastically decreased upon intensive illumination at 280 nm. Interestingly, the decrease of the near-UV ellipticity was found to fit a monoexponential function, the relaxation time (τ) of which is 183 ± 6 min (see the inset of Figure 5). The fairly good correspondence of this value with the relaxation time for the creation of free thiol groups strengthens the idea that both events are related to each other. In contrast to the near-UV spectrum, the far-UV CD spectrum of GLA changes only slightly upon irradiation. Therefore, we conclude that the tertiary structure of the protein, but not its secondary structure, is loosened by prolonged illumination.

Photoinduced Dimerization. In our search for an explanation of the relatively low thiol yield (1.83) upon prolonged illumination, we have found evidence that photolysis is accompanied by polymerization reactions. The existence of dimers, trimers, and some polymers was demonstrated by SDS−PAGE (not shown) and by gel filtration (Figure 6). Their relative amounts have been estimated from the surface of the elution peaks. After illumination for 6 h, the fractions are 0.66, 0.18, 0.07, and 0.09 for monomers, dimers, trimers, and polymers, respectively. The existence of oligomers suggests that some intermediately formed thiyl radicals from different GLA molecules combine to form intermolecular S−S bonds.

Photoinduced Bleaching of Tryptophan. After an initial increase and red shift, the yield of fluorescence of native LA diminishes upon prolonged illumination (Figure 4). It has been suggested that the fluorescence increase and red shift are related to a loss of the native structure (*8*). To obtain more information about the origin of the reduction of fluorescence, we inspected the changes in the absorption spectrum during illumination. The absorption spectra of irradiated GLA (Figure 7, dashed lines) conserve the typical feature of tryptophan as in the original protein (Figure 7, solid line), but gradually, a shoulder is formed on the red wavelength side of the absorption peak. Also, the baseline under the absorption peak becomes apparently elevated. The irradiated protein does not fluoresce upon excitation at wavelengths in the range of the newly formed shoulder (310−325 nm), and the excitation spectrum of the irradiated protein does not exhibit a similar shoulder. The latter observations ensure that only intact Trp groups are responsible for the observed fluorescence.

Mass Spectrometry. After irradiation for 3 h and subsequent treatment with iodoacetamide, the mass spectrum of GLA exhibited three peaks (data not shown). An unchanged GLA peak corresponding to approximately 50% of the total amount of protein is observed at 14 187 Da. The second and third peaks had masses of 14 243 and 14 300 Da, respectively, both accounting for approximately 25% of the total amount of protein. These two peaks correspond to the mass of GLA with one and two carbamidomethylated thiols, respectively. Although quantitation using ESI-MS is not always reliable, we consider the estimation of relative amounts to be valid, assuming that the ionization efficiency is only moderately affected by these modifications. MALDI analysis of the tryptic digests of the proteins revealed the origin of these free cysteines. Comparing the MALDI spectra of the digestion mixture obtained from intact GLA with the one obtained from irradiated and carbamidomethylated GLA revealed two significant differences (Figure 8). A new peak with a mass of 1091.7 Da appears which correlates with a peptide containing the carbamidomethylated Cys120 (see Table 1). Although the corresponding peptide with Cys6 (810 Da) was not found, we conclude that it was the Cys6−Cys120 disulfide bridge that had been broken during illumination. The second difference when comparing the

