

Original Research Article

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

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 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 (rupture 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 in 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-C α 2 to S γ -S γ and in the heavy chain 4.7 ± 0.3 Å from Trp-C α 2 to S γ -S γ [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 V_H 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 / 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 [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 4°C 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 -20°C.

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.

127 2.4 Western Blotting

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

132 2.5 Concentration Determination of the Purified scFv Fragment

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

138 2.6 Binding of scFv-82D6A3-wt and its Trp Mutants to VWF

139 Binding of scFv-82D6A3-wt and its Trp mutants to VWF was determined as described before
140 [17]. Briefly, a 96-well plate was coated with VWF and incubated with dilution series of
141 scFv's for 1.5 h at room temperature. Next, bound scFv's were detected by incubation with
142 anti-E Tag-HRP. Visualization was performed by adding H₂O₂ and OPD. The plate was
143 washed nine times with PBS, 0.1% Tween 20 between different incubation steps.

144 2.7 Illumination of scFv Protein Fragments

145 The scFv-82D6A3-wt and its Trp mutants were illuminated with 290 nm light in a quartz
146 cuvette of 0.4 cm path length (Perkin Elmer Instruments, Shelton, CT), using an Aminco-
147 Bowman® Series-2 spectrofluorimeter (Spectronic Instruments, Rochester, NY) equipped
148 with a 150W Xenon lamp. The excitation wavelength was centered at 290 nm with the slits
149 set to 16 nm band pass. A 0.5 ml sample of scFv (10 µg/ml in PBS) was illuminated at 4 °C
150 in the cuvette of the spectrofluorimeter. During illumination the solution was stirred with a
151 magnetic bar at 120 rpm. To avoid oxidation, all solutions were thoroughly **degassed under**
152 **vacuum at 2.39 kPa for 5 min**, prior to use and kept under N₂ atmosphere during
153 illumination. The illumination time was max. 3 hours; during illumination sampling were taken
154 at 0,1,2,3 hour.

155 Ferrioxalate actinometry showed that under these conditions the incident flux was 6×10^{14}
156 photons $\times$ sec⁻¹. Binding of the illuminated and non-illuminated scFv's to VWF was
157 determined as earlier described [17] and compared with the binding of non-illuminated scFv-
158 82D6A3-wt to VWF.

159 2.8 Fluorescence Spectroscopy

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

166 2.9 Determination of Evolved Thiols by Fluorescence Spectroscopy

167

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

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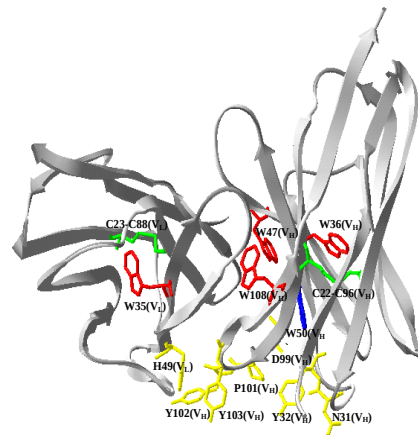
175 **3. RESULTS**

176

177 **3.1 Characterization of scFv-82D6A3-wt and its Trp Mutants**

178 **3.1.1 Expression and Purification**

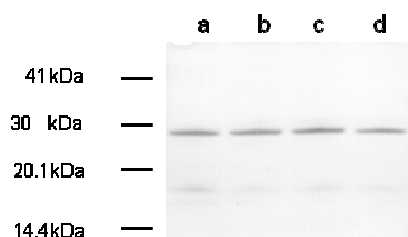
179 In this study, the scFv of 82D6A3, a mAb which specifically interacts with the A3-domain of
180 VWF [15], was used. In addition, we constructed three Trp mutants of scFv-82D6A3-wt. In
181 two mutants, either W35, adjacent to disulfide bond C23-C88 in V_L , or W36, adjacent to C22-
182 C96 in V_H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



183

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

190 The structure of the variable domains of 82D6A3 is deduced from the crystal structure of the
191 complex between the Fab-fragment of 82D6A3 and the VWF A3-domain (PDB entry 2ADF)
192 [15]. The mutants were named scFv-82D6A3-W35F(V_L) and scFv-82D6A3-W36F(V_H),
193 respectively. In a third mutant, scFv-82D6A3-W35F(V_L)/W36F(V_H), both the above
194 mentioned Trp residues were changed to Phe residues. The scFv's were expressed in *E.*
195 *coli* HB2151 cells and purified from the supernatant and periplasmic extract. SDS-PAGE
196 followed by Coomassie brilliant blue staining (Fig. 2) and Western blotting (data not shown)
197 indicated that the protein content of the samples used in the subsequent experiments
198 consisted of **nearly 90 % recombinant** scFv.

