

Original Research Article

Role of the Trp-disulfide triads in the UV light induced degradation of a monoclonal antibody scFv

ABSTRACT

Proteins are targets for photodegradation due to absorption of incident light by endogenous chromophores. In this work we study the role of Trp-disulfide triads in the light induced loss of immunoglobulin activity. The illumination tests were performed on a single chain variable fragment (scFv) of the monoclonal antibody 82D6A3, in which the variable domains of the heavy and the light chains are linked via (Gly4Ser)₃. The scFv binds to von Willebrand factor (VWF) indicating that an identical native structure as in the variable domains of the intact antibody is maintained. Upon illumination with near UV-B-light the scFv partially loses its capacity to bind 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 bridge of the V_L and V_H domain respectively, to Phe and compared the effects upon illumination of these mutants with those upon illumination of the wild type scFv. The results indicate that light induced excitation of W35(V_L) and of W36(V_H) mediates photolysis of the vicinal disulfide bonds. Although the more distant W47(V_H), W50(V_H) and W108(V_H) do not contribute to photolysis of the above mentioned disulfide bonds, the simultaneous substitution of W35(V_L) and of W36(V_H) nevertheless does not protect the scFv's affinity for VWF against illumination.

Keywords: *immunoglobulin, Trp-Phe mutants, tryptophan fluorescence, disulfide bonds, photolysis, aggregation, scFv.*

1. INTRODUCTION

The exposure of proteins to near-UV-B irradiation induces structural changes and alters their biological function [1]. The damage is initiated through photon absorption by the chromophoric amino acids. The indole nucleus is the most strongly near-UV-B absorbing group in proteins. Therefore, tryptophane (Trp, W) residues are primary components in the activation of protein photodegradation. Some decades ago it has been proposed that absorption of near-UV-B light by a Trp side chain initiates reductive splitting of disulfide bonds [2, 3]. Only recently it was demonstrated that such Trp-mediated photodegradation of protein disulfide bonds indeed occurs upon illumination of cutinase [4,5], α-lactalbumin [6,7] somatotropin [8] and lysozyme [9]. By analysis of the photoproducts of goat α-lactalbumin mutants with substituted Trp residues we demonstrated that the contribution of each Trp to 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 mouse IgG immunoglobulin were immobilized upon illumination, by covalent binding on a thiol reactive surface. The immobilized Fabs were oriented and conserved the native binding capacity to 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 von Willebrand factor (VWF), 82D6A3 [13, 14] which belongs to the immunoglobulin superfamily (IgSF) [15]. The hallmark feature of 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 – S-S triads [16]. Both variable domains of 82D6A3 contain such a Trp – S-S triad. In the variable domain of the heavy chain (V_H) this triad consists of Trp36 and of Cys22-Cys96, whereas in the variable domain of the light chain (V_L) this triad consists of Trp35 and of Cys23-Cys88. In order to eliminate the interference of the constant domains of the Ig, a single chain variable fragment (scFv) of 82D6A3 has been 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 – S-S triads. Moreover, it contains only three extra Trps which are all three located in the V_H domain [W47(V_H), W50(V_H) and W108(V_H)] and their distance from the upon mentioned SS-bridge is over 5 Å [16]. The above mentioned 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 phenylalanine (Phe, F) in the variable domain of the light (V_L) or heavy chain (V_H).

2. MATERIALS AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY

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. 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 4°C against PBS, the purity was checked in SDS-PAGE using a

85 Novex®10-20% Tricine gel followed by staining with Coomassie brilliant blue and stored at -
86 20°C.

87 **2.3 Construction, Expression and Purification of Tryptophan Mutants of scFv-** 88 **82D6A3**

89 Construction of Trp mutants, scFv-82D6A3-W36F(V_H), scFv-82D6A3-W35F(V_L) and scFv-
90 82D6A3-W35F(V_L)/W36F(V_H), was performed as described by Staelens et al. [17] using the
91 QuickChange® site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands)
92 and pCANTAB 5 E-scFv-82D6A3-wt-DNA. The DpnI-digested mutagenesis mixture was
93 immediately used to transform E. coli HB2151 cells according to the manufacturer's
94 instructions (RPAS expression module, GE Healthcare). Sequencing, expression, and
95 purification were performed as described [15]. After dialysis against PBS, purity was
96 checked as described above.