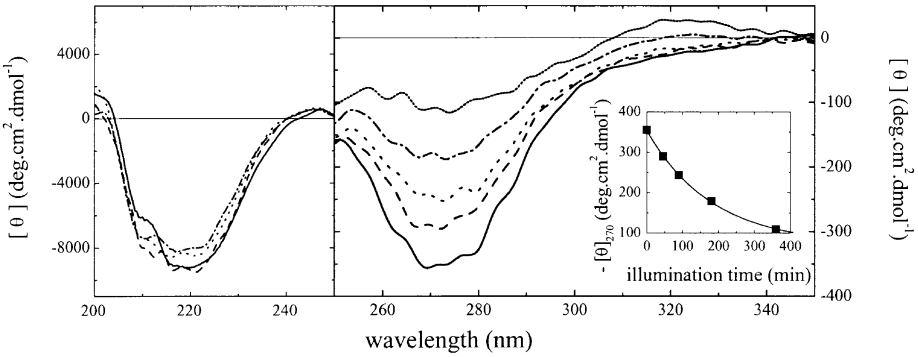

FIGURE 5: Circular dichroism spectra of apo-GLA in the far- and near-UV regions at different illumination times. The illumination periods are 0 (—), 45 (– – –), 90 (- - -), 180 (—•—), and 360 min (•••). The sample and illumination conditions are as described in the legend of Figure 3. The inset shows the ellipticity at 270 nm as a function of illumination time. The line through the experimental points represents the curve fitted according to a monoexponential function.

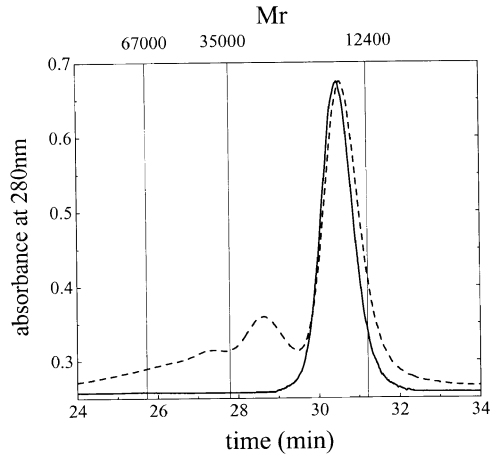

FIGURE 6: Analysis of LA oligomers by high-performance gel filtration of native (—) and illuminated GLA (– – –). The illuminated sample was irradiated for 6 h under the conditions described in the legend of Figure 3. The vertical lines represent the elution times of BSA (MW = 67.0 kDa), β-lactoglobulin (MW = 35.0 kDa), and cytochrome *c* (MW = 12.4 kDa), used as molecular mass markers.

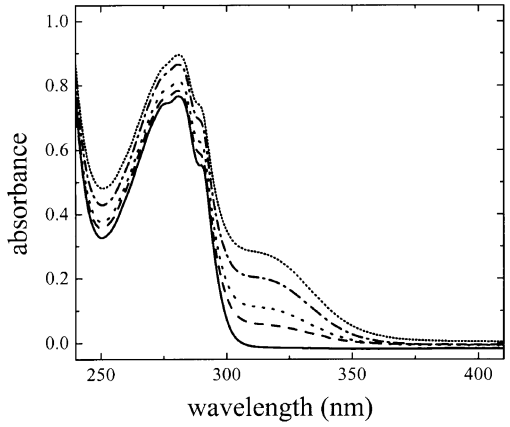

FIGURE 7: Absorption spectra of apo-GLA as a function of illumination time: 0 (—), 45 (– – –), 90 (- - -), 180 (—•—), and 360 min (•••). The sample and illumination conditions were as described in the legend of Figure 3.

MALDI spectra from the digestions of intact GLA with irradiated and carbamidomethylated GLA was the appearance of a new peak with a mass of 1554.0 Da for the protonated peptide. This mass approximately accounts for a heterofragment containing two peptides with Cys73, -61, and -77 being linked with the Cys61—Cys77 disulfide bridge (see Table 1). Clearly, this heterofragment results from a triple peptide that has lost a fragment through cleavage of the Cys73—Cys91 disulfide bond. In contrast to the Cys73—Cys91 bond, the Cys61—Cys77 bond is conserved during illumination. Evidence of the cleavage of the Cys73—Cys91 bond was also found in another sample. Before irradiation, the Cys6—Cys120 disulfide bridge of this GLA sample was cleaved by means of mild reduction with DTT (*21*) and the free thiol groups were carbamidomethylated. This so-called 3SS-CAM-GLA was irradiated for 5 h and treated again with iodoacetamide. The mass spectrum of irradiated 3SS-CAM-GLA resulted in three peaks with equivalent heights at 14 300, 14 358, and 14 415 Da (not shown). The first mass corresponds to that of intact 3SS-CAM-GLA, and the latter two correspond to the masses of the protein with one and two additional carbamidomethylated thiols, respectively. ESI-

