Ultraviolet Illumination-induced Reduction of α-Lactalbumin Disulfide Bridges

Eugene A. Permyakov,[1,2] Serge E. Permyakov,[1] Gintaras Y. Deikus,[1] Ludmila A. Morozova-Roche,[1,3] Valery M. Grishchenko,[2] Lina P. Kalinichenko,[2] and Vladimir N. Uversky[1,4]*

[1]*Institute for Biological Instrumentation of the Russian Academy of Sciences, Pushchino, Moscow region 142290, Russia*
[2]*Institute of Theoretical and Experimental Biophysics of the Russian Academy of Sciences, Pushchino, Moscow region 142290, Russia*
[3]*Department of Biochemistry, Umeå University, Umeå, SE 90187, Sweden*
[4]*Department of Chemistry and Biochemistry, University of California, Santa Cruz, California 95064*

ABSTRACT **Prolonged exposure of Ca^{2+}-loaded or Ca^{2+}-depleted human α-lactalbumin to ultraviolet light (270–290 nm, 1 mW/cm^2, for 2 to 4 h) results in a 10-nm red shift of its tryptophan fluorescence spectrum. Gel chromatography of the UV-illuminated samples reveals two non-native protein forms: (1) a component with a red-shifted tryptophan fluorescence spectrum; and (2) a component with kynurenine-like fluorescent properties. The first component has from 0.6 to 0.9 free DTNB-reactive SH groups per protein molecule, which are absent in the native protein and is characterized by slightly lowered Ca^{2+}-affinity (2×10^8 M^{-1} versus 8×10^8 M^{-1} for the native protein) and absence of observable thermal transition. The second component corresponds to the protein with photochemically modified tryptophan residues. It is assumed that the UV excitation of tryptophan residue(s) in α-lactalbumin is followed by a transfer of electrons to the S—S bonds, resulting in their reduction. Mass spectrometry data obtained for trypsin-fragmented UV-illuminated α-lactalbumin with acrylodan-modified free thiol groups reveal the reduction of the 61–77 and 73–91 disulfide bridges. The effect observed has to be taken into account in any UV-region spectral studies of α-lactalbumin. Proteins 2003;51:498–503.**

Key words: α-lactalbumin; tryptophan; cystein; disulfide bridge; photo-induced modification

INTRODUCTION

The intrinsic fluorescence method is one of the popular physical approaches that have been routinely used in numerous studies of proteins for decades (see, for example, Ref. 1). The method is connected with the exposure of proteins to ultraviolet irradiation, which is absorbed by aromatic chromophores. The light absorption causes transitions of the chromophores to electronically excited singlet states. Deactivation of the excited states may follow several ways: emission of fluorescence, conversion to triplet state, and then emission of phosphorescence, radiationless internal conversion, and various pseudo-chemical and chemical reactions. It is usually assumed that at UV illumination power levels used in most spectral instruments the effectiveness of the pseudo-chemical and chemical reactions for protein chromophores, resulting in protein structural changes is negligibly low. Nevertheless, for some chromophore environments these processes can be rather effective (see, for example, Ref. 2).

Many years ago we found that a prolonged illumination of Ca^{2+}-binding protein α-lactalbumin in water solution by 280 nm UV light resulted in significant spectral effects caused by changes in the protein structure due to disruption of specific S—S bridges in the protein. This observation was first reported on Conference for Young Scientists in Pushchino, Russia in 1996.[3] In the present article we represent the detailed description of the effect.

α-Lactalbumin is a very popular subject of inquiry used in numerous studies mostly because of its ability to form the so-called molten globule state under certain conditions (for review, see Ref. 4). It is a modifier protein in the lactose synthase (E.C. 2.4.1.22) complex. This small (M_r + 14,200 Da, 123 residues) globular protein contains 3 (human) or 4 (bovine) tryptophan residues and is a good object for fluorescence studies because of the fact that the fluorescence of tryptophan residues of α-lactalbumin is very sensitive to its conformation. The emission of Trp 60 is partly quenched by two nearby S—S bridges.[5]

α-Lactalbumin has one strong $Cà^{2+}$ binding site,[6] with a $K_{ass} \sim 5 \times 10^8$ M^{-1},[7] which also binds Mg^{2+} ($K_{ass} \sim 2 \times 10^3$ M^{-1}),[8] Na^+ ($K_{ass} \sim 100$ M^{-1}), K^+ ($K_{ass} \sim 10$ M^{-1}), Mn^{2+} ($K_{ass} \sim 2 \times 10^3$ M^{-1}), Cd^{2+}, Tb^{3+}. It also possesses a distinct Zn^{2+}-binding site with a binding constant for Zn^{2+} $K_{ass} \sim 5 \times 10^5$ M^{-1} and a few weaker sites.[9–11]

Native α-lactalbumin consists of two domains: a large α-helical domain and a small β-sheet domain, which are connected by a calcium-binding loop.[12,13] The α-helical domain is composed of three major α-helices (residues 5–11, 23–34, and 86–98) and two short 3_{10} helices (resi-

Grant sponsor: INTAS; Grant number: 01-2347 CA. Grant sponsor: Swedish Medical Research Council to L.A.M.-R.

