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Interaction of sulfonated anionic porphyrins with HIV glycoprotein gp120: photodamages revealed by inhibition of antibody binding to V3 and C5 domains

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Abstract

The key role of gp120 in the cellular entry of HIV makes this glycoprotein an attractive target for new drugs. Various polyanions bind to the positively charged V3 loop of gp120. Here, we consider a series of anionic porphyrins bearing two sulfonate groups and two carboxylic chains with various degree of esterification. These molecules carry an overall negative charge between 4 and 2. Upon activation by light, these compounds, known as photosensitizers, produce highly reactive oxygen species able to damage amino acid chains. The interactions of these molecules with the V3 loop and a positively charged area in the C5 region were investigated in the dark by using specific antibodies and ELISA protocols. Competitive inhibition of the anti-V3 antibody was observed with an increased efficiency for the esterified compounds. No evidence for binding to the C5 region was found. In contrast, when gp120 was irradiated with light in presence of the porphyrin prior to the addition of the antibody, strong inhibition of the anti-C5 antibody was observed revealing irreversible photo-damages in this region. No effect on the V3 loop was observed. Irradiations at two wavelengths made it possible to identify porphyrin monomers as the photoactive forms despite the presence of large excess of dimers in the incubation solution. It is suggested that porphyrins bound to the V3 loop could produce photo-damages at some distance, in particular within the C5 region that contains several photosensitive amino acids.

Keywords: Porphyrin; HIV; gp120; Polyanion; Photosensitizer; Antibody

1. Introduction

Most of the current drugs approved for the treatment of human immunodeficiency virus (HIV) infection are directed to the viral enzymes reverse transcriptase and protease (De Clercq, 2002). Notwithstanding their relative success in reducing morbidity, these drugs are not suited to prevent HIV entry into cells. In this respect, the first events in the HIV cycle, the adsorption and fusion steps, are receiving increasing attention (Blair et al., 2000; Stephenson, 2002). HIV entry into host cells depends on the sequential interaction of the gp120 envelope glycoprotein with the cellular receptor CD_4 (Wu et al., 1997) and one of the two co-receptors CCR5 and CXCR4 (Feng et al., 1996; Moore, 1997). Then, the gp41 glycoprotein that is non-covalently associated to gp120 undergoes conformational changes and mediates virus–cell membrane fusion (Jones et al., 1998; Sullivan et al., 1998).

The area involved in co-receptor binding has been identified (Kwong et al., 1998; Wyatt et al., 1998). It comprises highly positively charged regions including the gp120 V3 loop and conserved elements adjacent to this loop (Rizzuto et al., 1998). The HIV replication in vitro has long been shown to be inhibited by polyanions (Witvrouw et al., 1994) such as sulfated polysaccharides (Baba et al., 1988; Mitsuya et al., 1988; Schols et al., 1990), carboxylic derivatives (Neurath et al., 1991), oligonucleotides (Stein et al., 1993; Wyatt et al., 1994; Agatsuma et al., 1996) and anionic porphyrins (Neurath et al., 1992). The activity of these polyanions was found to depend on their number of charges (Witvrouw et al., 1994) suggesting binding to the V3 loop via electrostatic interactions. More recent analyses of the electrostatic potential of gp120 confirmed the key role of the V3 loop region (Kwong et al., 2000; Moulard et al., 2000). Although initial binding might depend on charge density (Kwong et al., 2000), specific structural interactions

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have also been shown to be important for some sulfated polysaccharides (Nishimura et al., 1998) and porphyrins (Debnath et al., 1994; Neurath et al., 1995).

In addition to five variable regions (V1–V5), gp120 possesses five conserved regions (C1–C5) (Starcich et al., 1986). Mutations in the C5 region lead to an almost complete dissociation of gp120 from gp41 (Helseth et al., 1991). Interacting sequences have been identified (Lopalco et al., 1993). As the virus–cell fusion depends on critical contacts between gp120 and gp41 (Maerz et al., 2001), the C5 region would be a good target for antiviral drugs. However, this region is poorly exposed in native viruses (Moore et al., 1994).