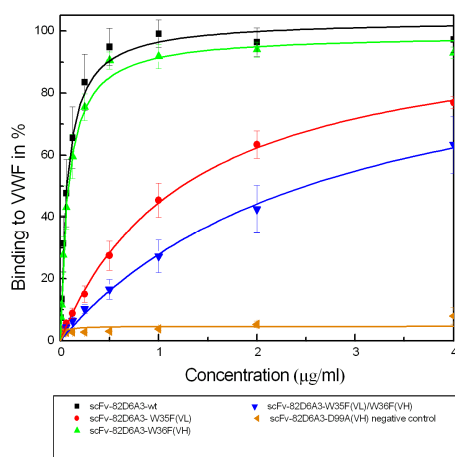


199

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

205 3.1.2 Affinity for VWF

206 The effect of the individual mutations on the binding of scFv to VWF coated to a 96-well
207 ELISA plate, is presented in Fig. 3. The EC₅₀ for the binding of scFv-82D6A3-wt to VWF is
208 2.2×10^{-9} M (0.06 $\mu\text{g/ml}$). Upon substitution of Trp36 from the heavy chain to Phe, the effect
209 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
210 contrast, the replacement of W35 in the light chain for Phe more significantly decreased the
211 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
212 expected, the simultaneous replacement of both Trp residues resulted in the largest
213 decrease of the scFv's affinity with an EC50 of scFv-82D6A3-W35F(V_L)/W36F(V_H) of $9.2 \times$
214 10^{-8} M (2.6 $\mu\text{g/ml}$).



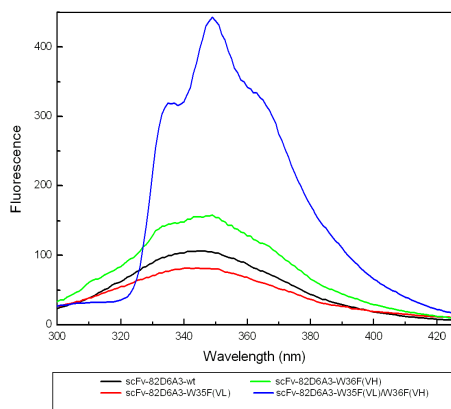
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216 **Fig. 3. Binding of scFv-82D6A3-wt and its Trp mutants to VWF.** Serial dilutions of purified wild
217 type scFv-82D6A3-wt (black) and mutant scFv-82D6A3-W35F(V_L) (red), scFv-82D6A3-W36F(V_H)
218 (green) and scFv-82D6A3-W35F(V_L)/W36F(V_H) (blue)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

224
225

3.1.3 Tryptophan Fluorescence

226 The fluorescence spectra of the scFv's at equal protein concentration ($\sim 4 \times 10^{-7}$ M) are
227 presented in Fig. 4. The fluorescence of scFv-82D6A3-wt slightly decreases upon
228 substitution of W35(V_L) but clearly increases upon substitution of W36(V_H). Surprisingly, the
229 fluorescence of the double mutant, scFv-82D6A3-W35F(V_L)/W36F(V_H), yields up to 4 times
230 the fluorescence of the wild type. The strong fluorescence increase upon substitution of
231 W35(V_L) in scFv-82D6A3-W36F(V_H) and of W36(V_H) in scFv-82D6A3-W35F(V_L),
232 respectively, implies (a) that both Trp residues are strongly quenched and (b) **that both**
233 **contribute** to indirect quenching of one or more of the remaining Trp residues: W47(V_H),
234 W50(V_H) and W108(V_H). These presumptions are based on the crystal data [15], W35(V_L) is
235 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.
236 Clearly, the direct contact of the considered Trp residues with a disulfide bridge enables the
237 energy or electron transfer [5]. The spectrum of scFv-82D6A3-W35F(V_L)/W36F(V_H) is
238 characterized by three distinct maxima. Therefore, each of the three Trp residues present in
239 scFv-82D6A3-W35F(V_L)/W36F(V_H) seems to interact in a specific way with its surroundings
240 and seems to emit the absorbed light energy in a distinct manner.



241

242 **Fig. 4. Fluorescence emission spectrum of scFv-82D6A3-wt and its Trp mutants.** The
243 concentration of all scFv fragments was 10 ± 2 µg /ml in PBS. The excitation wavelength was 290 nm,
244 the bandpass for both the excitation and emission slit was 4 nm. The spectra of scFv-82D6A3-wt
245 (black), scFv-82D6A3-W35F(V_L) (red), scFv-82D6A3-W36F(V_H) (green) and scFv-82D6A3-
246 W35F(V_L)/W36F(V_H) (blue) are given. The fluorescence spectra were corrected for inner filter effect
247 and blank spectrum (Raman peak of the water) was subtracted.
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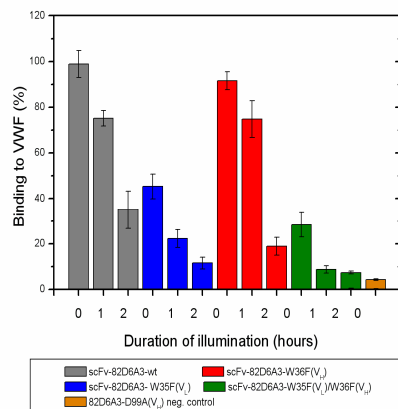
249 3.2 Effects Resulting from Illumination of Recombinant scFv Fragments