*Correspondence to: Vladimir N. Uversky, Department of Chemistry and Biochemistry, University of California, Santa Cruz, CA 95064. E-mail: uversky@hydrogen.ucsc.edu

Received 7 October 2002; Accepted 2 December 2002

dues 18–20 and 115–118). The small domain is composed of a series of loops, a small three-stranded antiparallel β-pleated sheet (residues 41–44, 47–50, and 55–56) and a short 3_{10} helix (77–80). The two domains are separated by a deep cleft. At the same time, the two domains are held together by the disulfide bridge between residues 73 and 91, forming the Ca^{2+}-binding loop.

Overall, the structure of the α-lactalbumin is stabilized by four disulfide bridges (6–120, 61–77, 73–91, and 28–111). It was shown by Demarest et al.[14] that crosslinking of peptides corresponding to the B- and D-helices by Cys28—Cys111 disulfide bond resulted in a dramatical increase in their structure. At the same time, there was very little enhancement of local structure due to the formation of the Cys6—Cys120 disulfide bond.[15] It is of interest that the three-disulfide form of α-lactalbumin, having a reduced Cys6—Cys120 disulfide bond, is similar to intact α-lactalbumin in secondary and tertiary structure.[16] The two-disulfide form of α-lactalbumin, having reduced Cys6—Cys120 and Cys28—Cys111 disulfide bonds, retains about half of the secondary and tertiary structure of the intact α-lactalbumin and seems to retain its β-domain, whereas the α-domain is unfolded[16] (see also Ref. 17).

In the present work we have shown that excitation of tryptophan residues in α-lactalbumin can result in pronounced structural changes in the protein due to reduction of its specific S—S bridges.

MATERIALS AND METHODS

Supplies and Chemicals

α-Lactalbumin (lot 128F-8140, bovine) was purchased from Sigma Chemical Co. (St. Louis, MO) or isolated and purified from human milk as described in Ref. 18. Sequencing grade trypsin from bovine pancreas was bought from Boehringer. All other chemicals were reagent grade or better. Solutions were prepared using distilled, demineralized water. Protein concentrations were evaluated spectrophotometrically, using an extinction coefficient $E_{1\%,280nm}$ + 20.1 for bovine protein and $E_{1\%,280nm}$ + 18.2 for human α-lactalbumin.[19] Molar extinction coefficient ϵ_{412nm} + 13,600 M^{-1} cm^{-1} was used for DTNB,[20] and ϵ_{391nm} + 20,000 M^{-1} cm^{-1} for acrylodan.[21]

Modification of α-Lactalbumin by UV Irradiation

Upon UV illumination of α-lactalbumin, the whole protein solution (20–40 μM, $[Ca^{2+}]$/[α-LA] + 1:1, 50 mM HEPES, 150 mM KCl, pH 7.8) in a 1-cm path length quartz cell was exposed to UV light from Hg-lamp SVD-120 and passed through the 270–290 nm band-pass filter. The UV illumination set up ensured the luminous flux of ~1 mW/cm^2. The temperature of the sample was kept at 20°C during illumination, and the solutions were continuously stirred.

The UV-illuminated protein was immediately subjected to fractionation on a Sephadex G-75 column (10 mM HEPES-KOH, pH 8.0). The content of free SH groups in resulting fractions was determined by DTNB test.[22] Five to 10 molar excess of DTNB over the protein was used; the absorbance at 412 nm was traced during 0.5 h.

Fluorescence Measurements

The fluorescence studies of Sephadex G-75 fractions were performed on a laboratory-made spectrofluorimeter, as described earlier.[23] Protein fluorescence was excited at 280.4 nm. All spectra were corrected for instrument spectral sensitivity. Temperature scans were performed stepwise, allowing the sample to equilibrate at each temperature for at least 5 min. The temperature was monitored directly inside the cell. The fraction of conversion from the native to the thermally denatured state was calculated from the plots of temperature dependence of emission intensity at a fixed wavelength as previously described.[1]

Calcium binding affinity of the protein was measured spectrofluorimetrically in experiments on EGTA titration of Ca^{2+}-loaded α-lactalbumin as described earlier.[7] The absolute accuracy of the resulting calcium binding constants exceeds $\pm\frac{1}{4}$ orders of their magnitudes, whereas the relative accuracy for the set of similar titration experiments is even better.

