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Photoexcitation of tryptophan groups induced reduction of disulfide bonds in hen egg white lysozyme

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Abstract

Prolonged exposure of hen egg white lysozyme to near-UV light results in an unusual fluctuating and red shift of fluorescence spectra. By chemical detection of free thiol groups with sulfhydryl reagent and spectroscopic measurement, it is found that the fluorescence changes are accompanied with the cleavage of disulfide bonds upon irradiation. However, tryptophan and the new generated free thiol groups are not stable within the protein and will further react during prolonged irradiation. The existence of photo-induced dimers is uncovered based on the fact that some intermediately formed thiol radicals in different lysozyme molecules can further form intermolecular S–S bonds. In addition, the decrease of UV absorption ability of lysozyme indicates that tryptophan will be reoxidated after the reduction of disulfide bonds. Then a conclusion is drawn that the disulfide bonds in lysozyme are only partially reduced and the reduction of Cys30-Cys115 may be preferential based on the structural analysis. Meanwhile, it is found that the tertiary structure of lysozyme is not very compact, and the secondary structure is also gradually disturbed during illumination.
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Keywords: Hen egg white lysozyme; UV light; Tryptophan; Disulfide bonds; Free thiol groups

1. Introduction

Prolonged exposure of proteins and enzymes to ultraviolet light may induce structural changes and even reduce or abolish their biological activity [1]. The radiation-induced damage to proteins in aqueous solutions has been extensively investigated over a period of years in both biophysical and biochemical researches. It is stated that the effect is initiated through photon absorption of the chromophoric amino acids [2]. Since the indole nucleus is the strongest near-UV absorbing group in proteins, tryptophan (Trp) residues play a main role in the activation of the protein photodegradation; and besides, the nature of these degradation reactions also depend on the microenvironment in protein molecules [2,3]. Among these researches, a phenomenon of photolysis is very interesting which occurs in proteins containing aromatic residues with thiol groups or disulfide bonds nearby. It is found that the near ultraviolet (UV) light absorption of Trp residues contributes to the reductive splitting of the nearby disulfide bonds in *Fusarium solani pisi* cutinase and goat alpha-lactalbumin [3–7]. At present, the spatial preference between the aromatic residues and the disulfide bonds in geometry of proteins has been extensively observed in hydrolytic enzymes, proteases and immunoglobulin superfamily, which seems conserved throughout the molecular evolution [8–11]. However, no more approved examples of photonic-induced reduction of disulfide bonds among them have been characterized in detail, aside from cutinase and lactalbumin. Considering the function of Trp residues as structure probes in protein conformations and the importance of disulfide bonds in maintaining the structural stability of proteins, it is essential to obtain the key information concerning the packing and structural features of them, which will be very useful for engineering the neighboring amino acids to obtain proteins meeting specific

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stability requirements [12]. Moreover, the photic activation phenomenon of disulfide bridges has been widely explored using the immobilization technology, and broad applications have been found in different areas of biotechnology, e.g., covalent drug–protein photoadducts, biosensors, and nanoparticles [10]. To better understand and apply this phenomenon, it is important to offer more information on protein photolysis and to investigate proper applications to it.

In the past years, we have studied the conformational changes of lysozyme. Because of the abundant and well-known molecular properties of lysozyme, it has been used extensively in kinds of radio and photobiological researches for investigating the protein folding mechanism as a model protein [13]. As shown in Fig. 1, hen egg white lysozyme (HEWL) is a two-domain globular protein containing 129 amino acids [14]. The alpha-domain is composed of three alpha-helices and one C-terminal 3_{10} helix, and the beta-domain is composed of one antiparallel three-stranded beta-sheet and one loop. Besides, lysozyme contains four pairs of disulfide bonds. Among these disulfide bonds, two of them (Cys6-Cys127, Cys30-Cys115) locate in the alpha-domain; one of them (Cys64-Cys80) locates in the beta-domain; and the other one (Cys76-Cys94) acts as a linkage between the two domains [15,16]. There are also six Trp residues within the globular protein. Among these chromophoric residues, Trp62, Trp63 and Trp123 are the most solvent-accessible ones [17]; and because of the high sensitivity to conformation changes, lysozyme is considered as a very good candidate for fluorescence studies. In certain conditions, a substantial fluorescence quenching of Trp residues can be observed during the fast phase of the refolding kinetics. The unusual fluorescence quenching is thought to be the result of disulfide bonds or aromatic residues, either Trp62 or Trp108, in the non-native conformations [17,18].