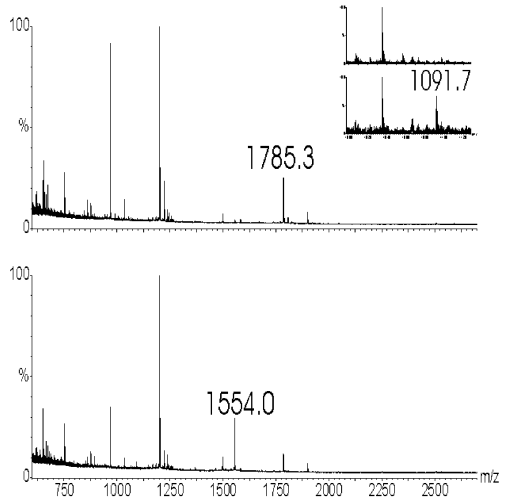

FIGURE 8: MALDI-TOF mass spectrum of the tryptic digest of apo-GLA (top) and of irradiated apo-GLA (bottom) followed by carbamidomethylation. The spectrum clearly shows a reduction in the magnitude of the peak corresponding to the disulfide-based heterofragment containing the Cys6—Cys120 disulfide (1785.3 Da). A new peak corresponding to a carbamidomethylated peptide containing Cys6 appears at 1091.7 Da (see the inset). Another new peak at 1554.0 Da appears that corresponds to the heterofragment containing the Cys61—Cys77 disulfide bond.

Table 1: Masses of Peptides Derived from GLA after Digestion with Trypsin[a]

mass (Da)	positions	peptide sequence	disulfide bridge
4681.13	17—58	DYGGVSLPE*W*V**C**TAFHTSGYD-TQAIVQNNDSTEYGLFQINNK	**28**—111
1581.73	80—93	FLDDDLTDDIV**C**AK	73—**91**
1199.65	99—108	VGINY*W*LAHK	
1033.49	115—122	LDQ*W*L**C**EK	6—**120**
1008.44	71—79	NI**C**NIS**C**DK	**73**—91, 61—**77**
967.41	63—70	DDQNPHSR	
752.35	6—11	**C**EVFQK	**6**—120
649.31	109—114	AL**C**SEK	28—**111**
617.34	1—5	EQLTK	
548.28	59—62	I*W***C**K	**61**—77

[a] The masses of peptides with a carbamidomethylated thiol can be calculated by adding 57.5 Da.

MS analysis of the trypsin digests of irradiated 3SS-CAM-GLA shows the appearance of two new masses (Figure 9). As in the prior model, these masses correspond to the products of the breaking of a triple peptide into a double peptide in which the Cys61—Cys77 bond is conserved and Cys73 is not carbamidomethylated (1553.0 Da) and into a single peptide with carbamidomethylated Cys91 (1639.0 Da). The formation of noncarbamidomethylated Cys73 and carbamidomethylated Cys91 was affirmed by ESI-MS/MS of the fragmented peptides.

DISCUSSION

The fluorescence changes offer a first indication that GLA is modified by irradiation at 280 or 295 nm. Under conditions of limited diffusion and in the absence of perturbation, the registered fluorescence spectra are not representative for the bulk of the solution (Figure 2). With stirring, identical changes are observed (Figure 4), although a longer and more intense illumination is required to observe the phenomenon. The illumination of native GLA is also accompanied by the