97 **2.4 Western Blotting**

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

102 **2.5 Concentration Determination of the Purified scFv Fragment**

103 The concentration of the purified scFv's was determined according to the Bradford method
104 using the Coomassie® Plus protein assay kit (Pierce, Rockford, IL). Briefly, dilution series of
105 purified scFv's and Albumin Standard in PBS were added to a 96-well plate (Greiner,
106 Frickenhausen, Germany). A fixed volume of Coomassie® Plus protein assay reagent was
107 added and absorbance was measured at 630 nm.

108 **2.6 Binding of scFv-82D6A3-wt and its Trp Mutants to VWF**

109 Binding of scFv-82D6A3-wt and its Trp mutants to VWF was determined as described before
110 [17]. Briefly, a 96-well plate was coated with VWF and incubated with dilution series of
111 scFv's for 1.5 h at room temperature. Next, bound scFv's were detected by incubation with
112 anti-E Tag-HRP. Visualization was performed by adding H₂O₂ and OPD. The plate was
113 washed nine times with PBS, 0.1% Tween 20 between different incubation steps.

114 **2.7 Illumination of scFv Protein Fragments**

115 The scFv-82D6A3-wt and its Trp mutants were illuminated with 290 nm light in a quartz
116 cuvette of 0.4 cm path length (Perkin Elmer Instruments, Shelton, CT), using an Aminco-
117 Bowman® Series-2 spectrofluorimeter (Spectronic Instruments, Rochester, NY) equipped
118 with a 150W Xenon lamp. The excitation wavelength was centered at 290 nm with the slits
119 set to 16 nm band pass. A 0.5 ml sample of scFv (10 µg/ml in PBS) was illuminated at 4 °C
120 in the cuvette of the spectrofluorimeter. During illumination the solution was stirred with a
121 magnetic bar at 120 rpm. To avoid oxidation, all solutions were thoroughly degassed prior to
122 use and kept under N₂ atmosphere during illumination. The illumination time was max. 3
123 hours; during illumination sampling were taken at 0,1,2,3 hour.

124 Ferrioxalate actinometry showed that under these conditions the incident flux was 6×10^{14}
125 photons $\times$ 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 4°C using an Aminco-Bowman® Series-2 spectrofluorimeter. The excitation wavelength was 290 nm, the bandpass for both the excitation and emission slit was 4 nm. The emission spectra were measured in the wavelength region 300-450 nm.

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 4°C 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 AND DISCUSSION

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 V_L, or W36, adjacent to C22-C96 in V_H, were substituted by a Phe (Fig. 1).

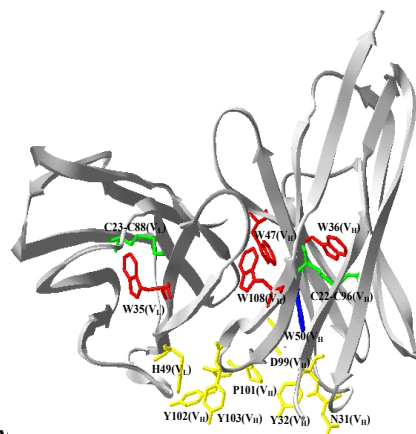
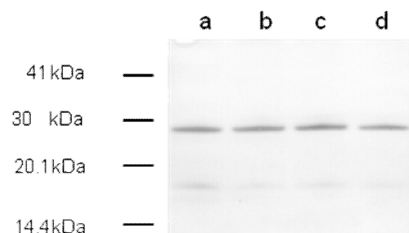


Fig. 1. Structure of the variable domains of 82D6A3 as deduced from the crystal structure of the complex between the Fab-fragment of 82D6A3 and the VWF A3-domain. (PDB entry 2ADF [15]). The Fig. was generated with PyMOL (<http://pymol.sourceforge.net/>). In red are the Trp residues, except for Trp50(V_H) 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 ca 90 % of recombinant scFv.