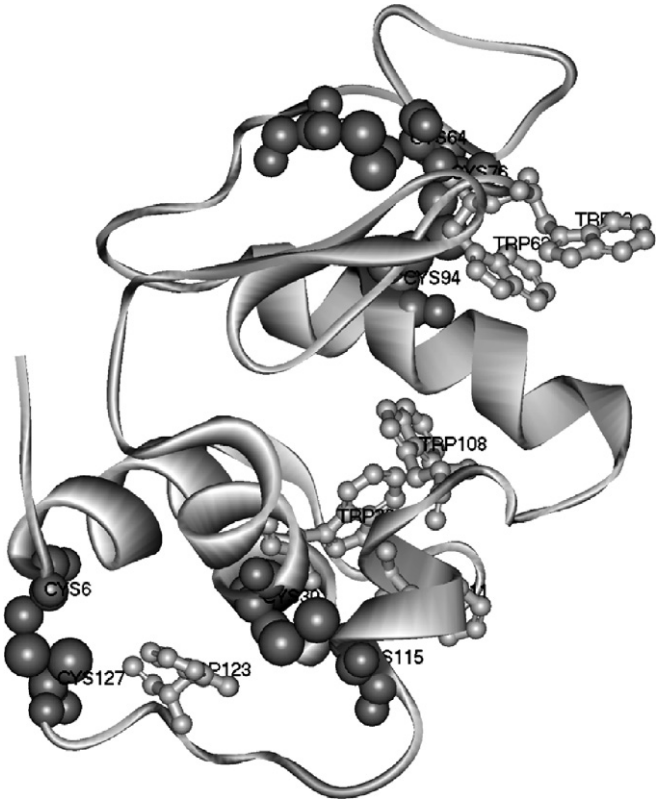

Fig. 1. The structure of hen egg white lysozyme with the four pairs of disulfide bonds and the six Trp residues.

In our studies, an interesting fluctuation and red shift in the fluorescence spectra are observed when lysozyme is illuminated by near-UV light. By using the chemical detection of free thiol groups with sulfhydryl reagent and spectroscopic measurements, we find that the fluorescence changes are accompanied with the cleavage of disulfide bonds upon illumination. In addition, the breakage of the disulfide bonds also affects the structural stability of HEWL; and the unfolding of secondary and tertiary structures in HEWL are described during prolonged illumination. To the best of our knowledge, this is the first report on photoexcitation of Trp groups induced reduction of disulfide bonds in hen egg white lysozyme.

2. Experimental

2.1. Materials

Hen egg white lysozyme and DTNB were purchased from Sigma Chemical Co. (St. Louis, USA). All other chemical reagents (Tris–HCl) used in our experiment were of analytical grade without further purification. All solutions were prepared using Milli-Q super purified water. The stock solutions of protein samples were prepared in 0.05 M Tris–HCl buffer (pH 8.0 at 25 °C), and the concentrations of proteins were determined spectrophotometrically. The 10 mM thiol specific reagent DTNB were dissolved in the absolute methanol, and kept in store for 2 weeks at 4 °C [9].