In all fluorescent experiments the sample illumination time and the UV-irradiation power level were minimized in order to avoid the UV-induced structural rearrangements in the protein molecule. Samples were illuminated only during measurement itself. The UV-induced spectral effects arising during the period of typical experiment were found to be negligible.

Labeling of Protein SH-Groups

The labeling of protein SH groups was performed as follows. α-Lactalbumin, after UV illumination, was treated by acrylodan to label formed free thiol groups.[21] The excess of the reagent was removed via dialysis. The remaining S—S bonds of acrylodan-labeled protein were reduced by DTT treatment in presence of 8 M urea. The formed free SH groups were chemically modified by 2-iodoacetamide. The rest of the reagents were removed by dialysis.

Tryptic Digestion and MS Analysis

Tryptic digestion of acrylodan-iodoacetamide-modified α-lactalbumin was carried out in 100 mM Tris-HCl, pH 8.5. Sequencing grade trypsin was added to 10 mg/ml protein solution in an amount equal to 2% (w/w). A second lot of trypsin (2%, w/w) was added after 5 h, and the digestion was allowed to proceed for an additional 5 h. All incubations were performed at 37°C.

Tryptic peptides were separated on a HPLC Alltech ALLTIMA C18 column. Resulting fragments were further purified by HPLC Spectrophysics C8 column and analyzed by mass spectrometry using a MicroMass Quattro-2 instrument.

RESULTS

UV Illumination Induces Structural Modification of α-Lactalbumin

Figure 1 shows the tryptophan fluorescence spectra of Ca^{2+}-loaded human α-lactalbumin in aqueous solution (50 mM HEPES, 150 mM KCl, pH 7.8) before and after 4-h exposure to UV light at 280 nm at 20°C. It is clearly seen that the prolonged illumination of the protein sample by

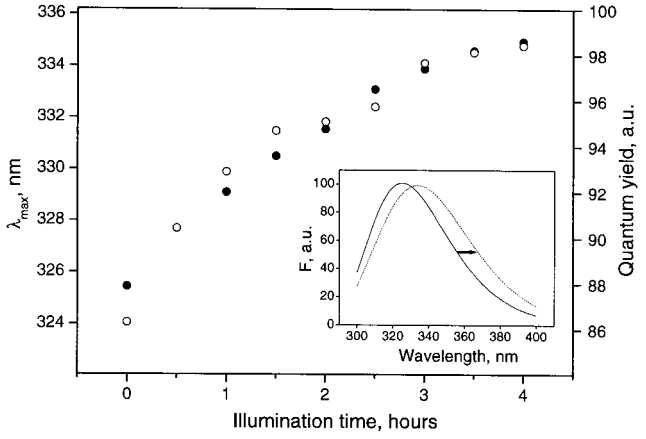

Fig. 1. Time course of the changes in tryptophan fluorescence of the Ca^{2+}-loaded human α-lactalbumin (35 μM, $[Ca^{2+}]/[\alpha\text{-LA}] + 1{:}1$) in water solution (50 mM HEPES, 150 mM KCl, pH 7.8), caused by UV illumination (Hg-lamp emission, passed through 270–290 nm band-pass filter; 1 mW/cm^2) of the sample at 20°C. (●), fluorescence spectrum maximum position, λ_{max}; (○), relative fluorescence quantum yield, estimated by area under experimental spectrum. The insert represents the initial (solid line) and final (dashed line) spectra. Fluorescence was excited at 280.4 nm.

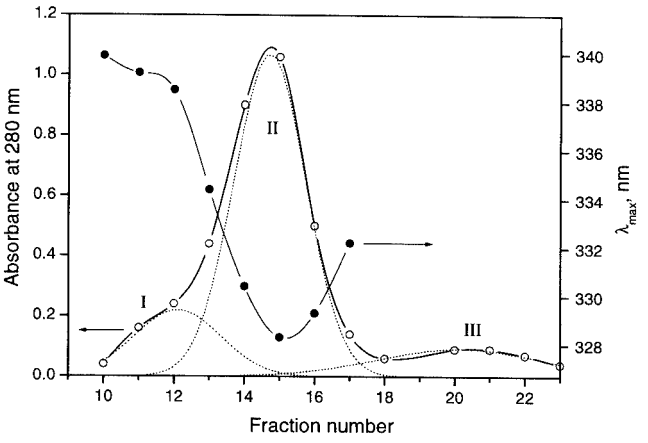

Fig. 2. Gel chromatography of the UV-illuminated Ca^{2+}-loaded human α-lactalbumin ($[Ca^{2+}]/[\alpha\text{-LA}] + 1{:}1$) on a Sephadex G-75 column (10 mM HEPES-KOH, pH 8.0). Protein elution was monitored by absorbance at 280 nm (○) and tryptophan fluorescence (fluorescence spectrum maximum position, ●). Absorbance curve is deconvoluted into three components: I, II, and III (Gauss curves, dotted lines). Fluorescence was excited at 280.4 nm.

