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Role of the Trp-disulfide Triads in the UV Light Induced Degradation of a Monoclonal Antibody scFv

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Authors' contributions

This work was carried out in collaboration between all authors. Authors IH, ZM and HD designed the study and made together of the interpretation of the results, finally prepared with author EI the corrected version of the manuscript. Authors EI, SS, AV performed the experiments with proteins and their fluorescence analysis, literature searches, wrote the protocol and the first draft of the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: Proteins are targets for photodegradation due to absorption of incident light by endogenous chromophores, e.g aromatic side chains. In this work we study the role of Trp-disulfide triads in the light induced loss of immunoglobulin activity.
Study Design: We investigated a single chain variable fragment (scFv) of the Trp-disulfide triad containing monoclonal antibody 82D6A3. The scFv binds to von Willebrand factor (VWF) and upon illumination with near UV-B-light the scFv partially loses its binding capacity to VWF. In order to relate this observed degeneration to the specific Trp-disulfide triads, we mutated W35(V_L) and W36(V_H) which are in direct contact with the disulfide

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bridge of the V_L and V_H domain respectively, to Phe and compared the effects upon illumination of these mutants and the wild type scFv.

Methodology: We constructed and expressed three mutants, then tested the binding affinity of wild type and mutants to VWF. To study whether illumination caused protein fragmentation (rapture of disulfide bridge, structural changes, number of evolved thiols) we performed fluorescence spectroscopy, western blot and SDS-PAGE.

Results: Upon illumination with near UV-B-light the scFv partially lost its capacity to bind to VWF, indicating that the structure, orientation or the accessibility of the paratope is changed; while disulfide bonds were broken in the wild type and the monosubstituted mutants and dimers and multimers/higher aggregates were formed.

Conclusion: The results indicate that light induced excitation of W35(V_L) and W36(V_H) mediates photolysis of the vicinal disulfide bonds. And although the more distant W47(V_H), W50(V_H) and W108(V_H) do not contribute to photolysis of the disulfide bonds, the simultaneous substitution of W35(V_L) and of W36(V_H) nevertheless did not protect the scFv's affinity for VWF against illumination.

Keywords: Immunoglobulin; Trp-Phe mutants; tryptophan fluorescence; disulfide bonds; photolysis; aggregation; scFv.

1. INTRODUCTION

It is known that near-UV-B irradiation induces structural changes and may reduce or even abolish the biological function of proteins and enzymes [1]. Proteins are targets for photo-destruction due to absorption of incident light by endogenous chromophores (such as amino acid side chains or bound prosthetic groups) within the protein structure. The major chromophoric amino acids present in proteins are tryptophan (Trp, W), tyrosine (Tyr, Y), phenylalanine (Phe, F), histidine (His, H), cysteine (Cys, C) and cystine. The other major amino acids and peptide bonds do not absorb light in the near-UV-B region. Among the major chromophoric amino acids, Trp through its indole nucleus is the most strongly near-UV-B absorbing group in proteins with the largest molar absorption coefficient. Therefore, tryptophan residues are primary components in the activation of protein photodegradation. Both fluorescence and phosphorescence of tryptophan is widely used to probe the structure and dynamic processes in proteins. Some decades ago it has been proposed that absorption of near-UV-B light by Trp initiates reductive splitting of disulfide bonds [2,3]. It was also observed that a prolonged illumination of α-lactalbumin in water solution by UV light at 280 nm resulted in some unusual fluorescence spectral effects. Despite the ample information available about the impact of UV light on amino acids in solutions, little is known about Trp-mediated photolysis of disulfide bonds in proteins. Only recently it was demonstrated that Trp-mediated photodegradation of disulfide bonds indeed occurs upon illumination of cutinase [4,5], α-lactalbumin [6,7], somatotropin [8] and lysozyme [9]. Prompers et al. and Neves-Petersen et al. [4,5] demonstrated that the unusual fluorescence behavior indeed results from structural changes in the protein due to the breakage of a disulfide bridge (SS-bridge), which is in direct contact with the Trp residue. Prompers et al. [4] assumed that the cleavage is mediated by the excited Trp. Possibly the induced disruption involves electron transfer from the excited-state Trp to the disulfide bridge which is known to be a strong quencher [8]. Another possible mechanism [5] is that the excited-state dipole moment of the Trp as a result of near-UV-B illumination induces a dipole moment jn the adjacent disulfide bond with possibly energy transfer from Trp to the disulfide bond inducing vibrational modes ultimately leading to its disruption.

By analysis of the photoproducts of goat α-lactalbumin mutants where single Trp residues were replaced by phenylalanine, we demonstrated that the contribution of each Trp to the photolytic breakage of disulfide bonds strongly depends on the microenvironment of both the Trp and the disulfide bond [10,11]. The photochemical reaction between a Trp residue and the disulphide bridge provides a biotechnological application as well since e.g. the UV-B light mediated immobilization of proteins on solid surfaces can serve as a base for biosensors [12]. In this report, Fab-fragments of a mouse IgG immunoglobulin were immobilized upon illumination by covalent binding to a thiol reactive surface. The immobilized Fabs were oriented and conserved their native binding capacity for their specific antigen. The reaction mechanism behind the immobilization of the Fab fragment involved light-induced breakage of a specific disulfide bridge which is located near a Trp residue.

The present study is directed to further explore Trp mediated photolysis in bio-active proteins and to define the functional damage which it provokes. We used a monoclonal antibody against human VWF, 82D6A3 [13,14] with known sequence and crystal structure and that contains Trp-disulfide triads, which belongs to the immunoglobulin superfamily (IgSF) [15]. The hallmark feature of the IgSF is their immunoglobulin (Ig) fold motif, which is characterized by a domain of 70 to 110 amino acids folded in the form of two parallel β-sheets, packed face to face and connected by a single disulfide bond. For nearly all Igs, the two above mentioned S-atoms within this motif form a characteristic triad with a single Trp in their close vicinity: the so-called Trp–SS triads, where the mid-point distance in the V-domain of the light chain is 4.6±0.3 Å from Trp-Cη2 to Sγ-Sγ and in the heavy chain 4.7±0.3 Å from Trp-Cη2 to Sγ-Sγ [16].

Both variable domains of 82D6A3 contain such a Trp–SS triad. In the variable domain of the heavy chain (V_H) this triad consists of Trp36 and Cys22-Cys96 (Trp–SS bridge distance 4.9 Å), whereas in the variable domain of the light chain (V_L) this triad consists of Trp35 and Cys23-Cys88 (Trp–SS bridge distance 4.0 Å). In order to eliminate the interference of the constant domains of the Ig, a single chain variable fragment (scFv) of 82D6A3 was constructed by overlap-PCR starting from the DNA coding for the variable domains of the Ig [15]. This scFv-82D6A3 (28 kDa), consisting of the Ig's V_L and V_H domain linked via a $(Gly4Ser)_3$ chain, retains an excellent affinity for VWF, indicating that the paratope for VWF-interaction is well conserved. The scFv does not contain disulfide bridges besides those of the two Trp–SS triads. Moreover, it contains only three extra Trp all located in the VH domain [W47(V_H), W50(V_H) and W108(V_H)] and their distance from the upon mentioned SS-bridges is over 5 Å [15]. These characteristics make scFv-82D6A3 a very well suited agent to explore the protein degeneration induced by near-UV-B excitation of Trp residues. In order to be in a position to relate the observed degeneration to a specific Trp-disulfide interaction, we compared the effects resulting from photodegradation of wild type scFv-82D6A3 (scFv-82D6A3-wt) with those obtained upon illumination of scFv mutants, scFv-82D6A3-W35F(V_L), scFv-82D6A3-W36F(V_H) and scFv-82D6A3-W35F(V_L)/W36F(V_H), with the number indicating the position of the Trp that is mutated to Phe in the variable domain of the light (V_L) or heavy chain (V_H) as was done in the case of the GLA protein in our earlier study [11].