2.2. Irradiation of the samples

Each time, 3 ml of the HEWL sample (1 mg/ml at pH 8.0 buffer) was firstly added to the 1 cm × 1 cm cuvette, which then was illuminated by the fluorescence spectrophotometer (Jasco FP-6500, Tokyo, Japan) with a thermostatic cuvette holder. During the illumination, the solutions were stirred at 120 rpm to reach homogeneous state. The excitation wavelength was centered at 280 nm. To make sure of adequate photon flux, UV light was passed through 10 nm wide band-pass filter. Spectroscopic studies were carried out after illumination at 25 °C.

2.3. Detection of free thiol groups of HEWL upon irradiation

Free thiol groups were detected with the Ellman assay upon reaction with DTNB. An excess of DTNB (100 μl

DTNB stock solution at a concentration of 10 mM) was added to 900 μl of HEWL solution after illumination. The concentration of free thiol groups was determined with DTNB using absorbance value at 412 nm, a molar extinction coefficient of 13,600 M^{-1} cm^{-1} for TNB^- [3].

2.4. Fluorescence spectroscopy measurement

The intrinsic fluorescence spectra were measured on a fluorescence spectrophotometer (Jasco FP-6500) with a cuvette of 1 cm light path at 25 °C. All fluorescence spectra were collected at the protein concentration of 1.0 mg/ml. For emission spectra, the excitation wavelength was at 280 nm and the emission spectra were recorded in the wavelength range of 295–400 nm; the bandwidths of excitation and emission slits were set as 3 nm and 3 nm, respectively. Each spectrum was the average of 5 scans.

2.5. Circular dichroism spectroscopy measurement

All circular dichroism (CD) spectra were recorded on a Jasco-810 (Tokyo, Japan) spectrophotometer under constant nitrogen flush at 25 °C. The far-UV CD spectra of proteins (0.5 mg/ml) were recorded from 190 to 250 nm with a 1 mm path-length cuvette. The near-UV CD spectra of proteins (1.0 mg/ml) were recorded from 250 to 300 nm with a 1 mm path-length cuvette. Spectra were collected with a scan speed of 200 nm/min and a response time of 1 s. Each spectrum was the average of 5 scans.

2.6. Mass spectrometry

The irradiated samples reacted with DTNB were desalted by gel filtration on a PD-10 column (Amersham) and subsequently analyzed by mass spectrometry. The MALDI mass spectra for HEWL were performed on a MALDI-TOF-MS (autoflex, Bruker) spectrometry. Protein samples were prepared in a solution containing SA (in acetone containing 0.1% TFA, 5 μl). A portion (2 μl) of this solution was loaded onto the sample target and the solvent was removed by air-drying. Finally, the loaded target was transferred to the mass spectrometry with optimal laser intensity for analysis.

2.7. Absorption spectroscopy measurement

The absorption spectroscopy measurement was performed on an ultraviolet–visible–near recording spectrophotometer (Shimazu UV-3100, Japan) at 25 °C. The absorption of the released TNB^- at 412 nm was recorded with a 1 mm path-length cuvette. The absorption spectra of proteins (1 mg/ml) were recorded over the wavelength range of 240–400 nm with a 1 cm path-length cuvette, and collected at a scanning speed of 200 nm/min. Each spectrum was the average of 5 scans.

3. Results

3.1. Fluorescence behavior of HEWL on illumination

At the neutral condition and room temperature, an unusual fluorescence behavior is observed upon illumination of HEWL with near-UV light at 280 nm. The wavelength and the intensity at the maximal emission peak are shown in Fig. 2 as a function of the illumination time. The fluorescence intensity is normalized to the absorbance at the excitation wavelength, in order to remove the effect of Trp photodegradation on the fluorescence intensity and show only the effect of conformational changes. It is clearly seen that a progressive red shift of the maximal emission peak is observed by prolonged illumination of HEWL and it is up to 10 nm at the time of 180 min. However, the intensity change of the maximal emission peak is not monotonic with the illumination time. The fluorescence intensity increases quickly with the increment of the photon flux in the first 30 min, then the intensity reaches a plateau for about 30 min; after that, the intensity increases quickly again till about 110 min. In the following time, the fluorescence intensity comes to decrease till the end of the measurement. The emission peak of fluorescence spectra is indicative of the solvent-accessible surface area and the surrounding polarity of the fluorophores within HEWL [19–21]. The red shift of emission peaks indicates that the photolysis has resulted in the structural damage of HEWL. The increase of fluorescence intensity is considered relating to the more exposure of Trp to water in an expanded conformation, which is consistent with the red shift of emission peak [7]. However, the subsequent decrease of fluorescence intensity seems elusive, which may be due to either the photodestruction of the fluorophores or the