Because of its key role in the first step of the HIV infection cycle, gp120 is a potential target for prophylactic treatment and biological fluid decontamination. Although the safety of blood transfusion has been significantly improved during the past decade (Dodd et al., 2002), this remains a problem especially in developing countries (Moore et al., 2001). Photodynamic treatments that involve the generation of very short-lived reactive species upon light irradiation of a photosensitizer (Spikes, 1982) have been considered for decontamination of plasma (Mohr et al., 1997) and platelet concentrates (Margolis-Nunno et al., 1997). Porphyrins (Matthews et al., 1991; North et al., 1994), chlorins (Grandadam et al., 1995) and phthalocyanines (Margolis-Nunno et al., 1996) have been shown to inactivate HIV upon excitation by light. However, treatment of blood products faces the challenge of elimination of pathogens without affecting the function of transfused components. This could be achieved by better targeting of the photosensitizer (Vever-Bizet et al., 1999).

Although some anionic porphyrins have been shown to interact with the V3 loop of gp120 and to inhibit HIV replication, little attention has been paid to the activity of these compounds upon activation by light (Neurath et al., 1992, 1995; Debnath et al., 1994). In the present study, specific interactions in the dark of a series of anionic porphyrins with gp120, as well as the light-induced effects, were investigated by using two antibodies directed toward the V3 loop and a positively charged epitope in the C5 region, respectively.

2. Materials and methods

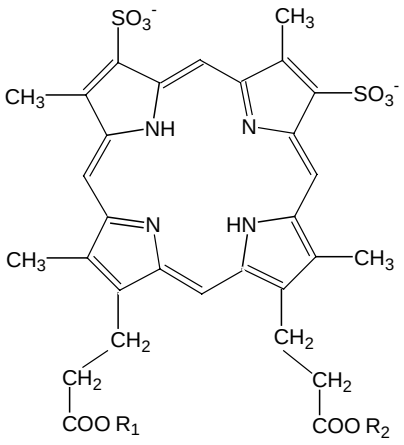
2.1. Chemistry

The structures of the porphyrins used in the present study are shown in Fig. 1. Deuteroporphyrin disulfonic acid (DS) and its dimethyl ester derivative (DS-DM) were purchased from Porphyrin Products (Logan, UT). The ester was found to be contaminated by mono- and non-esterified derivatives and was purified by anion exchange chromatography (Dairou et al., 2002). Deuteroporphyrin DS monomethyl ester (DS-MM), two regioisomers, noted (a) and (b) and diethyl ester (DS-DE) were prepared from DS-DM and purified as described elsewhere (Dairou et al., 2002). The concentration of these compounds was expressed in terms of porphyrin unit, even when they were dimerized (see Section 3). Dulbecco phosphate-buffered saline (PBS) adjusted to pH 7.4 was purchased from Life Technologies (Paisley, UK).

2.2. Light irradiation

The optical spectra were recorded by using an Uvikon 923 (Bio-Tek, Milano, Italy).

Irradiations were carried out using a 1000 W mercury–xenon arc mounted in a lamp housing equipped with a rear reflector and a 3-in. diameter fused silica condenser of *f*/0.7 aperture (Oriel, Statford, CT). After elimination of infrared light by a water filter, the beam was focused at the entrance of a monochromator (Bentham M300, Reading, UK). The entrance and exit slits were set at 5 mm. The mercury spectrum lines at 405 or 366 nm were selected. The exit beam was reflected to the bottom by a 45° mirror. A secondary 250 mm focal lens was used to produce an almost collimated beam. It was possible to irradiate, from



Porphyrin	R1	R2	Charge	Kd (M^{-1})
DS	H	H	4	$1.42 \pm 0.03 \times 10^{6}$
DS – MM a	H	CH_3	3	$1.02 \pm 0.1 \times 10^{7}$
DS – MM b	CH_3	H	3	$1.05 \pm 0.03 \times 10^{7}$
DS – DM	CH_3	CH_3	2	$4.45 \pm 0.06 \times 10^{7}$
DS - DE	C_2H_5	C_2H_5	2	$7.78 \pm 0.2 \times 10^{7}$