In view of the important impact of the near-UV-B irradiation on the structure and function of proteins, it is of the utmost importance to further unravel the conditions of Trp-mediated photolysis of disulfide bonds. Some newer information can be obtained by studying the scFv protein. Our second goal was to study the influence of near-UV-B light on the variable domain of an Ig and to test the effect of the mutation and illumination on the binding capacity to VWF of the protein fragments generated.

2. MATERIALS AND METHODS

2.1 Reagents

Horseradish peroxidase [HRP] labeled anti-E Tag antibodies [anti-E Tag-HRP] were purchased from GE Healthcare (Munich, Germany). Human full-length VWF purified from donor plasma displaying a normal multimeric pattern was purchased from Red Cross, Belgium. Bacti casein peptone, Bacti yeast extract and Bacti purified agar were purchased from BioTrading (Keerbergen, Belgium). Primers for the construction of the mutants were purchased from Eurogentec (Seraing, Belgium). Plasmid DNA was prepared using the NucleoSpin® Plasmid Quick Pure kit (Machery-Nagel, Düren, Germany), 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarine [CPM] from Molecular Probes (Eugene, OR). Novex®10-20% Tricine gel was purchased from Invitrogen (Carlsbad, California) and ortho-phenylenediamine [OPD] from Sigma (St. Louis, MO).

2.2 Prokaryotic Expression and Purification of scFv- 82D6A3-wt

The construction of scFv-82D6A3-wt (European Bioinformatics Institute Database accession number AJ965435) and its expression as a soluble protein in the periplasm of Escherichia coli (*E. coli*) HB2151 cells using the pCANTAB 5 E vector (GE Healthcare) has been previously described [17]. The scFv-82D6A3-wt was purified from the periplasmic extract and the supernatant as described using a HiTrap anti-E Tag column (GE Healthcare). After dialysis overnight at 4ºC against PBS, the purity was checked in SDS-PAGE using a Novex®10-20% Tricine gel followed by staining with Coomassie brilliant blue and stored at -20ºC.

2.3 Construction, Expression and Purification of Tryptophan Mutants of scFv-82D6A3

Construction of Trp mutants, scFv-82D6A3-W36F(V_H), scFv-82D6A3-W35F(V_L) and scFv-82D6A3-W35F(V_L)/W36F(V_H), was performed as described by Staelens et al. [17] using the QuickChange® site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands) and pCANTAB 5 E-scFv-82D6A3-wt-DNA. The DpnI-digested mutagenesis mixture was immediately used to transform E. coli HB2151 cells according to the manufacturer's instructions (RPAS expression module, GE Healthcare). Sequencing, expression and purification were performed as described [15]. After dialysis against PBS, purity was checked as described above.

2.4 Western Blotting

Equal volumes of elution fractions containing scFv-82D6A3-wt or its mutants were loaded on a 10-20% SDS-PAGE gradient gel and transferred to a nitrocellulose membrane (Schleicher and Schuell Bio Science, Dassel, Germany). Detection of the all scFv fragments [scFv's] was performed with anti-E Tag-HRP antibody solution (1/5000) as described [17].

2.5 Concentration Determination of the Purified scFv Fragment

The concentration of the purified scFv's was determined according to the Bradford method [18] using the Coomassie® Plus protein assay kit (Pierce, Rockford, IL). Briefly, dilution series of purified scFv's and Albumin Standard in PBS were added to a 96-well plate

(Greiner, Frickenhausen, Germany). A fixed volume of Coomassie® Plus protein assay reagent was added and absorbance was measured at 630 nm.

2.6 Binding of scFv-82D6A3-wt and its Trp Mutants to VWF

Binding of scFv-82D6A3-wt and its Trp mutants to VWF was determined as described before [17]. Briefly, a 96-well plate was coated with VWF and incubated with dilution series of scFv's for 1.5 h at room temperature. Next, bound scFv's were detected by incubation with anti-E Tag-HRP. Visualization was performed by adding H_2O_2 and OPD. The plate was washed nine times with PBS, 0.1% Tween 20 between different incubation steps.

2.7 Illumination of scFv Protein Fragments

The scFv-82D6A3-wt and its Trp mutants were illuminated with 290 nm light in a quartz cuvette of 0.4cm path length (Perkin Elmer Instruments, Shelton, CT) using an Aminco-Bowman® Series-2 spectrofluorimeter (Spectronic Instruments, Rochester, NY) equipped with a 150W Xenon lamp. The excitation wavelength was centered at 290 nm with the slits set to 16 nm band pass. A 0.5 ml sample of scFv (10µg/ml in PBS) was illuminated at 4ºC in the cuvette of the spectrofluorimeter. During illumination the solution was stirred with a magnetic bar at 120 rpm. To avoid oxidation, all solutions were thoroughly degassed under vacuum at 2.39 kPa for 5 min, prior to use and kept under N_2 atmosphere during illumination. The illumination time was max. 3 hours during illumination sampling were taken at 0,1,2,3 hour.

Ferrioxalate actinometry [19] showed that under these conditions the incident flux was $6x10^{14}$ photons x sec^{-1}. Binding of the illuminated and non-illuminated scFv's to VWF was determined as earlier described [17] and compared with the binding of non-illuminated scFv-82D6A3-wt to VWF.

2.8 Fluorescence Spectroscopy

Fluorescence measurements were carried out immediately after illumination. Steady state fluorescence spectra were acquired at 4ºC using an Aminco-Bowman® Series-2 spectrofluorimeter. The excitation wavelength was 290nm, the bandpass for both the excitation and emission slit was 4 nm. The emission spectra were measured in the wavelength region 300-450 nm. The fluorescence spectra were corrected for inner filter effect and blanc spectrum (Raman peak of the water) was subtracted.

2.9 Determination of Evolved Thiols by Fluorescence Spectroscopy

The measurements were performed after each illuminated period of scFv fragments at a concentration of 0.35 µM protein. Evolved thiols generated during illumination of scFv's were quantified by reaction with 2 µM CPM at pH 7.0 and 4ºC based on the fluorescence increase of the CPM-protein adduct [20] (excitation at 386 nm, emission at 469 nm). The fluorescence increase of CPM upon binding with free thiols was calibrated with a dilution series of 2-mercapto-ethanol.

3. RESULTS

3.1 Characterization of scFv-82D6A3-wt and its Trp Mutants

3.1.1 Expression and purification

In this study, the scFv of 82D6A3, a mAb which specifically interacts with the A3-domain of VWF [15] was used. In addition, we constructed three Trp mutants of scFv-82D6A3-wt. In two mutants, either W35, adjacent to disulfide bond C23-C88 in VL or W36, adjacent to C22-C96 in VH were substituted by a Phe (Fig. 1).