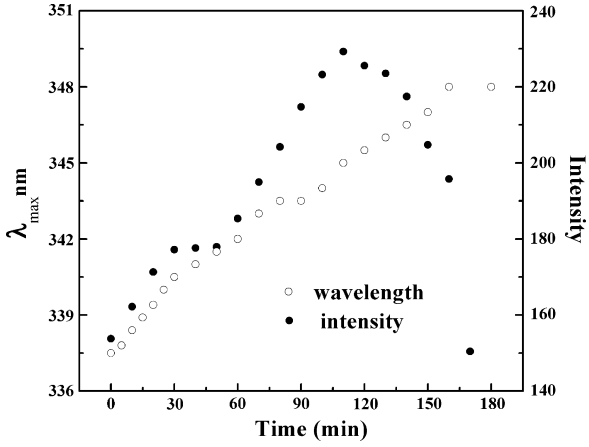

Fig. 2. The wavelength and the intensity at the maximal emission peak of fluorescence spectra as a function of the illumination time. The fluorescence spectra of HEWL are measured in aqueous solution (0.05 M/L Tris–HCl buffer, pH 8.0) illuminated by UV light of 280 nm with a broad bandpass of 10 nm. The fluorescence intensity is normalized to the absorbance at the excitation wavelength, in order to remove the effect of Trp photodegradation on the fluorescence intensity and show only the effect of conformational changes.

florescence quenching of some groups during the illumination as the results of the conformational change.

In order to obtain more information about the action of Trp residues during irradiation, the fluorescence spectra excited at 295 nm are also involved. It is worth noting that the absorption by any other chromophores except Trp residues is excluded in the case of 295 nm excitation [3]. The results resemble to those from the measurement with the excitation wavelength at 280 nm, and only the intensity of fluorescence spectra is less pronounced than upon excitation at 280 nm due to the absence of absorption from non-Trp aromatic residues.

3.2. Detection of free thiol groups

The unusual spectral signals allow us to focus on the structural information of HEWL. As shown in Fig. 1 and Table 1, one can see that the closely spaced triads of residues aromatic/Cys-Cys are rich in HEWL. It is well known that Trp residue shows a preference for disulfide bonds in the spacial environment, and the prone contacts between sulfur atoms and the aromatic ring give rise to the fluorescence decay [12]. Moreover, the photic activation phenomenon of disulfide bridges in the alpha-lactalbumin protein has been reported [3]. Because lysozyme and alpha-lactalbumin protein have a high degree of homology in both sequence and structure of them, the appearance of tryptophan-mediated photoreduction of disulfide bonds in HEWL seems reasonable [22,23]. In order to verify that, the detection of the free thiol groups among the photoproducts is performed based on the reaction of thiol groups with DTNB. Usually DTNB is used for the quantitative analysis of Cys residues in proteins since the absorbance value of the released TNB^- is proportional to the number of free thiol groups [3,13,23]. The absorbance spectra of the released TNB^- are measured using the UV/vis spectrophotometer, with an excess of DTNB added in HEWL solution after illumination. Fig. 3 shows the absorbance signal of the released TNB^-, from which it is found that the absorption is very low before illumination, while as the illumination begins, the absorption increases quickly till about 60 min. The increase of the absorption intensity at 412 nm implies the increasing production of the free thiol groups. Considering native HEWL does not contain any