UV light at room temperature causes about 10-nm red shift of the maximum of its tryptophan fluorescence spectrum. The time course of the spectral changes during the UV illumination of the protein solution demonstrates progressive red spectral shift and increase in relative fluorescence quantum yield (area under the spectrum). Similar UV light-induced red spectral shift is also characteristic for Ca^{2+}-free human α-lactalbumin in water solution (in excess of EGTA). The same results are achieved upon excitation at 296.7 nm, but tyrosine residues are not excited. Thus, the spectroscopic effects observed imply that the protein undergo some structural changes as a result of excitation of its tryptophan residues.

In order to separate different protein forms, arising as a result of the UV illumination, the illuminated protein was subjected to gel filtration. Figure 2 represents elution profile of the UV-illuminated Ca^{2+}-loaded human α-lactalbumin on a Sephadex G-75 column (10 mM HEPES-KOH, pH 8.0), traced by absorbance at 280 nm and tryptophan fluorescence (fluorescence maximum position, λ_{max}). One can clearly see on the chromatogram the presence of at least three chromatographic components: I, fractions 10 to 11 with a red-shifted tryptophan fluorescence spectrum (λ_{max} = 339–340 nm); II, fractions 15 to 16 with the tryptophan fluorescence spectrum, characteristic for native protein (λ_{max} = 328–329 nm); and III, fractions 18 to 23, possessing nontryptophan fluorescence.

It is evident that the last fractions correspond to the protein with tryptophan chromophores photochemically destroyed by the exposure to UV light. Upon excitation at 313 nm the component III demonstrates emission with λ_{max} = 417 nm, which resembles the emission of *N*-formylkynurenine, one of the major products of photooxidation of Trp residue in solution, in peptides and proteins (for review, see Ref. 24). The second component seems to correspond to the native protein, whereas the first component seems to be the most interesting α-lactalbumin form, possessing somewhat changed conformation, which is characterized by increased accessibility of tryptophan residues to the solvent.

Ca^{2+}-binding Properties of Native and UV-modified α-Lactalbumins

We characterized Ca^{2+}-binding properties of the component I. The binding of calcium ions to the α-lactalbumin components was studied by means of the so-called "back titration" experiments.[7] Figure 3 represents results of spectrofluorimetric titration of the Ca^{2+}-loaded α-lactalbumin components I and II by the strong Ca^{2+} chelator EGTA at pH 8.0 and temperature 41°C. In the case of the component II (with native spectral parameters) the gradual addition of EGTA causes 12-nm red shift of tryptophan fluorescence spectrum and an increase in relative fluorescence quantum yield, i.e., effects typical for native α-lactalbumin.[7] At the same time, the EGTA-titration of component I results in an opposite effect in relative fluorescence quantum yield without noticeable spectral shifts. The experimental curves for relative fluorescence quantum yield for both components were fitted by theoretical curves computed according to the one-site binding scheme,[7] and the best fit was achieved when the binding constant was 2×10^8 M^{-1} for the component I and 8×10^8 M^{-1} for the component II, i.e., the UV-changed component has slightly lower affinity to Ca^{2+} compared with the native-like component.

Thermal Stability of Native and UV-modified Forms of Human α-Lactalbumin

The thermal stability of component I was also analyzed. Figure 4 compares the temperature dependencies of tryptophan fluorescence parameters for apo-forms of the native protein component I. There are no thermally induced spectral changes in the UV-modified protein, except gradual