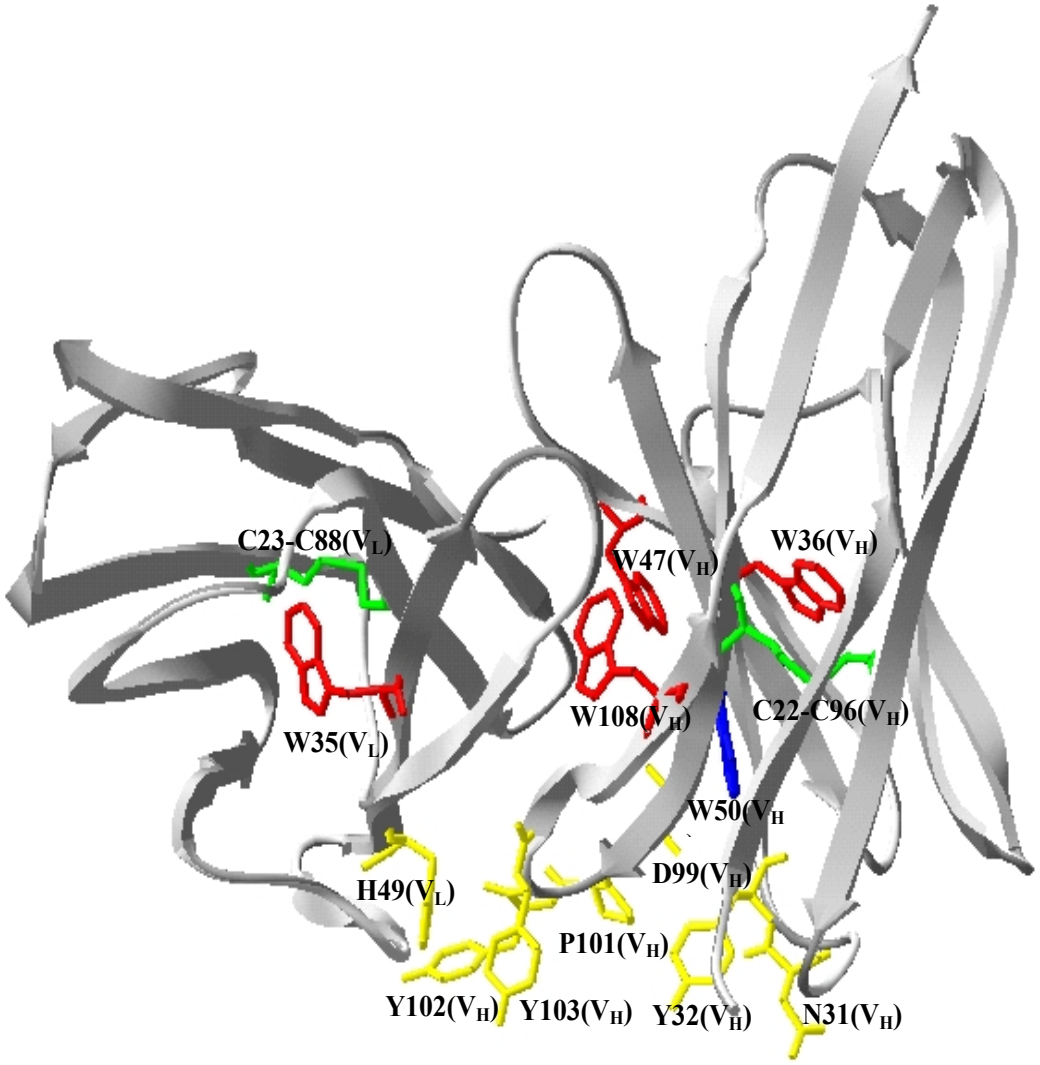

Fig. 1. Structure of the variable domains of 82D6A3

Ribbon representation of the variable domains of 82D6A3 as deduced from the crystal structure of the complex between the Fab-fragment of 82D6A3 and the VWF A3-domain. (PDB entry 2ADF [15]). The Fig.1 was generated with PyMOL (http://pymol.sourceforge.net/). In red are the Trp residues, except for Trp50(V_H) which is part of the paratope (in blue), in yellow are the other amino acids of the paratope and in green are the disulfide bridges.

The structure of the variable domains of 82D6A3 is deduced from the crystal structure of the complex between the Fab-fragment of 82D6A3 and the VWF A3-domain (PDB entry 2ADF) [15]. The mutants were named scFv-82D6A3-W35F(V_L) and scFv-82D6A3-W36F(V_H), respectively. In a third mutant, scFv-82D6A3-W35F(V_L)/W36F(V_H), both the above mentioned Trp residues were changed to Phe residues. The scFv's were expressed in *E. coli* HB2151 cells and purified from the supernatant and periplasmic extract. SDS-PAGE followed by Coomassie brilliant blue staining (Fig. 2) and Western blotting (data not shown) indicated that the protein content of the samples used in the subsequent experiments consisted of nearly 90% recombinant scFv.

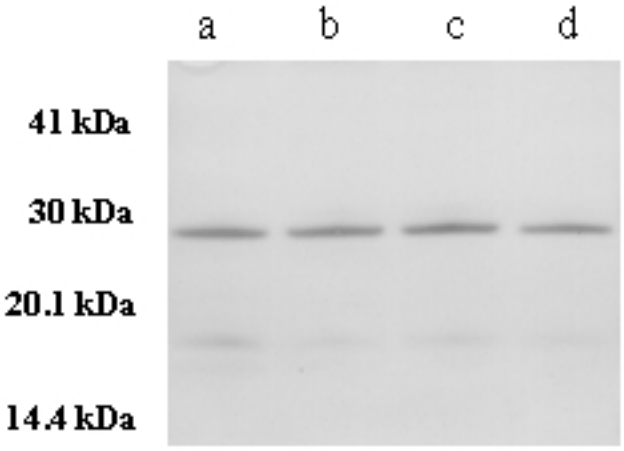

Fig. 2. Expression of scFv-82D6A3-wt and its Trp-mutants

(a) scFv-82D6A3-wt, (b) mutants scFv-82D6A3-W35F(V_L), (c) scFv-82D6A3-W36F(V_H) and (d) scFv-82D6A3- W35F(V_L)/ W36F(V_H) were expressed in E. coli HB2151 and purified from the supernatant and periplasm by affinity chromatography on a HiTrap anti-E Tag column. Fractions containing 10±2µg protein/ml were loaded on a 10-20% SDS-PAGE gradient gel and purity was checked by Coomassie brilliant blue staining.

3.1.2 Affinity for VWF

The effect of the individual mutations on the binding of scFv to VWF coated to a 96-well ELISA plate is presented in Fig. 3. The EC_{50} for the binding of scFv-82D6A3-wt to VWF is $2.2x\ 10^{-9}$ M (0.06 µg/ml). Upon substitution of Trp36 from the heavy chain to Phe, the effect on the affinity is minor; the EC_{50} of scFv-82D6A3-W36F(V_H) is $3.7x10^{-9}$ M (0.1 µg/ml). In contrast, the replacement of W35 in the light chain for Phe more significantly decreased the affinity for VWF with an EC_{50} of scFv-82D6A3-W35F(V_L) of $4.6x10^{-8}$ M (1.25 µg/ml). As expected, the simultaneous replacement of both Trp residues resulted in the largest decrease of the scFv's affinity with an EC50 of scFv-82D6A3-W35F(V_L)/W36F(V_H) of $9.2x\ 10^{-8}$ M (2.6 µg/ml).