Table 1
Shortest distances (Angstroms) between the indole rings and disulfide bonds in HEWL

	Cys6-Cys127	Cys30-Cys115	Cys64-Cys80	Cys76-Cys94
Trp28	13.3	9.1	15.6	10.8
Trp62	28.6	17.2	6.8	8.0
Trp63	28.3	20.1	6.9	3.8
Trp108	21.1	9.0	11.6	7.4
Trp111	16.4	4.1	19.4	16.0
Trp123	8.7	3.4	24.4	22.3

Distance shorter than 5 Å refers to a close van der waals contact.

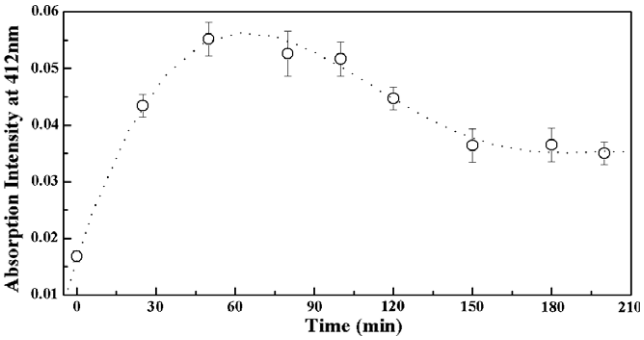

Fig. 3. The absorbance signal at 412 nm of the released TNB^- on the reaction of lysozyme with DTNB as a function of the UV illumination time.

free thiol groups and nothing but free thiol groups are responsible for the reduction of DTNB, photoexcitation of Trp groups in HEWL leads to the breaking of disulfide bonds. Although the absorption intensity is higher than that without illumination, the absorption at 412 nm of the released TNB^- is still lower compared to that of fully reduced HEWL. According to the proportional relationship between the absorbance value and the number of free thiol groups, there are only one or two pairs of free thiol groups per protein molecule formed in the photo-induced reactions.

However, in the following time, there is some decrease of the absorption at 412 nm, which indicates the reduction of the free thiol groups. To make clear the reason of relatively low yield of thiol groups during illumination, the irradiated HEWL samples are desalted and analyzed by mass spectrometry. The MALDI mass spectrum of the irradiated HEWL sample shows two peaks. One of the peaks at 14,163 Da corresponds to the native unchanged HEWL. Another small peak of dimers at 28,518 Da is also observed, which indicates some intermediately formed thiol radicals in different HEWL molecules can further form intermolecular S–S bonds. To a certain extent, the possibility of intermolecular polymerization reactions of HEWL during illumination will result in the reduction of thiol groups and the decrease of absorption intensity after 60 min.

3.3. Photo-induced bleaching of tryptophan

As the fluorescence spectrum is related to the UV absorption of Trp residues and the irradiation effect is initiated through photon absorption of the chromophoric amino acids, to tell more information about the photolysis of HEWL, the UV absorption spectra of HEWL during illumination are offered in Fig. 4. In this figure we can see that the absorption intensity of irradiated HEWL gradually decrease with the illumination time. In the inset of Fig. 4, the relaxation time of the UV absorbance at 280 nm is also shown. The UV absorbance at 280 nm correlates with the absorbance feature of aromatic residues

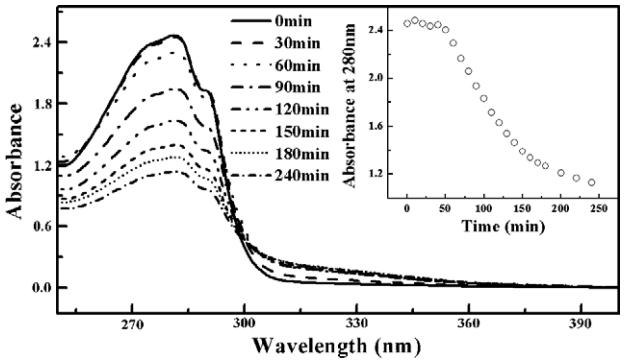

Fig. 4. The UV absorption spectra of lysozyme at different illumination time. The inset shows the absorption at 280 nm in time course of illumination.