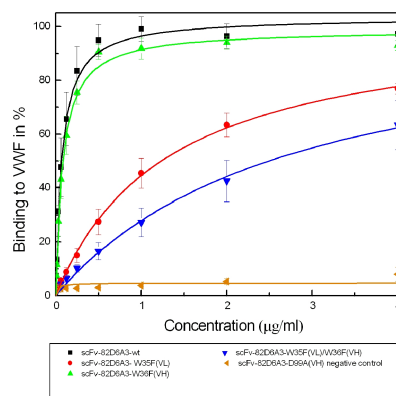
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Fig. 2. Expression of scFv-82D6A3-wt and its Trp-mutants. scFv-82D6A3-wt (a) and mutants scFv-82D6A3-W35F(V_L) (b), scFv-82D6A3-W36F(V_H) (c), scFv-82D6A3-W35F(V_L)/W36F(V_H) (d) were expressed in *E. coli* HB2151 and purified from the supernatant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

180 **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 EC₅₀ for the binding of scFv-82D6A3-wt to VWF is 2.2×10^{-9} M (0.06 $\mu\text{g/ml}$). Upon substitution of Trp36 from the heavy chain to Phe, the effect on the affinity is minor; the EC₅₀ of scFv-82D6A3-W36F(V_H) is 3.7×10^{-9} M (0.1 $\mu\text{g/ml}$). In contrast, the replacement of W35 in the light chain for Phe more significantly decreased the affinity for VWF with an EC₅₀ of scFv-82D6A3-W35F(V_L) of 4.6×10^{-8} M (1.25 $\mu\text{g/ml}$). 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 9.2×10^{-8} M (2.6 $\mu\text{g/ml}$).

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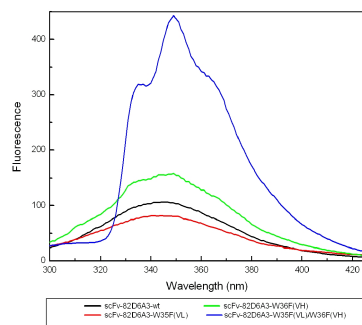
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192 **Fig. 3. Binding of scFv-82D6A3-wt and its Trp mutants to VWF.** Serial dilutions of purified wild
193 type scFv-82D6A3-wt and mutant scFv-82D6A3- W35F(V_L), scFv-82D6A3-W36F(V_H) and scFv-
194 82D6A3-W35F(V_L)/W36F(V_H) were added to VWF-coated wells, and bound scFv was detected with
195 anti-E Tag-HRP. Visualization was performed by adding H₂O₂ and OPD. The maximal absorbance
196 calculated from the fitted curve obtained with scFv-82D6A3-wt was arbitrarily set as 100% binding. The
197 inactive mutant scFv-82D6A3-D99A(VH) was used as a negative control [17]. All data points are the
198 mean $\pm$ S.E.M. of single measurements in at least five independent experiments.

199

200 3.1.3 Tryptophan Fluorescence

201 The fluorescence spectra of the scFv's at equal protein concentration ($\sim 4 \times 10^{-7}$ M) are
202 presented in Fig. 4. The fluorescence of scFv-82D6A3-wt slightly decreases upon
203 substitution of W35(V_L) but clearly increases upon substitution of W36(V_H). Surprisingly, the
204 fluorescence of the double mutant, scFv-82D6A3-W35F(V_L)/W36F(V_H), yields up to 4 times
205 the fluorescence of the wild type. The strong fluorescence increase upon substitution of
206 W35(V_L) in scFv-82D6A3-W36F(V_H) and of W36(V_H) in scFv-82D6A3-W35F(V_L),
207 respectively, implies (a) that both Trp residues are strongly quenched and (b) that both
208 substituted residues contribute to indirect quenching of one or more of the remaining Trp
209 residues: W47(V_H), W50(V_H) and W108(V_H). These presumptions are based on the crystal
210 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
211 C22-C96 in V_H. Clearly, the direct contact of the considered Trp residues with a disulfide
212 bridge enables the energy transfer or electron transfer [5]. The spectrum of scFv-82D6A3-
213 W35F(V_L)/W36F(V_H) is characterized by three distinct maxima. Therefore, each of the three
214 Trp residues present in scFv-82D6A3-W35F(V_L)/W36F(V_H) seems to interact in a specific
215 way with its surroundings and seems to emit the absorbed light energy in a distinct manner.