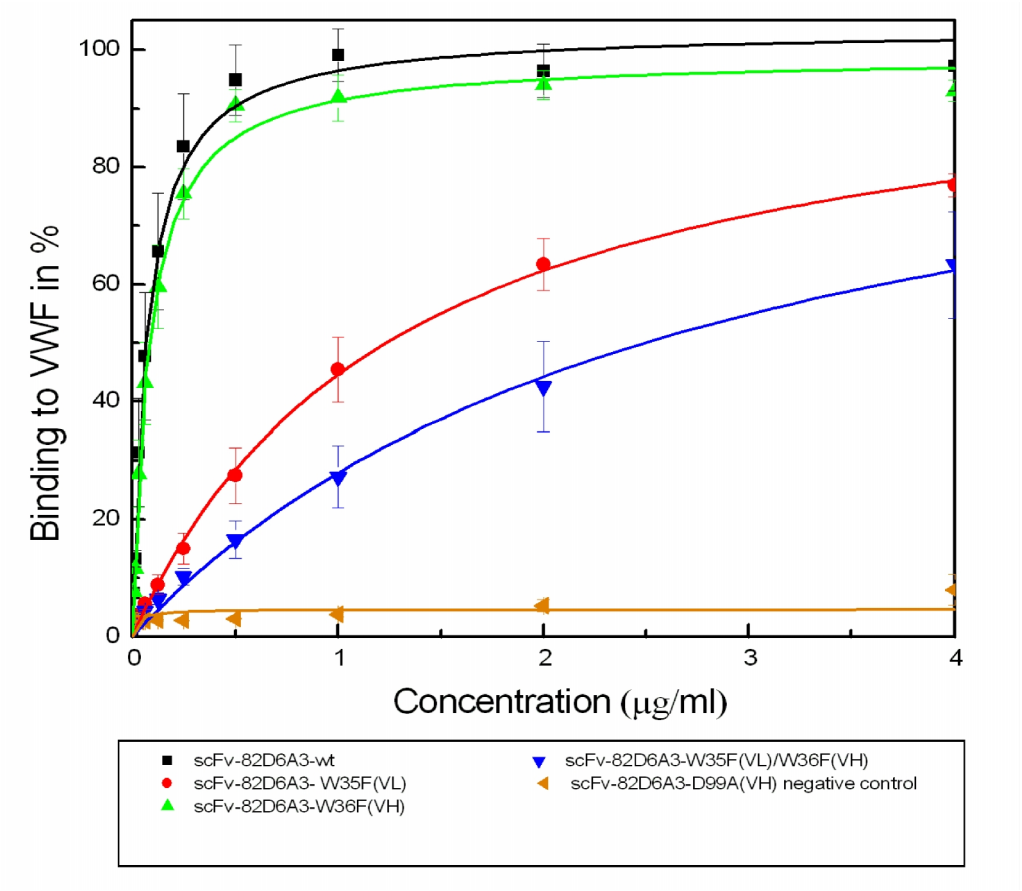

Fig. 3. Binding of scFv-82D6A3-wt and its Trp mutants to VWF

Serial dilutions of purified wild type scFv-82D6A3-wt (black) and mutant scFv-82D6A3-W35F(V_L) (red), scFv-82D6A3-W36F(V_H) (green) and scFv-82D6A3-W35F(V_L)/W36F(V_H) (blue) were added to VWF-coated wells and bound scFv was detected with anti-E Tag-HRP. Visualization was performed by adding H_2O_2 and OPD. The maximal absorbance calculated from the fitted curve obtained with scFv-82D6A3-wt was arbitrarily set as 100% binding. The inactive mutant scFv-82D6A3-D99A(V_H) (orange) was used as a negative control [17]. All data points are the mean ± S.E.M. of single measurements in at least five independent experiments.

3.1.3 Tryptophan fluorescence

The fluorescence spectra of the scFv's at equal protein concentration (~ 4x10-7M) are presented in Fig. 4. The fluorescence of scFv-82D6A3-wt slightly decreases upon substitution of W35 (V_L) but clearly increases upon substitution of W36(V_H). Surprisingly, the fluorescence of the double mutant, scFv-82D6A3-W35F (V_L)/W36F (V_H), yields up to 4 times the fluorescence of the wild type. The strong fluorescence increase upon substitution of W35 (V_L) in scFv-82D6A3-W36F(V_H) and of W36(V_H) in scFv-82D6A3-W35F(V_L), respectively, implies (a) that both Trp residues are strongly quenched and (b) that both contribute to indirect quenching of one or more of the remaining Trp residues: W47(V_H), W50(V_H) and W108(V_H). These presumptions are based on the crystal data [15], W35(V_L) is adjacent (3.6 Å) to C23-C88 in V_L and W36(V_H) is adjacent (4.9 Å) to C22-C96 in V_H. Clearly, the direct contact of the considered Trp residues with a disulfide bridge enables the energy or electron

transfer [5]. The spectrum of scFv-82D6A3-W35F(V_L)/W36F(V_H) is characterized by three distinct maxima. Therefore, each of the three Trp residues present in scFv-82D6A3-W35F(V_L)/W36F(V_H) seems to interact in a specific way with its surroundings and seems to emit the absorbed light energy in a distinct manner.

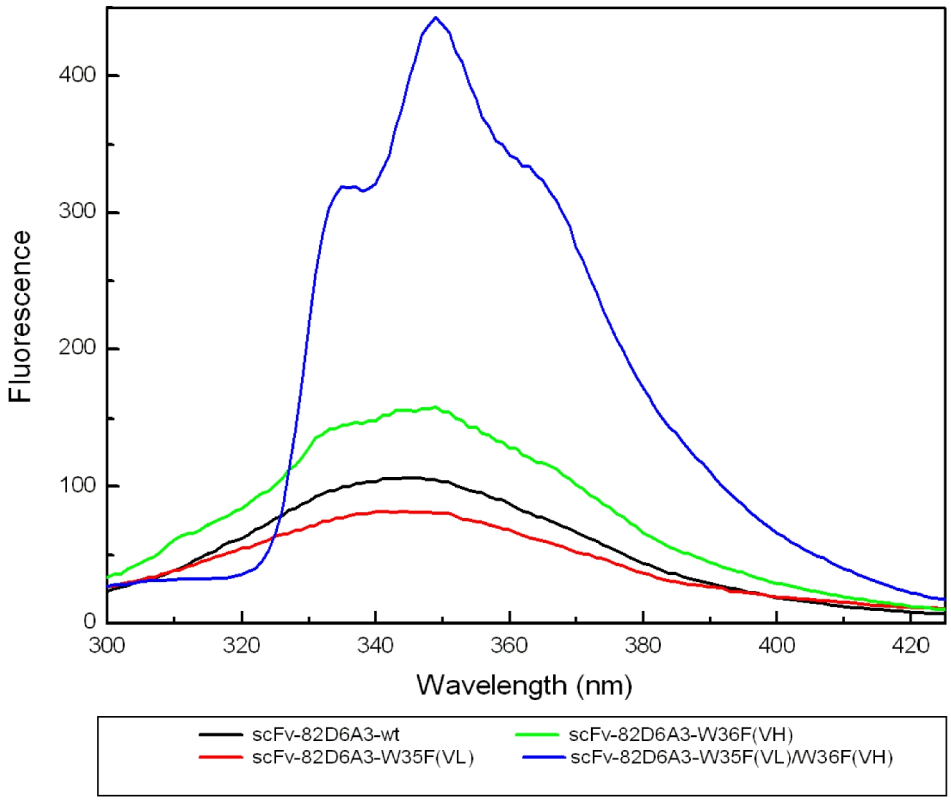

Fig. 4. Fluorescence emission spectrum of scFv-82D6A3-wt and its Trp mutants

The concentration of all scFv fragments was 10±2 µg /ml in PBS. The excitation wavelength was 290 nm, the bandpass for both the excitation and emission slit was 4 nm. The spectra of scFv-82D6A3-wt (black),scFv-82D6A3-W35F(V_L) (red), scFv-82D6A3-W36F(V_H) (green) and scFv-82D6A3-W35F(V_L)/W36F(V_H) (blue) are given. The fluorescence spectra were corrected for inner filter effect and blanc spectrum (Raman peak of the water) was subtracted