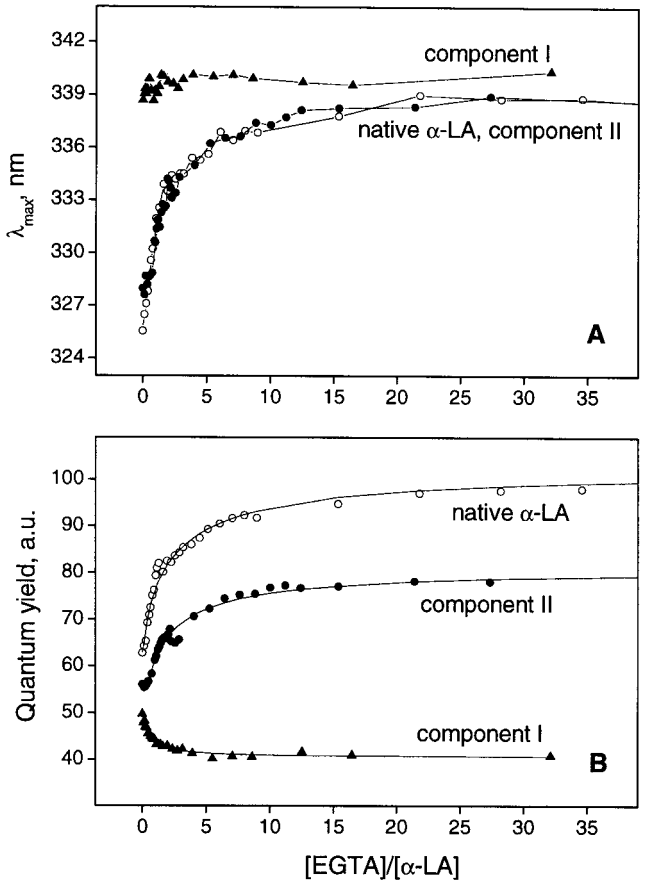

Fig. 3. Spectrofluorimetric EGTA titration of the Ca^{2+}-loaded α-lactalbumin components I (▲, 6.3 μM) and II (●, 8.2 μM) by EGTA at pH 8.0 (10 mM HEPES-KOH) and temperature 41°C. Data for the native protein (○) are shown for comparison. (A) Tryptophan fluorescence spectrum maximum position; (B) relative fluorescence quantum yield. Solid lines (B) are theoretical, obtained using the scheme of competition of EGTA and the protein for Ca^{2+}. Fluorescence was excited at 280.4 nm.

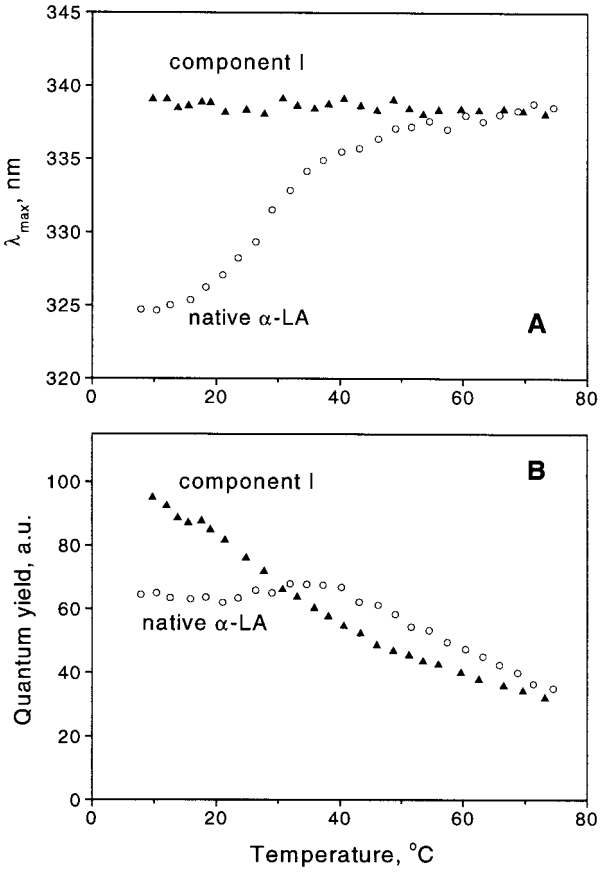

Fig. 4. Temperature dependence of tryptophan fluorescence parameters for α-lactalbumin component I (▲, 5.9 μM), compared with those for the native protein (○, 7.8 μM), in absence of Ca^{2+}. [EGTA]/[protein] + 30–120; pH 8.0, 10 mM HEPES-KOH. (A) Fluorescence spectrum maximum position; (B) fluorescence quantum yield change. Fluorescence was excited at 280.4 nm.

decrease in the relative fluorescence quantum yield, whereas the melting of native protein results in a prolonged red spectral shift and stepped decrease in relative fluorescence quantum yield, which reflects the thermally induced denaturation of the protein. Thus, the apo-form of component I seems to be mostly in denatured conformation.

UV illumination-induced Reduction of α-Lactalbumin Disulfide bridges

To elucidate the nature of the unusual properties of component I, we studied its reaction with the thiol-specific reagent DTNB.[20] The reaction was monitored by the appearance of a characteristic absorption band at 412 nm. It turned out that component I reacts with DTNB justifying presence of free SH groups accessible to DTNB. The quantitative analysis of free Cys content gave 0.6 to 0.9 of free SH groups per protein molecule. On the contrary, the native-like component II did not show presence of any free SH groups, as it is supposed to be for the native α-lactalbumin. The unexpectedly low value of thiol content in the UV-modified fraction can be due to the several reasons, such as inter- or *intra*molecular recombination of a part of free SH groups or trivial oxidation of cysteines. Importantly, our SDS-PAGE data did not reveal any oligomeric forms, which rejects the possibility of *inter*molecular recombination of the SH groups.