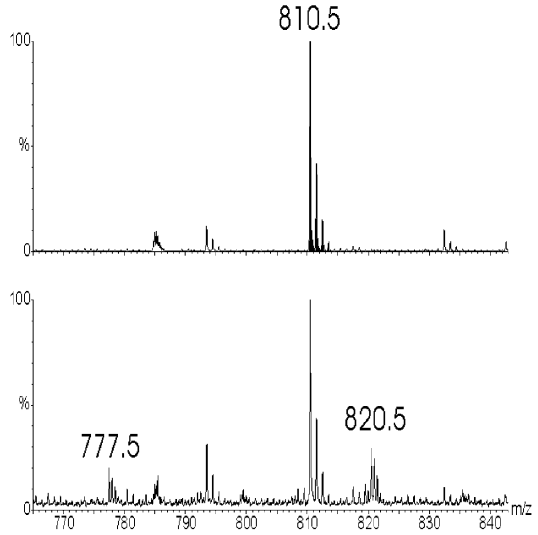

FIGURE 9: Electrospray mass spectrum of the tryptic digestion of 3SS-CAM-GLA (top) and of irradiated 3SS-CAM-GLA (bottom) showing two new peaks for doubly charged ions. The peaks correspond to masses of 1555.0 and 1641.0 Da, respectively, for the charged peptides, i.e., 1553.0 and 1639.0 Da, respectively, for the noncharged peptides.

appearance of free thiol groups (Figure 3). The rate at which free thiol groups are formed apparently decreases in an exponential way. The disulfide−thiol conversion within the protein is visibly related to the change in the tertiary conformation of native GLA (Figure 5, inset). Breakage of disulfide bonds does not directly disturb the elements of secondary structure (Figure 5) but allows the possibility that these formerly linked elements can migrate away from each other. α-Lactalbumins possess the characteristic that the partially folded state, with loosened tertiary structure and native secondary structural elements, is relatively stable under nondenaturing conditions (*24*). The quantum yield for the creation of free thiol groups is smaller upon illumination with 272−288 nm light than upon illumination at 292−308 nm. This indicates that also within the former wavelength range the photoreduction of disulfide bonds is predominantly mediated by Trp residues.

Upon prolonged irradiation of GLA, up to 1.84 free thiol groups are detected (Figure 3). The mass spectrum of a GLA sample that was irradiated for 3 h and subsequently treated with iodoacetamide consists of three components: a non-carbamidomethylated, a singly carbamidomethylated, and a doubly carbamidomethylated component (not shown). In agreement with the chemical analysis of the number free thiol groups, the mass spectrum indicates that up to two free thiol groups are formed. It could be expected that the two free thiol groups refer to the cleavage of a single disulfide bound. Therefore, the presence of a large fraction of singly carbamidomethylated protein in the mass spectrum is surprising. The occurrence of this species suggests either that the cleavage of a disulfide bond by photolysis does not necessarily result in the formation of two free thiol groups or that steric hindrance excludes interaction with an external reagent. A comparison of the mass spectra of the tryptic digests from GLA that was labeled either after photolysis or after chemical reduction (3SS-CAM-GLA) excludes the latter possibility. Indeed, the peak with a mass of 1091.7 Da in the mass spectrum of photolyzed GLA (Figure 8, inset) correlates with a protonated peptide containing carbamidomethylated Cys120, clearly proving the cleavage of the Cys6−Cys120 disulfide bond. However, no peak with a mass of 810 Da is found, which would be correlated with a GLA peptide with carbamidomethylated Cys6. In contrast to these findings, the fragments with free thiols at both Cys6 and Cys120 are clearly detected in the spectrum of proteolyzed 3SS-CAM-GLA (not shown).