3.2 Effects Resulting from Illumination of Recombinant scFv Fragments

3.2.1 Affinity for VWF

In order to study the influence of near-UV-B illumination on the variable domains of Ig's, we illuminated the scFv-82D6A3-wt and its mutants with light of 290 nm. The effect of illumination on the binding of the scFv to VWF was studied by ELISA. The samples containing 10±2 µg/ml in PBS of scFv-82D6A3-wt, scFv-82D6A3-W35F(V_L), scFv-82D6A3-W36F(V_H) or scFv-82D6A3-W35F(V_L)/ W36F(V_H) were illuminated (max. 2 hours) 0-2 hours under the conditions mentioned in Materials and Methods. VWF coated plate wells were incubated with non-illuminated or with illuminated wild type scFv-82D6A3 and mutants W35F(V_L), W36F(V_H) and W35F(V_L)/W36F(V_H). All samples were diluted to a concentration

of 4 µg/ml and added to VWF coated wells in a dilution series and bound scFv was detected with anti-E Tag-HRP antibodies as described in Fig. 3. The same maximal absorbance as used in Fig. 3 was arbitrarily (at 1 µg /ml based on inflexion points) set to 100% binding (Fig. 5a). The binding of a sample containing scFv-82D6A3-D99A(V_H) was used as a negative control. After 1 and 2 hours of irradiation, the binding of the scFv-82D6A3-wt to coated VWF decreased by 24 (±4)% and 65 (±5)%, respectively (Fig. 5a). These results indicate that illumination significantly alters the conformation or the accessibility of the paratope of the scFv. Furthermore, the binding of all mutant scFv's significantly decreased upon prolonged irradiation. In Fig. 5b are presented the reduction of intact scFv's upon illumination. For each scFv mutant, the non-illuminated species is considered to be 100% intact. The % of intact scFv in illuminated samples is deduced from the concentration of non-illuminated scFv (Fig. 3) needed to bind to the same extent to VWF as illuminated scFv does.

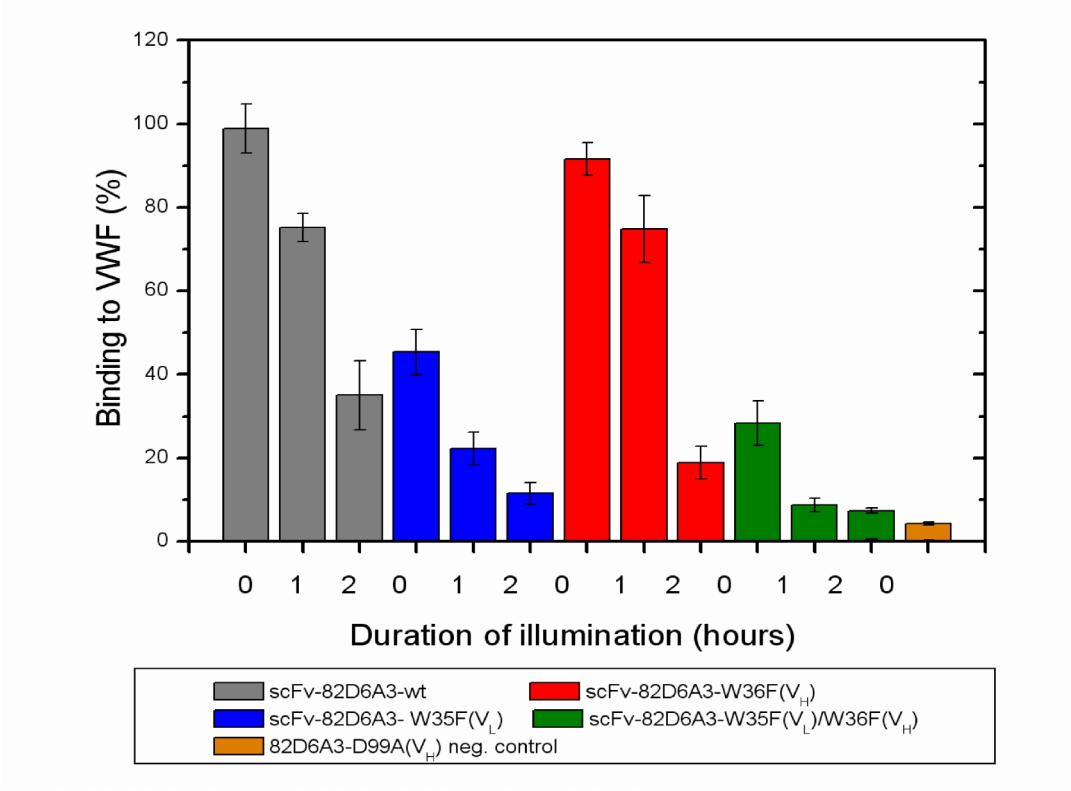

Fig. 5a. The effect of illumination on the VWF binding of scFv's

The binding to VWF of all fragments at 1 µg/ml, scFv-82D6A3-wt (grey), scFv-82D6A3-W35F(V_L) (blue), scFv-82D6A3-W36F(V_H) (red) and scFv-82D6A3- W35F(V_L)/ W36F(V_H) (green) after 0h, 1h and 2 h illumination is presented . 82D6A3-D99A(V_H) (orange) was used as a negative control. Data are the mean ± SEM of single measurements in at least five independent experiments

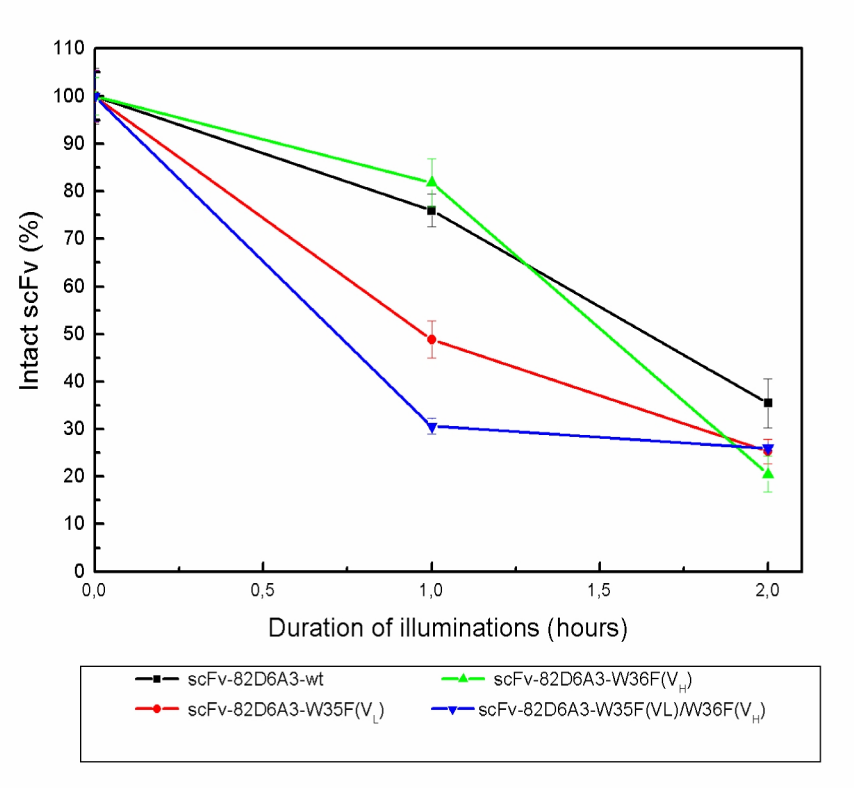

Fig. 5b. Reduction of intact scFv upon illumination

The binding to VWF of 1 µg/ml scFv-82D6A3-wt (black), mutants scFv-82D6A3-W35F(V_L) (red), scFv-82D6A3-W36F(V_H) (green) or scFv-82D6A3-W35F(V_L)/W36F(V_H) blue) are presented in function of the illumination time. For each scFv mutant, the non-illuminated species is considered to be 100% intact. The percentage of intact scFv in illuminated samples is deduced from the concentration of non-illuminated scFv (Fig. 3) needed to bind to VWF to the same extent (data of Fig. 5a)