250 3.2.1 Affinity for VWF

251 In order to study the influence of near-UV-B illumination on the variable domains of Ig's, we
252 illuminated the scFv-82D6A3-wt and its mutants with light of 290 nm. The effect of
253 illumination on the binding of the scFv to VWF was studied by ELISA. The samples
254 containing 10 ± 2 µg/ml in PBS of scFv-82D6A3-wt, scFv-82D6A3-W35F(V_L), scFv-82D6A3-
255 W36F(V_H) or scFv-82D6A3-W35F(V_L)/W36F(V_H) were illuminated (max. 2 hours) 0-2 hours
256 under the conditions mentioned in Materials and Methods. VWF coated plate wells were
257 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.

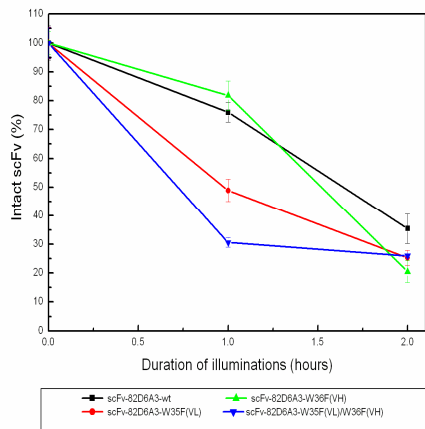
As a consequence, replacement of Trp36 in V_H and of Trp35 in V_L does not significantly protect the scFv's against irradiation induced loss of affinity neither in single nor in double mutants.



274

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.

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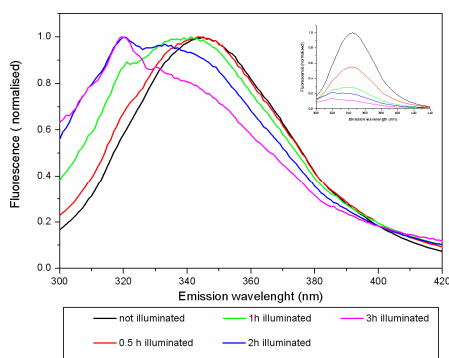


280

281 **Fig. 5b. Reduction of intact scFv upon illumination.** The binding to VWF of 1 µg/ml scFv-82D6A3-
 282 wt (black), mutants scFv-82D6A3-W35F(V_L) (red), scFv-82D6A3-W36F(V_H) (green) or scFv-82D6A3-
 283 W35F(V_L)/W36F(V_H) (blue) are presented in function of the illumination time. For each scFv mutant,
 284 the non-illuminated species is considered to be 100% intact. The percentage of intact scFv in
 285 illuminated samples is deduced from the concentration of non-illuminated scFv (Fig. 3) needed to bind
 286 to VWF to the same extent (data of Fig. 5a).
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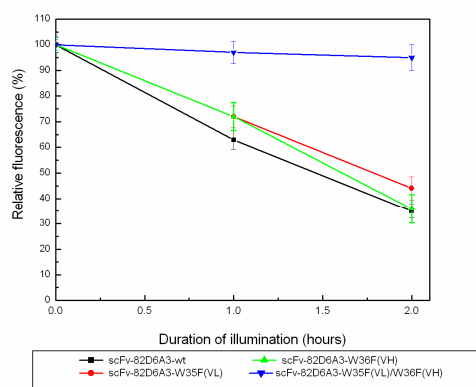
288 **3.2.2. Tryptophan Fluorescence**

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



301

302 **Fig. 6a. Effect of illumination on the steady state Trp fluorescence.** Normalized fluorescence
 303 spectrum of scFv-82D6A3-wt after illumination with 290nm UV-B light. The spectra were taken at 0,
 304 0.5, 1, 2 and 3h. The protein concentration was 20 µg/ml and illumination conditions are as described
 305 in 2.7 Materials and methods. The inset shows the relative fluorescence intensity of illuminated scFv-
 306 82D6A3-wt after 0, 0.5, 1, 2 and 3 hour.