The formation of only a single free thiol group is also evidenced within the peptide fragments derived from the photolytic cleavage of the Cys73−Cys91 bond. The fragment of photolyzed, blocked, and digested GLA with a molecular mass of 1639.0 Da correlates with a peptide in which the free thiol of Cys91 has reacted with iodoacetamide (Figure 9). In the same digest, the fragment with a mass of 1553.0 Da correlates with a peptide that contains a noncarbamidomethylated Cys73. No fragment is found that points to the existence of a carbamidomethylated Cys73. In an analogous experiment, Prompers et al. (*4*) treated irradiated cutinase with DTNB. The mass spectrum of the reaction mixture exhibited a peak corresponding to the mass of this enzyme having only one derivatized thiol group, but lacked the peak that corresponds to two complexed thiol groups. Here again, the cleavage of a disulfide bridge induced by photoexcitation of a Trp residue resulted in a single free thiol. Although a lack of accessibility of one of the thiol groups cannot be ruled out, it is striking that each analysis of thiol groups from a Trp-mediated, photoinduced cleavage of a disulfide bridge yields only one reactive thiol group. The formation of only one free thiol group, therefore, appears to be an inherent property of Trp-mediated photolysis.

According to Bent and Hayon (*24*), the rupture of a disulfide bridge upon UV illumination of Trp results in the formation of a thiolate ion and a thiyl radical according to the following scheme:

$$\mathrm{TrpH} \xrightarrow{h\nu} \mathrm{TrpH}^{\bullet+} + \mathrm{e}^- \quad (2)$$

$$\mathrm{RSSR} + \mathrm{e}^- \rightarrow (\mathrm{RSSR})^{\bullet-} \quad (3)$$

$$(\mathrm{RSSR})^{\bullet-} \rightarrow \mathrm{RS}^{\bullet} + \mathrm{RS}^- \quad (4)$$

The presence of a free thiol within the protoproducts provides evidence that proton transfer from the tryptophan cation radical to the thiolate ion is enabled:

$$\mathrm{TrpH}^{\bullet+} + \mathrm{RS}^- \rightarrow \mathrm{Trp}^{\bullet} + \mathrm{RSH} \quad (5)$$

Some intermolecular radical coupling results in dimerization and even trimerization of GLA molecules (Figure 6). Intramolecular radical coupling presumably can result in the sulfenylation of the Trp that is involved:

$$\mathrm{Trp}^{\bullet} + \mathrm{RS}^{\bullet} \rightarrow \mathrm{TrpSR} \quad (6)$$

The formation of a shoulder at the red wavelength side of the absorption peak of Trp (Figure 7) is in agreement with the existence of such modified Trp residues. The red-shifted shoulder is evidence of an increased level of delocalization of electrons of the aromatic indole nucleus (*26*). The substitution of a hydrogen of the indole ring with an alkylsulfenyl group enables such extra delocalization.

A more thorough inspection of the mass spectra of the tryptic fragments from irradiated and carbamidomethylated

Table 2: Shortest Distances (Angstroms) between the Indole Rings and the Different Disulfide Bridges in GLA[a]

	Cys6−Cys120	Cys28−Cys11	Cys61−Cys77	Cys73−Cys91
Trp26	14.5	8.9	15.9	10.9
Trp60	28.6	18.2	6.4	3.9
Trp104	20.7	9.0	11.7	7.7
Trp118	9.8	3.4	24.5	22.0

[a] A distance shorter than 5 Å refers to a close van der Waals contact.

GLA further supports the idea that products are formed according to that scheme. Indeed, the mass peak of 1553.0 Da (Figures 8 and 9) is readily ascribed to a heterofragment containing two peptides linked with the Cys61−Cys77 disulfide bridge and including Cys73. The mass further indicates that after cleavage of the Cys73−Cys91 disulfide bridge, Cys73 is not reactive to iodoacetamide. Interestingly, Tryp60 also belongs to the above-mentioned heterofragment (Table 1), enabling the possibility of radical-induced formation of a thioether linkage between Cys73 and Trp60 within the heterofragment. On further refinement, we learn that the mass of the heterofragment is ~4 Da smaller than the result obtained by adding the masses of the individual peptides (1008.4 and 548.3 Da; see Table 1). Clearly, the disulfide bond linking Cys61 with Cys77 is responsible for a loss of two H atoms, representing 2 Da. The remaining 2 Da refers to the loss of another pair of H atoms which, therefore, supports the hypothesis that Trp60 is linked to Cys73.