As a consequence, replacement of Trp36 in V_H and of Trp35 in V_L does not significantly protect the scFv's against irradiation induced loss of affinity neither in single nor in double mutants.

3.2.2 Tryptophan fluorescence

The loss of activity upon irradiation of scFv-82D6A3 is accompanied by changes in the steady state fluorescence spectra. Fig. 6a represents the changes in the steady state Trp fluorescence spectra. The inset shows the photoinduced bleaching of tryptophan: The yield of Trp fluorescence of scFv-82D6A3-wt diminishes upon prolonged illumination. Fig. 6b represents the percentage of integrated fluorescence of scFv-82D6A3-wt and of its mutants as a function of the illumination time. We detected a significant decrease and blue shift of the fluorescence after prolonged irradiation of wild type scFv and of the single Trp mutants, scFv-82D6A3-W35F(V_L) and scFv-82D6A3-W36F(V_H). Surprisingly, irradiation of the double mutant scFv-82D6A3-W35F(V_L)/W36F(V_H) does not change its fluorescence. Since, as mentioned above, W47(V_H), W50(V_H) and W108(V_H) contribute to the fluorescence of this double mutant, these remaining Trp residues and their immediate surroundings apparently are not affected by irradiation.

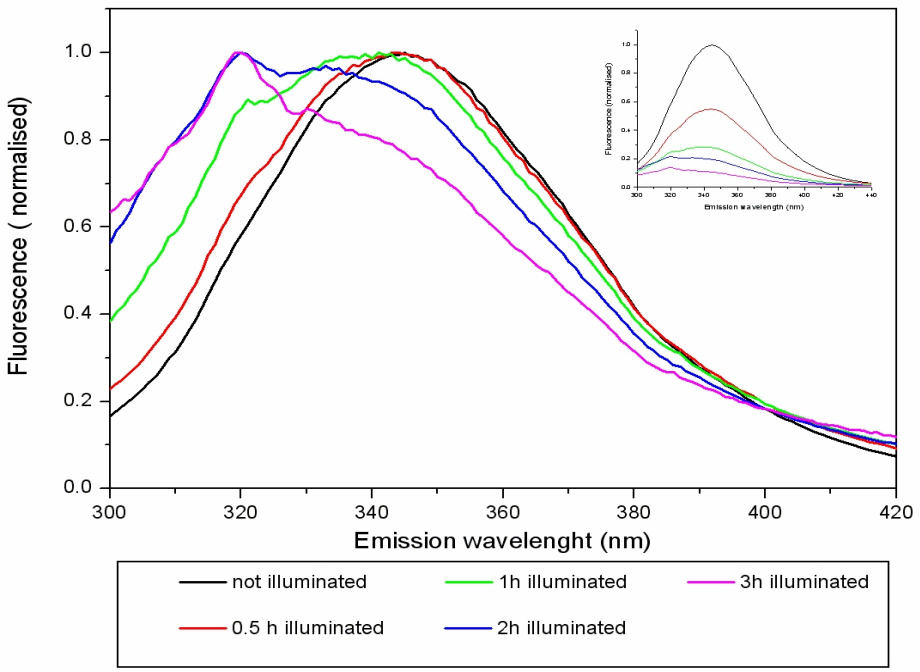

Fig. 6a. Effect of illumination on the steady state Trp fluorescence

Normalized fluorescence spectrum of scFv-82D6A3-wt after illumination with 290nm UV-B light. The spectra were taken at 0, 0.5,1, 2 and 3h. The protein concentration was 20 µg/ml and illumination conditions are as described in 2.7 Materials and methods. The inset shows the relative fluorescence intensity of illuminated scFv-82D6A3-wt after 0,0.5,1,2 and 3 hour

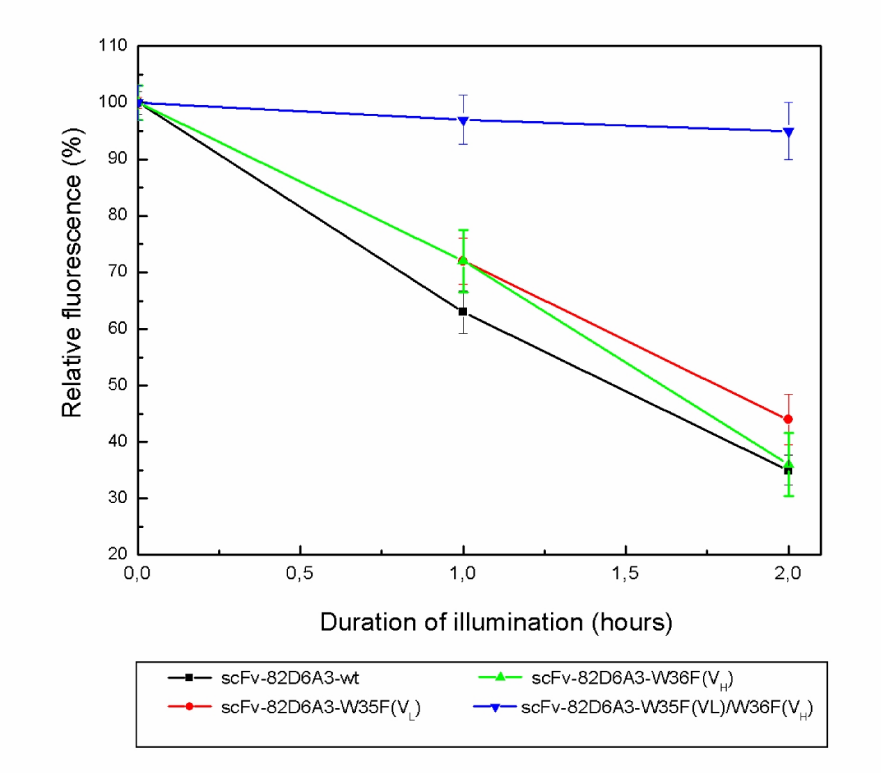

Fig. 6b. Effect of illumination on the intrinsic fluorescence

The percentage of the total integrated fluorescence, compared with the total integrated fluorescence observed at zero time is plotted as a function of the illumination time for scFv-82D6A3-wt (black), scFv-82D6A3-W35F(V_L) (red), scFv-82D6A3-W36F(V_H) (green) and scFv-82D6A3-W35F(V_L)/W36F(V_H) (blue). The data points are presented as mean ± SEM of at least three independent experiments.