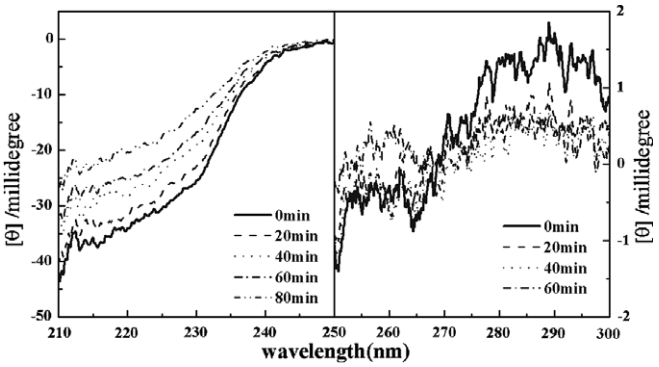

Fig. 5. Circular dichroism spectra of lysozyme in the far-UV and near-UV regions during illumination.

(Trp, Tyr, etc.) with UV light. It is interesting that the decrease of the absorbance at 280 nm behaves a sigmoidal transition. At the first 60 min of the illumination, the absorption value at 280 nm is almost constant; subsequently, a transition appears with the large decrease of absorption value. After 180 min, the absorption value decreases slowly till the end of the measurement. Ultraviolet light has high energy to arouse Trp and Tyr residues in HEWL to excited states. The direct electron transfer from the excited Trp to nearby disulfide bonds can retard photodegradation by the generation of free thiol groups [3,19]. However, the photolysis of chromophores will proceed once the breakage of disulfide bonds occurs. The acute decrease of absorption ability indicates that Trp may be reoxidated on illumination after the reduction of disulfide bonds.

3.4. Radiation-induced conformational changes of HEWL

The unfolding of proteins can be induced by thermal, chemical denaturation as well as UV radiation. The structure of HEWL is stabilized by four pairs of disulfide bonds and the loss of disulfide bonds can result in the destruction of HEWL structure, which can be known from the facts that the free thiol groups of irradiated HEWL molecules are free accessible to DTNB and the red shift of fluorescence spectrum. In order to make sure the conformational changes of HEWL during illumination, the far- and near-UV CD spectra are shown in Fig. 5. The far-UV signal mainly shows the change of the secondary structure in HEWL, while the near-UV signal of HEWL shows the tertiary environment of aromatic residues [24]. As shown in Fig. 5, the signal intensity of the far-UV CD spectra are decreased gradually and the peak of the near-UV CD spectra disappear rapidly on illumination, which indicate not only the tertiary structure but also the secondary structure of HEWL are disturbed by UV light irradiation. The result is different from photo-induced damage on alpha-lactalbumin which is disturbed on its tertiary structure rather than the secondary structure [3].

4. Discussion

The fluorescence changes offer an indication that the conformational structure of HEWL has been altered by illumination. Based on spectroscopic measurements and structural analyses, the photoexcitation of Trp groups induced the reduction of disulfide bonds in hen egg white lysozyme has been characterized in detail. According to the radiation mechanism, the process is usually mediated by a transfer of photo-ionization electrons to disulfide bond and induced the S–S bond destruction in the following manners [3]:

$$\mathrm{TrpH} \xrightarrow{h\nu} \mathrm{TrpH}^{\cdot+} + e^- \quad (1)$$

$$\mathrm{RSSR} + e^- \longrightarrow (\mathrm{RSSR})^{\cdot-} \quad (2)$$

$$(\mathrm{RSSR})^{\cdot-} \longrightarrow \mathrm{RS}^{\cdot} + \mathrm{RS}^- \quad (3)$$