It is reasonable to suggest that the observed UV-induced changes in the properties of α-lactalbumin result from the reduction of disulfide bonds as a consequence of energy transfer from excited nearby tryptophan chromophores. Usually, this mechanism is mediated by the transfer of photo-ionization electrons to S—S bond, followed by S—S bond destruction (see, for example, Ref. 2):

$$\mathrm{Trp} + h\nu \rightarrow \mathrm{Trp}^* \rightarrow \cdot\,\mathrm{Trp}^+ + e^- \quad (1)$$

$$e^- + \text{—S—S—} \rightarrow \text{—}S^- + \cdot\,S\text{—} \quad (2)$$

In order to find out which of the four S—S bridges in native human α-lactalbumin are reduced during the UV irradiation, we labeled the free thiol groups of the protein in the following manner. α-Lactalbumin, after UV illumination, was immediately chemically modified by acrylodan to mark the formed free SH groups . Then the remains of

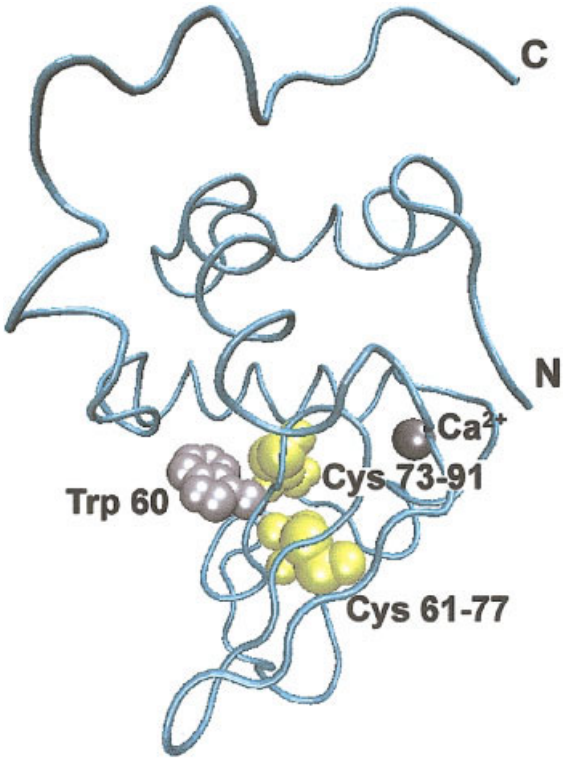

Fig. 5. Location of Trp 60 and S—S bridges 73–91 and 61–77 in human α-lactalbumin molecule (1HML file of Protein Data Bank).

the S—S bonds of the acrylodan-labeled protein were reduced by DTT in the presence of 8 M urea, and the newly formed SH groups were modified by 2-iodoacetamide. The resulting protein was cleaved by trypsin. The tryptic fragments were separated using reverse-phase HPLC and analyzed by mass spectrometry. The found that masses 1464 and 1836 Da correspond to acrylodan-labeled peptides 71–79 and 80–93, containing fully modified Cys73 and Cys77, and Cys91, respectively. Thus, the data obtained indicate that UV illumination results in disruption of both S—S bridges 73–91 and 61–77.

DISCUSSION

We have obtained comparable results for bovine α-lactalbumin (data not shown). Thus, our data suggest that UV illumination may induce reduction of the disulfide bridges in human and bovine α-lactalbumins. Taking into account that the Cys73—Cys91 bond connects two structural domains of α-lactalbumin, its reduction most likely will result in severe destabilization of the protein structure, which is observed for component I (see Fig. 4). On the basis of the proximity of Trp60 to the both disulfide bonds 73–91 and 61–77 (Fig. 5), one can assume that just Trp60 supplies them with electron, resulting in their reduction.

We did not find any significant UV-induced effects, except trivial photo destruction, in the case of hen egg lysozyme, a protein homologous in the sequence and tertiary structure to α-lactalbumin. This fact shows that, in spite of higher photosensitivity of cystines compared with tryptophans (see for example, Ref. 25), tryptophans, but not cystines, are the primary targets of photochemical action of UV light in the case of α-lactalbumin.