Fig. 1. Structure and dimerization constant (K_d) of the compounds used.

above, 16 wells of a 96-well plate with homogeneous light distribution. The uniformity of light intensity was controlled by scanning the working area with a power meter (Scientech, Boulder, CO) equipped with a 1.5 cm diameter diaphragm. Typical light intensities were 2.62 mW cm^{-2} at 405 nm and 4.65 mW cm^{-2} at 366 nm corresponding to incident flux of photons of 5.34×10^{15} photons cm^{-2} and 8.57×10^{15} photons cm^{-2}, respectively. These intensities were reduced with the appropriate optical neutral glass filters (Schott, Mainz, Germany) when necessary. The light intensity was controlled before each set of experiments.

2.3. Virology

Recombinant HIV-1 gp120 IIIB purchased from Intracel Corporation (Frederick, MD) was produced in a baculovirus expression system. The murine IgG1 anti-gp120 monoclonal antibody (mAb) 13105100 was purchased from ABI (Columbia, MD). It was raised against the peptide IRIQRGPGRAFVTI corresponding to the sequence of the V3 loop flanking the GPGR apical motif. The mAb 9201 (Du Pont de Nemours, Boston, MA), raised against the peptide MRDNWRSELIKY, was directed to an epitope located on the α5 helix in the C5 terminal region of gp120. An anti-mouse goat IgG antibody conjugated to horseradish peroxidase (Bio-Rad, Benicia, CA) was used to reveal the binding of the mouse antibody with gp120. The peroxidase substrate was 3,3′,5,5′-tetramethylbenzidine (TMB). The one-step turbo TMB-ELISA kit from Pierce (Rockford, IL) was used.

Enzyme-linked immunosorbent assays (ELISA) were performed using 96-well microtiter plates (Immulon 4, Dynex Technologies Inc, Chantilly, VA). The procedure to reveal binding of antibodies to gp120 was as follows. The plates were coated with 50 μl of a gp120 solution (1 μg/ml) in PBS. After 2 h at room temperature, the wells were washed three times with PBS containing 0.05% Tween-20 (PBS-Tween) and postcoated overnight at 4 °C with 150 μl of 2% non-fat dry milk solution in PBS, as a blocking agent. Then, the wells were washed three times with PBS-Tween. Control wells were treated in the same way without added gp120. Then, 50 μl of a solution of mAb 13105100 or mAb 9201 in PBS were added. After 15-min incubation at 20 °C, the wells were washed three times before the addition of 50 μl of a 1/1000 solution of goat anti-mouse IgG antibody conjugated to horseradish peroxidase. After 60 min, the wells were washed and 50 μl of the TMB solution were added according to the Manufacturer's recommendation. The enzyme reaction was allowed to progress at 20 °C for 5 min before it was stopped by addition of 50 μl of 1 M sulfuric acid. The binding of mAbs was quantified by measuring absorbance at 450 nm, with an ELISA plate analyzer (Dynatech Laboratories, Chantilly, VA). Typical absorbance measurements versus the mAb concentration are reported in Fig. 2. Saturation curves with half values of 0.075 and 0.167 μg/ml are observed for mAb 13105100 and mAb 9201, respectively.

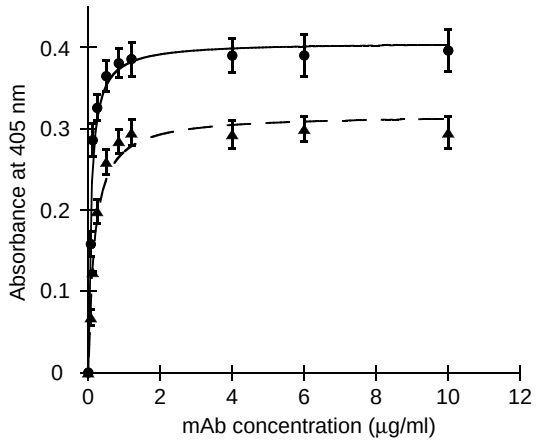


Fig. 2. Antibody binding to gp120 measured by ELISA. The absorbance of the TMB product is plotted vs. the concentration of mAb directed to gp120 according to the protocol described in the text: (●) mAb 13105100; (▲) mAb 9201. Data are fitted by a function of the form $y = ax/(x + b)$ where a corresponds to the absorbance value obtained at saturation and b is the mAb concentration for half-saturation (∼mAb dissociation constant; 0.075 and 0.167 μg/ml for mAb 13105100 and mAb 9201, respectively). The absorbance values are corrected for controls. In both cases, the absorbance of the controls was 0.05 ± 0.01.