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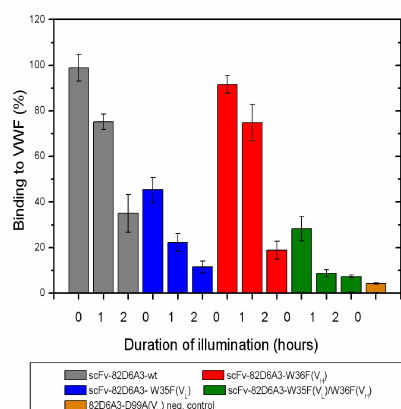
Fig. 4. Fluorescence emission spectrum of scFv-82D6A3-wt and its Trp mutants. The concentration of all scFv fragments was $10 \pm 2 \mu\text{g/ml}$ in PBS. The excitation wavelength was 290 nm, the bandpass for both the excitation and emission slit was 4 nm. The spectra of scFv-82D6A3-wt, scFv-82D6A3-W35F(V_L), scFv-82D6A3-W36F(V_H) and scFv-82D6A3-W35F(V_L)/W36F(V_H) are given. The fluorescence spectra were corrected for inner filter effect and blanc spectra (Raman peak of the water) were 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 $10 \pm 2 \mu\text{g/ml}$ 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 $4 \mu\text{g/ml}$ 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 $1 \mu\text{g/ml}$ 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 $24 (\pm 4) \%$ and $65 (\pm 5) \%$, 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.

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.

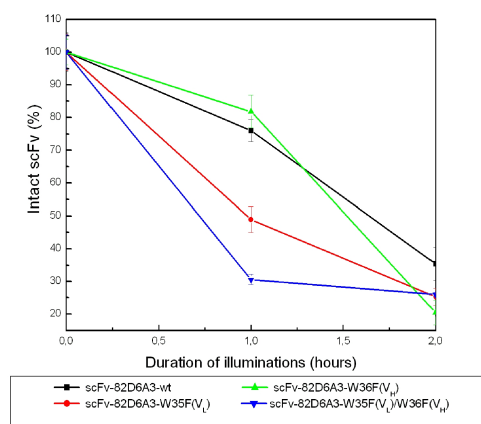


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Fig. 5a. The effect of illumination on the VWF binding of scFv's. The binding to VWF of all fragments at $c = 1 \mu\text{g/ml}$, scFv-82D6A3-wt, scFv-82D6A3-W35F(V_L), scFv-82D6A3-W36F(V_H) and

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scFv-82D6A3- W35F(V_L)/ W36F(V_H) after 0h, 1h and 2 h illumination is presented . 82D6A3-D99A(V_H) was used as a negative control. The data points are the mean $\pm$ SEM of single measurements in at least five independent experiments.



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Fig. 5b. Reduction of intact scFv upon illumination. The binding to VWF of 1 μ g/ml scFv-82D6A3-wt, mutants scFv-82D6A3-W35F(V_L), scFv-82D6A3-W36F(V_H) or scFv-82D6A3-W35F(V_L)/W36F(V_H) 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).

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263 **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 tryptophane: 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.

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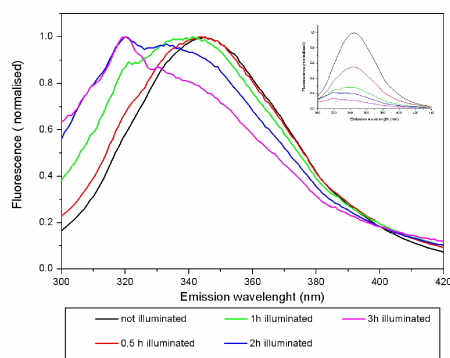
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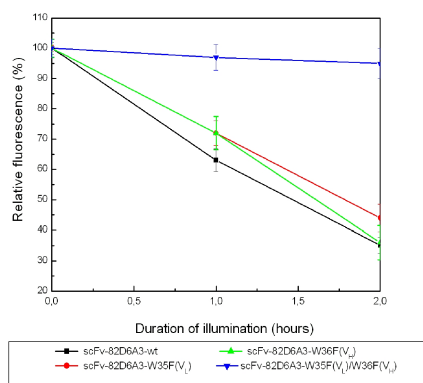
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285 **Fig. 6a. Effect of illumination on the steady state Trp fluorescence.** Normalized fluorecence
 286 spectrum of scFv-82D6A3-wt after illumination with 290nm UV-B light. The spectras were
 287 taken at 0, 0.5, 1, 2 and 3h. The protein concentration was 20 μ g/ml and illumination conditions are as
 288 described in 2.7 Materials and methods. The inset shows the relative fluorescence intensity of
 289 illuminated scFv-82D6A3-wt after 0,0.5,1,2 and 3 hour.