3.2.3 Evolved thiols

The increase of the CPM fluorescence observed during illumination when this probe was mixed with either wild type scFv-82D6A3-wt (Fig. 7) or single mutants of scFv clearly proves that evolved thiols were formed upon illumination of these fragments. The concentration of evolved thiols after 1-hour of illumination amounted to 0.15±0.05 thiols/scFv unit. In contrast, the fluorescence of CPM in a solution containing the double mutant, scFv-82D6A3-W35F(V_L)/W36F(V_H) did not increase upon illumination (Fig. 7), indicating that here no free thiols are formed upon illumination.

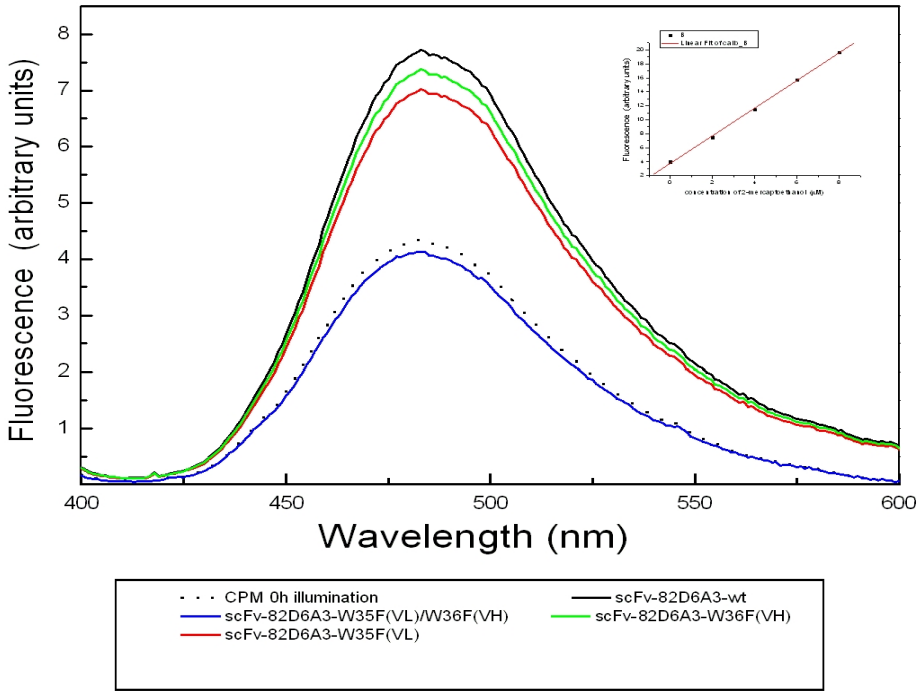

Fig. 7. Detection of the evolved thiols

The fluorescence spectra of CPM (black dotted) are taken without and with protein fragments: scFv-82D6A3-wt (black), scFv-82D6A3-W35F(V_L) (red), scFv-82D6A3-W36F(V_H) (green) and scFv-82D6A3-W35F(V_L)/W36F(V_H) (blue). The inset shows the calibration of 2 µM CPM solution in the presence of 2-mercaptoethanol. The excitation wavelength was 386 nm, the bandpass for both the excitation and emission slit was 4 nm

3.2.4 Light induced aggregation and/or polymerization.

The above data referring to creation of free thiols upon illumination of scFv-82D6A3-wt, scFv-82D6A3-W35F(V_L) and of scFv-82D6A3-W36F(V_H) but not upon illumination of the double mutant, scFv-82D6A3-W35F(V_L)/W36F(V_H), point to disruption of one or both disulfide bridges when the residues W35 and W36 are present in the V_L and V_H domains, respectively. However, the fraction of free thiols obtained after one hour of illumination is small and does not tend to reach the 2 or 4 thiols/scFv unit which would be expected when one or two disulfide bridges are disrupted. Vanhooren et al. demonstrated that intermediately formed thiyl radicals can give rise to creation of new linkages [10]. The newly formed linkages may be intra- as well as intermolecular. Here, we examined the appearance of multimers by SDS-PAGE and Western blotting in the absence and in the presence of 1,4-dithiothreithol, respectively. After illumination of scFv-82D6A3-wt and subsequent electrophoresis under non-reducing conditions, the monomer band at 28 kDa decreased in intensity and new bands of 56 kDa and of higher molecular weight appeared in the Western blots (Fig. 8). However, under reducing conditions the intensity of the higher molecular weight bands did not fully disappear, indicating that reducible and non reducible bonds are formed between monomers of wild type scFv-82D6A3. These partially reducible disulfide bonds indicate that multimers are present or that aggregation might occur. Similar results were obtained with the mutants' scFv-82D6A3-W35F(V_L) and scFv-82D6A3-W36F(V_H).

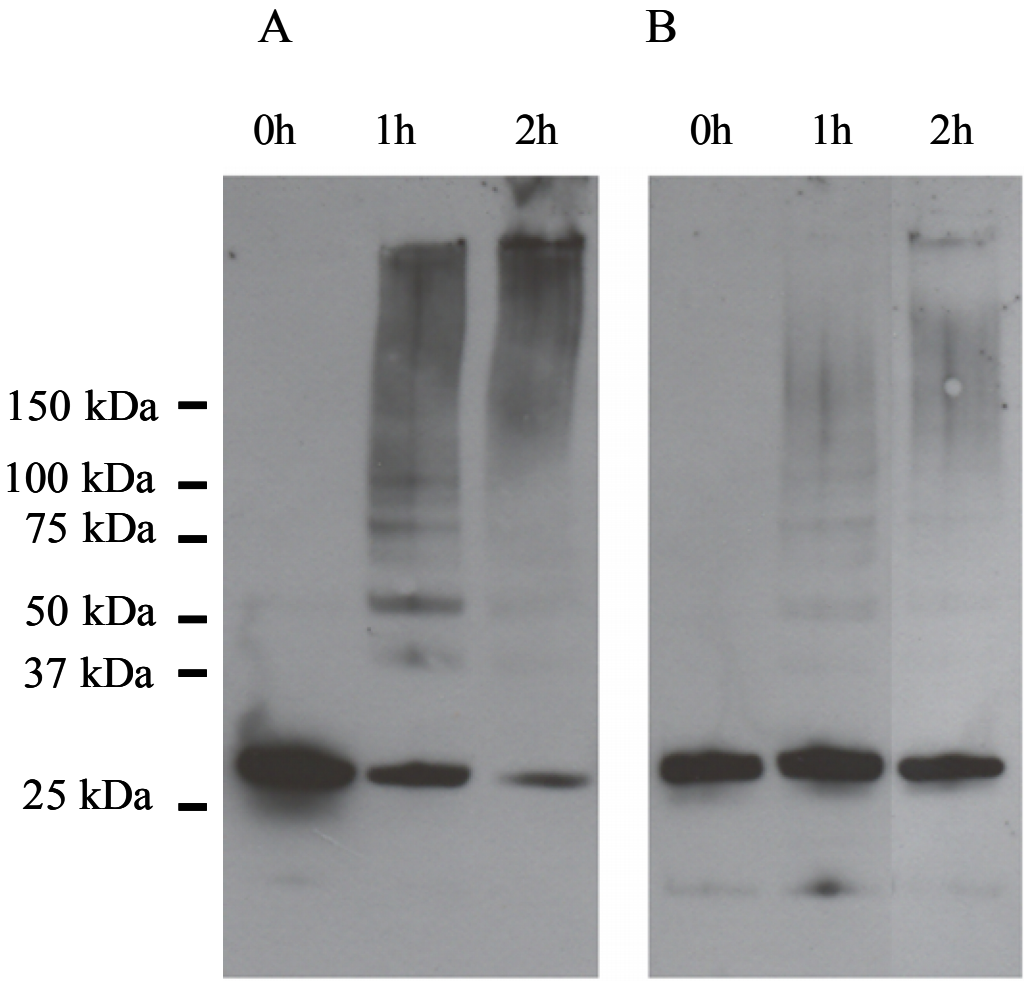

Fig. 8. Effect of illumination on formation of multimers

Samples containing 10µg/ml scFv-82D6A3-wt were illuminated for respectively 0, 1 and 2 hours. A fixed volume of illuminated samples was loaded on a 10-20 % SDS-PAGE gradient gel in the absence (A) and presence of 1,4-dithiothreithol (B). The proteins were electroblotted to a nitrocellulose membrane and the presence of scFv with E-Tag was detected with anti-E Tag-HRP antibodies.