Finally, it is of interest to remark that the intramolecular sulfenylation of Trp explains why the above-mentioned peptide fragment lacks thiol reactivity and why, more generally, only a single reactive thiol group is generated upon photolysis of a disulfide bond via the absorption of near-UV light by a nearby Trp residue.

Up to now, we have been discussing the nature of the Trp-mediated photolysis of disulfide bonds. Next, we want to focus on the specific disulfide bonds that are being attacked. GLA contains four Trp groups and four disulfide bridges. Near-UV irradiation of intact GLA results in the photolysis of only two of these bonds: Cys73−Cys91 and Cys6−Cys120. An inspection of the crystal structure of GLA [PDB entry 1HFY (*5*)] indicates that the Cys73−Cys91 disulfide is in direct contact with the indole ring of Trp60. This readily explains the transfer of an electron from Trp60 to the Cys73−Cys91 disulfide and the preference for cleavage of that bond. Within the crystal, position 4 of the indole ring of Trp60 is oriented toward the S atom of Cys73. Therefore, it seems likely that the photochemical reactions (eqs 2−6) are initiated by the resonance structures with high electron density at the indolyl C4 atom. As a consequence, the hydrogen at that position may also preferentially be substituted for the sulfenyl radical resulting from Cys73. Interestingly, the recent structure analysis of "quinohemoprotein amine dehydrogenase" revealed the existence a thioether bond between the Sγ atom of a Cys residue and the indolyl C4 of a modified Trp (*27*, *28*).

With regard to the Cys6−Cys120 disulfide, in crystallized GLA the shortest inter-residue distance between the Cys6−Cys120 disulfide group and the nearest Trp (Trp118) is 9.8 Å (see Table 2). Also, the crystal structure shows a direct van der Waals contact between the indole of Trp118 and the Cys28−Cys111 disulfide group (shortest inter-residue distance of 3.4 Å; see Table 2). Therefore, the Trp-mediated photolysis of the Cys6−Cys120 disulfide bond is not expected. Presumably, moments of direct contact, suited for electron transfer between excited Trp118 and the Cys6−Cys120 disulfide, are enabled by the enhanced mobility of the structural elements within a protein in solution. Moreover, the cleavage of the Cys6−Cys120 disulfide bond upon excitation of Trp118 is promoted by the weakness of this disulfide bond. From high-resolution X-ray data of crystallized baboon LA, it has been deduced that the poor strength of the Cys6−Cys120 disulfide mainly results from torsion created on the five successive covalent bonds of the Cys−Cys side chain (*21*, *29*). The resolution of the available X-ray data for GLA and for most other LAs exceeds 1.8 Å and, therefore, is not sufficient for an accurate calculation of the torsion energy of the Cys6−Cys120 side chain (*21*). However, the weakness of that disulfide bond in GLA is obvious from its extraordinarily fast reduction when the protein is treated with chemical reducing agents.

Besides the weakness of that Cys6−Cys120 disulfide, the orientation of Trp118 may also be important for the preference for photoreduction. Indeed, within the crystal structure, Trp118, although relatively distant, is oriented in such a way that the indolyl C4 atom is directed toward the Sγ atoms of Cys6. As stated above, this may be the optimal orientation for an electron transfer from excited Trp and for the formation of a thioether linkage.

In conclusion, the results of this study suggest that Trp-mediated photoreduction of a disulfide bond within proteins is more generally enabled and is not restricted to the specific microenvironment found in cutinase (*4*, *30*). A direct contact between the excited Trp and the considered disulfide bond seems to be favorable for the induction of photolysis. However, the mutual comparison of the reactivity of the Cys6−Cys120 and Cys28−Cys111 disulfides and of their relative position with respect to Trp118 indicates that other factors are also important. The Trp-mediated photoreduction of a disulfide bond preferentially results in the formation of a single free cysteine. We found evidence that the second Cys residue can form a thioether linkage with the indole group of the mediating Trp residue.

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