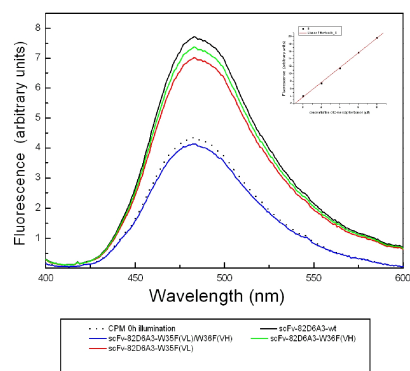


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291 **Fig. 6b. Effect of illumination on the intrinsic fluorescence.** The percentage of the fluorescence,
 292 compared with the fluorescence observed at zero time, is plotted as a function of the illumination time
 293 for scFv-82D6A3-wt, scFv-82D6A3-W35F(V_L), scFv-82D6A3-W36F(V_H) and scFv-82D6A3-
 294 W35F(V_L)/W36F(V_H). The data points are presented as mean $\pm$ SEM of at least three independent
 295 experiments.
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297 **3.2.3. Evolved Thiols**

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



305

306 **Fig. 7. Detection of the evolved thiols.** The fluorescence spectra of CPM are taken without and with
 307 protein fragments: scFv-82D6A3-wt, scFv-82D6A3-W35F(V_L), scFv-82D6A3-W36F(V_H) and scFv-
 308 82D6A3-W35F(V_L)/W36F(V_H). The inset shows the calibration of 2 μ M CPM solution in the presence of
 309 2-mercaptoethanol. The excitation wavelength was 386 nm, the bandpass for both the excitation and
 310 emission slit was 4 nm.

311

312 **3.2.4. Light Induced Aggregation and/or Polymerisation.**

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

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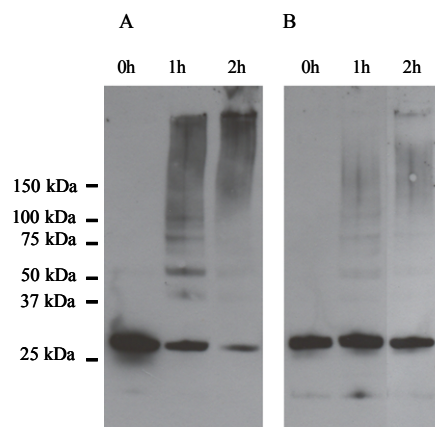


Fig. 8. Effect of illumination on formation of multimers. Samples containing 10µg/ml scFv-82D6A3-wt were illuminated for respectively 0, 1 and 2 hours. A fixed volume of illuminated samples was loaded on a 10-20 % SDS-PAGE gradient gel in the absence (A) and presence of 1,4-dithiothreitol (B). The proteins 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 scFv15. 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(V_H), 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 [16] 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 Trp-residue [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 Trps 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 (±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 detected free thiol groups.

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 resulting 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, light-induced immobilization would not change the activity of the protein.

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405 **4. CONCLUSION**

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407 However, our results demonstrate that also the disulfide bridges of the Trp-disulfide triads in
408 the interior of the V_H and V_L domains of antibodies might be sensitive to light-induced
409 breakage. Moreover, the loss of affinity for VWF upon mutation and moreover upon
410 illumination of scFv-82D6A3-W35F(V_L)/W36F(V_H) suggests that additional reactions in the
411 variable fragments of Ig's, other than photocleavage of disulfide bonds, contribute to the light
412 induced loss of affinity for the antigen. Our data suggest that UV-B-light can in addition
413 induce the formation of multimers / aggregation next to Trp – S-S triad mediated
414 photodegradation of scFv's. As a consequence, light induced immobilization of Fab
415 fragments on a thiol reactive surface may require a strict limitation of the applied amount of
416 photons to avoid a substantial loss of affinity for the antigen.

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