Similar UV irradiation-induced effects were observed earlier for human plasma fibronectin.[26] Exposure of 21- or 40-kDa gelatin binding fragments of plasma fibronectin as well as of entire protein to 280-nm light caused more than 10-nm red shift of tryptophan fluorescence spectrum and generated two, four, and six free sulfhydryl groups per molecule, respectively. The authors proposed that these effects result from structural changes caused by the breakage of disulfide bonds as a consequence of energy transfer from nearby tryptophans. The same conclusion was drawn from the study of UV-induced structural changes in cutinase from *Fusarium solani pisi*.[27,28]

Moreover, recent study by Vanhooren et al.[29] on UV-induced modifications of goat α-lactalbumin gave analogous results: photoexcitation of protein tryptophan residues resulted in a disruption of its S—S bonds. Remarkably, the authors report the breakage of the Cys6—Cys120 and Cys73—Cys91 bonds. Although the reduction of Cys73-Cys91 bond, observed also in our study for human α-lactalbumin, might be easily explained by the direct contact between this bond and the indole ring of Trp60, simplifying the electron transfer from excited Trp to S—S bond, the reduction of Cys6—Cys120 disulfide, which was not observed for human protein, seems to be not so obvious phenomenon.

In fact, the shortest inter-residue distance between the Cys6—Cys120 disulfide group and the nearest Trp (Trp118) is 9.8 Å. The authors suggested that the electron transfer could occur because of the Trp118 approaching of the Cys6—Cys120 bond as a result of the increased mobility of protein structural elements in solution. Other factors presumably favoring the disruption of Cys6—Cys120 disulfide are as follows: (1) its highly reactive properties with respect to the chemical reducing agents; (2) the positional relationship of Trp118 and this S—S bond, optimal for the electron transfer. Because we did not find the reduction of the Cys6—Cys120 bond for human α-lactalbumin, possessing the same tryptophan residues except for the lacking Trp26, one can assume that the reduction of Cys6—Cys120 bond in goat protein could be due to the electron transfer from the photoexcited Trp26 residue, but not from the Trp118. Although shortest inter-residue distance between the Cys6—Cys120 and Trp26 is 14.4 Å (PDB entry 1HFY),[30] it could be significantly shortened if Trp26 exists in the different rotamer forms. Our estimations show that in this case the distance can be as low as 8 Å.

Another intriguing observation is that Cys61—Cys77 bond of goat α-lactalbumin, located in the close proximity to Trp60 residue (6.5 Å; similar to human protein), but it does not suffer from the UV illumination, in contrast to the human α-lactalbumin. This fact implies that the electron transfer from excited tryptophan residues to disulfide bonds depends not only on distance between donor and acceptor, but also on some other factors.

The results of the present study should be taken into account in any fluorescent measurement on α-lactalbumin

samples. Prolonged exposure of α-lactalbumin 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 in its turn can lead to artifacts and complications in interpretation of experimental data. For this reason during the ordinary fluorescence measurements in our study only small part of the sample has been illuminated by single spectral line of Hg lamp for a short period of time required to measure the spectrum (for 2–3 min), whereas to analyze the effects of illumination, the whole sample has been subjected for several hours to the UV light passed through interference filter instead of monochromator.

Furthermore, our experience with the Perkin-Elmer LS-50B spectrofluorometer (with a xenon lamp with the continuous spectrum) shows that pronounced UV-induced spectral changes are induced in α-lactalbumin during a single spectrum measurement (this takes ~2–3 min), when majority of the sample is illuminated (i.e., when the narrow cells and an experimental setup with the large widths of excitation monochromator slits, from 5 to 10 nm, have been used). Thus, present article should serve as a warning for other researchers to avoid, if possible, similar experimental conditions for α-lactalbumin fluorescence measurements.