4. DISCUSSION

In order to study the influence of near-UV-B light on the variable domain of an Ig, illumination-tests were performed on the single chain variable fragment (scFv) of the mAb 82D6A3, in which the V_L and V_H domains are linked via a $(Gly4Ser)_3$ linker. This scFv-82D6A3 inhibits the VWF-collagen interaction through competitive binding to the VWF A3-domain, indicating that an identical native structure as in the Fv of the intact antibody is maintained in the scFv [15]. Upon illumination with near UV-B-light the scFv partially loses its capacity to bind to VWF (Fig. 5), indicating that the structure, orientation or the accessibility of the paratope is changed. The paratope residues that are essential for antigen binding were previously defined as N31(V_H), Y32(V_H), D99(VH), P101(V_H), Y102(V_H) and Y103(V_H) [15]. These residues are located in two loops and form one continuous patch on the surface

of the antibody. W50(V_H) and H49(V_L), situated adjacent to this patch, play ancillary roles in binding. In the interior of both the V_H and V_L, two triads consisting of a Trp residue and a disulfide bond are located at a distance [15] of about 12 Å from the antigen binding patch. It is known that UV-B-light can induce changes in the protein structure due to the disruption of disulfide bonds upon excitation of a nearby Trpresidue [4-10]. To obtain a more explicit assessment of the impact of the Trp-disulfide triads upon the photodegradation of scFv-82D6A3, we systematically substituted one or both of the triad Trp by Phe: scFv-82D6A3-W35F(V_L), scFv-82D6A3-W36F(V_H) and scFv-82D6A3-W35F(V_L)/W36F(V_H). Accordingly, in our study disulfide bonds are broken upon illumination of scFv-82D6A3-wt (Fig. 7) and also of the mutants' scFv-82D6A3-W35F(V_L) and scFv-82D6A3-W36F(V_H). The number of free thiols [0.15 ($\pm$0.05) free thiols/scFv unit after 1 hour of illumination] however is small compared to the loss of affinity observed after the same illumination time. On the other hand, we observed that upon illumination of scFv-82D6A3-wt, dimers and multimers/higher aggregates are formed of which a larger part is linked by reducible bonds (Fig. 8). This result refers to either intermolecular disulfide bonds created by recombination of thiyl radicals intermediately formed during the irradiation of scFv-82D6A3-wt or a UV-B-light induced multimerisation/aggregation of scFv-82D6A3 fragments. It partially explains the low yield of free thiol groups detected.

In contrast to the findings for scFv-82D6A3-wt and for the single Trp mutants, scFv-82D6A3-W35F(V_L) and scFv-82D6A3-W36F(V_H), no light-induced formation of free thiols could be detected in the double mutant scFv-82D6A3-W35F(V_L)/W36F(V_H) (Fig. 7). Indeed, in agreement with the intense fluorescence (Fig. 4) the three Trp residues remaining in scFv-82D6A3-W35F(V_L)/W36F(V_H) are not in contact with the disulfide bond in the V_L nor with the one in the V_H domain and therefore, do not transfer an appreciable amount of their excitation energy to these bonds. Unfortunately, the simultaneous substitution of W35(V_L) and of W36(V_H) did not protect the affinity of the mutant for VWF, as it already resulted in a reduced binding to VWF in the non-illuminated sample, which decreased further upon illumination (Fig. 5a, 5b). Therefore, in addition to the effects which are induced by light absorption of W35(V_L) and of W36(V_H), the light absorption of W47(V_H), W50(V_H) and/or W108(V_H) seems to create specific alterations in the structure, folding, orientation or the accessibility of the paratope of scFv-82D6A3-W35F(V_L)/W36F(V_H). Earlier, Creed [3] described that excited Trp can initiate several processes including photoionization, intersystem crossing and intramolecular proton- and charge transfer. The final photoproduct largely depends on the nature of the close environment surrounding the excited Trp. Anyhow, as irradiation of the double mutant did not lead to changes in the fluorescence spectrum (Fig. 6), the reduction of the affinity of scFv-82D6A3-W35F(V_L)/W36F(V_H) for VWF seems not to be caused by damage of either W47(V_H), W50(V_H) or W108(V_H), which ensure the major part of the light absorption.

In contrast to our scFv, which only consists of the linked V_H and V_L domains of an IgG, a Fab fragment consists of four domains, C_H, V_H, C_L and V_L linked with hydrophobic contacts and with one highly conserved disulfide bridge located between its C_H and C_L domain. The latter disulfide bridge is solvent accessible and is located far from the antigen binding site, and according to Neves-Petersen et al. [12], this disulfide bridge is particularly susceptible to light-induced breakage allowing immobilization of Fab fragments. The authors believe that as a consequence of the large distance between the susceptible S-S bond and the antigen binding site (distance more than 13 Å), light-induced immobilization would not change the activity of the protein. However, our results demonstrate that also the disulfide bridges of the Trp-disulfide triads in the interior of the V_H and V_L domains of antibodies might be sensitive to light-induced breakage. Moreover, the loss of affinity for VWF upon mutation and

additionally upon illumination of scFv-82D6A3-W35F(V_L)/W36F(V_H) suggests that additional reactions in the variable fragments of Ig's, other than photocleavage of disulfide bonds, contribute to the light induced loss of affinity for the antigen. Our data suggest that UV-B-light furthermore can induce the formation of multimers/aggregates next to Trp – SS triad mediated photodegradation of scFv's. As a consequence, light induced immobilization of Fab fragments on a thiol reactive surface may require a strict limitation of the applied amount of photons to avoid a substantial loss of affinity for the antigen. Our results show that our studied protein fragment (scFv82D6A3) couldn't be applied as a good biosensor which could of course be due to the small size of the scFv. We were surprised, because the most of IgG proteins usually can serve as a good biosensor.

5. CONCLUSION

Summarizing, in this work we observed that the effects induced by photon absorption by W35(V_L) and W36(V_H) of the scFv of the monoclonal antibody 82D6A3 are clearly different from the ones seen following photon absorption by W47(V_H), W50(V_H) and W108(V_H). Light induced excitation of W35(V_L) and of W36(V_H) mediates the formation of free thiols, although the number of free thiols is small when compared to the loss of affinity observed after the same illumination time. On the other hand we observed the formation of new intermolecular disulfide bonds resulting in dimers and multimers/higher aggregates. After simultaneous substitution of W35(V_L) and W36(V_H), the affinity of scFv-82D6A3 for VWF still decreased upon illumination, although as expected, no free thiol groups could be detected.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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