The electron transfer rate constant is related to the Trp/S–S bridge distance [3,19]. At the native state of lysozyme, the effective electron transfer from Trp residues to the closest disulfide bonds will result in some fluorescence quenching. Once protein unfolds during the illumination, both the emission intensity and peak of fluorescence spectra will increase as shown in Fig. 2. However, Trp residues are separated from the nearby disulfide bridges when HEWL adopts an incompact conformation, which is unfavourable to the electron transfer between Trp residues and disulfide bonds. Thus the photodegradation of Trp residues occurs after 60 min, especially after the disruption of the disulfide bonds. Although the UV absorption ability of Trp residues decreases after 60 min as shown in Fig. 4, the fluorescence intensity will increase till 110 min due to more exposure of the Trp residues and less fluorescence quenching caused by the disulfide bonds. At last, the fluorescence intensity starts to decrease due to the photo-induced bleaching of Trp residues.

The formation of free thiol groups are detected by sulfhydryl reagent and mass spectrometry as shown in Fig. 3. However, the yield of free thiol groups in the sample is very low and the products will be reoxidized during the prolonged illumination. The relatively low yields of the free

thiol groups in UV-modified structure of HEWL are correlated to the specific environment within HEWL and the nature of Trp and Cys residues. Now we give our reason based on two facets. Firstly, we focus on the specific disulfide bonds which are being attacked. HEWL contains six Trp residues and four pairs of disulfide bridges. From Fig. 1 and Table 1, we can see that the disulfide bond Cys30-Cys115 is superior neighbor to Trp123 and Trp111 residues (shortest inter-residue distance of 3.4 Å) which are accessible to the solvent, thus this pair of disulfide bond has the highest tendency to break up. Another pair of disulfide bond Cys76-Cys94 as the linkage of the active sites in the two domains is also potentially reduced, because it is close to Trp63 residue (shortest inter-residue distance of 3.8 Å). As to others, the possibility of reduction of disulfide bonds within HEWL are low due to either the long distance between Trp residues and disulfide bonds or the burial of the Trp residues in the interior of HEWL. Moreover, during the unfolding process, the increase of the distance between Trp residues and the nearby disulfide bonds may also block the electron transfer at some extent. Hence, the disulfide bonds in HEWL are partially reduced during the irradiation and the generated quantity of free thiol groups is a bit low. Secondly, the reduction of disulfide bonds is not the end of the photolytic reaction, the new generated free thiol groups in HEWL are not stable and will further react to form intermolecular S–S bonds. Therefore, only one or two pairs of free thiol groups per protein molecule form during the photo-induced reactions.

Prolonged exposure of proteins to UV light may induce structural damages. Breakage of disulfide bonds directly disturbs the structure of HEWL. The red shift and the initial increase of the fluorescence intensity in fluorescence spectra indicate the partially unfolding behavior of HEWL. From the far- and near-UV CD spectra shown in Fig. 5, we can find that the protein unfolds with the loss of secondary and tertiary structure. The results should be taken into account in X-ray scatting, UV absorbance and fluorescence spectroscopy when dealing with lysozyme samples. Prolonged exposure of lysozyme solution to high-power UV light, especially in small quartz cells, can cause spectral changes due to the light-induced breakage of disulfide bonds, which can lead to artifacts and complications in interpretation of experimental data.

5. Conclusions

In summary, prolonged exposure of HEWL with near-UV light results in a high probability of disrupting disulfide bridges mediated by endogenous excited tryptophan residues. This appears to be another example of tryptophan-mediated photoreduction of disulfide bonds in proteins. We believe that these observations are important for the investigation of radiation-induced damage on proteins and will have far-reaching implications to improve the immobilization method in the science of protein immobilization.

Acknowledgments

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