REFERENCES

1. Permyakov EA. Luminescent spectroscopy of proteins. Boca Raton, Ann Arbor, London, Tokyo: CRC Press; 1993.
2. Rubin AB Biophysics. Vol. 2. Moscow: University Press; 2000.
3. Permyakov SE, Deikus GU, Grishchenko VM, Veprintsev DB, Permyakov EA. Pushchino, Russia: Proceedings of Conference for Young Scientists; 1996, p 68.
4. Permyakov EA, Berliner LJ. α-Lactalbumin: structure and function. FEBS Lett 2000;473:269–274.
5. Ostrovsky AV, Kalinichenko LP, Emelyanenko VI, Klimanov AV, Permyakov EA. Environment of tryptophan residues in various conformational states of alpha-lactalbumin studied by time-resolved and steady-state fluorescence spectroscopy. Biophys Chem 1988;30:105–112.
6. Hiraoka Y, Segawa T, Kuwajima K, Sugai S, Murai N. α-Lactalbumin: a calcium metalloprotein. Biochem Biophys Res Commun 1980;95:1098–1104.
7. Permyakov EA, Yarmolenko VV, Kalinichenko LP, Morozova LA, Burstein EA. Calcium binding to α-lactalbumin: structural rearrangement and association constant evaluation by means of intrinsic protein fluorescence changes. Biochem Biophys Res Commun 1981;100:191–197.
8. Permyakov EA, Kalinichenko LP, Morozova LA, Yarmolenko VV, Burstein EA. Lactalbumin binds magnesium ions: study by means of intrinsic fluorescence technique. Biochem Biophys Res Commun 1981;102:1–7.
9. Murakami K, Berliner LJ. A distinct zinc binding site in the α-lactalbumins regulates calcium binding. Is there a physiological role for this control? Biochemistry 1983;22:3370–3374.
10. Permyakov EA, Shnyrov VL, Kalinichenko LP, Kuchar A, Reyzer IL, Berliner LJ. J Protein Chem 1991;10:577–584.
11. Ren JS, Stuart DI, Acharya KR. α-Lactalbumin possesses a distinct zinc binding site. J Biol Chem 1993;268:19292–19298.
12. Acharya KR, Ren J, Stuart DI, Phillips DC, Fenna RE. Crystal structure of human α-lactalbumin at 1.7 Å resolution. J Mol Biol 1991;221:571–581.
13. Acharya KR, Stuart DI, Phillips DC, McKenzie HA, Teahan CG . Models of the three-dimensional structures of echidna, horse, and pigeon lysozymes: calcium-binding lysozymes and their relationship with α-lactalbumins. J Protein Chem 1994;13: 569–584.
14. Demarest, SJ., Fairman R, Raleigh DP. J Mol Biol 1998;283:279–291.
15. Moriarty DF, Demarest SJ, Robblee J, Fairman R, Raleigh DP. Local interactions and the role of the 6–120 disulfide bond in α-lactalbumin: implications for formation of the molten globule state. Biochim Biophys Acta 2000;1476:9–19.
16. Hendrix T, Griko YV, Privalov PL. Energetics of structural domains in α-lactalbumin. Protein Sci 1996;5:923–931.
17. Wu LC, Schulman BA, Peng ZY, Kim PS. Disulfide determinants of calcium-induced packing in alpha-lactalbumin. Biochemistry 1996;35:859–863.
18. Kaplanas RI, Antanavichyus AI. Isolation and purification of α-lactalbumin, the component of lactosesynthetase. Biokhimia (Moscow) 1975;40:584–590.
19. Nozaka M, Kuwajima K, Nitta K, Sugai S, Detection and characterization of the intermediate on the folding pathway of human α-lactalbumin. Biochemistry 1978;17:3753–3758.
20. Riddles PW, Blakeley RL, Lerner B. Ellman's reagent: 5,5′-dithiobis(2-nitrobenzoic acid)—a reexamination. Anal Biochem 1979;94:75–81.
21. Prendergast FG, Meyer M, Carlson GL, Iida S, Potter JD. Synthesis, spectral properties, and use of 6-acryloyl-2-dimethylaminonaphthalene (Acrylodan). A thiol-selective, polarity-sensitive fluorescent probe. J Biol Chem 1983;258:7541–7544.
22. Boyne AF, Ellman GL. A methodology for analysis of tissue sulfhydryl components. Anal Biochem 1972;46:639–653.
23. Permyakov EA, Burstein EA, Sawada Y, Yamazaki I. Luminescence of phenylalamine residues in superoxide dismutase from green pea. Biochim Biophys Acta 1977;491:149–154.
24. Creed D. The photophysics and photochemistry of the near-UV absorbing amino-acids.1. Tryptophan and its simple derivatives. Photochem Photobiol 1984;39:537–562.
25. Smith KC, Hahawalt PC. Molecular photobiology. New York, London: Academic Press; 1969.
26. Miles AM, Brew SA, Ingham KC, Smith RL. Photoinduced structural changes in the collagen/gelatin binding domain of fibronectin. Biochemistry 1995;34:6941–6946.
27. Prompers JJ, Hilbers CW, Pepermans HAM. Tryptophan mediated photoreduction of disulfide bond causes unusual fluorescence behaviour of Fusarium solani pisi cutinase. FEBS Lett 1999;45: 409–416.
28. Weisenborn PCM, Meder H, Egmond MR, Visser TJWG, van Hoek A. Photophysics of the single tryptophan residue in Fusarium solani cutinase: evidence for the occurrence of conformational substates with unusual fluorescence behaviour. Biophys Chem 1996;58:281–288.
29. Vanhooren A, Devreese B, Vanhee K, van Beeumen J, Hanssens I. Photoexcitation of tryptophan groups induces reduction of two disulfide bonds in goat α-lactalbumin. Biochemistry 2002;41: 11035–11043.
30. Pike AC, Brew K, Acharya KR. Crystal structures of guinea-pig, goat and bovine α-lactalbumin highlight the enhanced conformational flexibility of regions that are significant for its action in lactose synthase. Structure 1996;4:691–703.