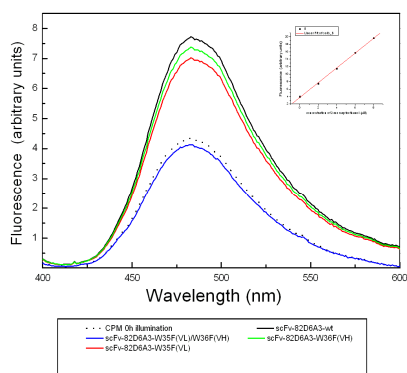


307

308 **Fig. 6b. Effect of illumination on the intrinsic fluorescence.** The percentage of the total integrated
 309 fluorescence, compared with the total integrated fluorescence observed at zero time, is plotted as a
 310 function of the illumination time for scFv-82D6A3-wt (black), scFv-82D6A3-W35F(VL) (red), scFv-
 311 82D6A3-W36F(VH) (green) and scFv-82D6A3-W35F(VL)/W36F(VH) (blue). The data points are
 312 presented as mean ± SEM of at least three independent experiments.
 313

314 **3.2.3. Evolved Thiols**

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



322

323 **Fig. 7. Detection of the evolved thiols.** The fluorescence spectra of CPM (black dotted) are taken
 324 without and with protein fragments: scFv-82D6A3-wt (black), scFv-82D6A3-W35F(V_L) (red), scFv-
 325 82D6A3-W36F(V_H) (green) and scFv-82D6A3-W35F(V_L)/W36F(V_H) (blue). The inset shows the
 326 calibration of 2 μM CPM solution in the presence of 2-mercaptoethanol. The excitation wavelength was
 327 386 nm, the bandpass for both the excitation and emission slit was 4 nm.
 328

329 **3.2.4. Light Induced Aggregation and/or Polymerisation.**

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

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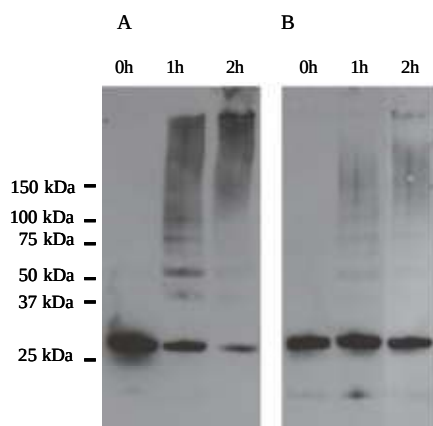


Fig. 8. Effect of illumination on formation of multimers. Samples containing 10µg/ml scFv-82D6A3-wt were illuminated for respectively 0, 1 and 2 hours. A fixed volume of illuminated samples was loaded on a 10-20 % SDS-PAGE gradient gel in the absence (A) and presence of 1,4-dithiothreitol (B). The proteins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

407 Anyhow, as irradiation of the double mutant did not lead to changes in the fluorescence
408 spectrum (Fig. 6), the reduction of the affinity of scFv-82D6A3-W35F(V_L)/W36F(V_H) for VWF
409 seems not to be caused by damage of either W47(V_H), W50(V_H) or W108(V_H), which ensure
410 the major part of the light absorption.

411 In contrast to our scFv, which only consists of the linked V_H and V_L domains of an IgG, a Fab
412 fragment consists of four domains, C_H, V_H, C_L and V_L linked with hydrophobic contacts and
413 with one highly conserved disulfide bridge located between its C_H and C_L domain. The latter
414 disulfide bridge is solvent accessible and is located far from the antigen binding site, and
415 according to Neves-Petersen et al. [12], this disulfide bridge is particularly susceptible to
416 light-induced breakage allowing immobilization of Fab fragments. The authors believe that
417 as a consequence of the large distance between the susceptible S-S bond and the antigen
418 binding site (distance more than 13 Å), light-induced immobilization would not change the
419 activity of the protein. However, our results demonstrate that also the disulfide bridges of the
420 Trp-disulfide triads in the interior of the V_H and V_L domains of antibodies might be sensitive
421 to light-induced breakage. Moreover, the loss of affinity for VWF upon mutation and
422 additionally upon illumination of scFv-82D6A3-W35F(V_L)/W36F(V_H) suggests that additional
423 reactions in the variable fragments of Ig's, other than photocleavage of disulfide bonds,
424 contribute to the light induced loss of affinity for the antigen. Our data suggest that UV-B-
425 light furthermore can induce the formation of multimers / aggregates next to Trp – SS triad
426 mediated photodegradation of scFv's. As a consequence, light induced immobilization of
427 Fab fragments on a thiol reactive surface may require a strict limitation of the applied amount
428 of photons to avoid a substantial loss of affinity for the antigen. Our results show that our
429 studied protein fragment (scFv82D6A3) couldn't be applied as a good biosensor which could
430 of course be due to the small size of the scFv. We were surprised, because the most of IgG
431 proteins usually can serve as a good biosensor.

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435 5. CONCLUSION

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

446

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