The experiments involving porphyrins were carried out as follows. Solutions of porphyrins in PBS (25 μl) were added to control and gp120-coated wells and allowed to react at room temperature. After incubation, the plates were irradiated by monochromatic light or kept in the dark for the same time. In most experiments, the incubation and irradiation times were 20 and 10 min, respectively. Then, mAb 13105100 or mAb 9201 was added. In most cases, the mAb concentration was 1 μg/ml, a value that corresponds to the beginning of the plateau of the curves shown in Fig. 2. In some experiments, the mAb concentration was increased to 10 μg/ml in order to check competition between antibodies and porphyrins. Two protocols were used for dark or light irradiation experiments. In the first protocols (D_I, L_I), the porphyrin solution was left in contact with the anti-gp120 antibodies. In the second protocols the porphyrin was removed before mAb addition (D_{II}, L_{II}). In some experiments, three washes were added at this step. The volume and concentration of the added mAb solutions were adjusted so that the final experimental conditions were the same for the two protocols. After 15-min incubation at 20 °C, the wells were washed three times and processed as indicated above. Experiments were performed at least in triplicate. The protocols and the epitope of monoclonal antibodies are recapitulated in Fig. 3.

3. Results

3.1. Solution properties of sulfonated deuteroporphyrins

The sulfonated porphyrins with the structure depicted in Fig. 1 have been shown to dimerize in aqueous solutions

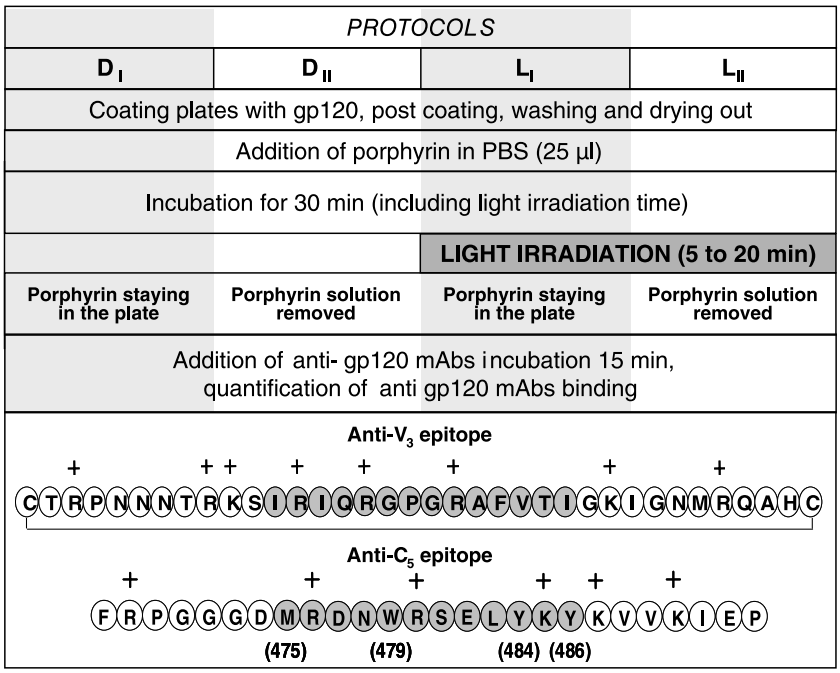


Fig. 3. (Top) Successive steps in ELISA protocols used for dark and light irradiation experiments. (Bottom) Amino acid sequences of the gp120 V3 loop and the C terminal domain for the HIV-1 strain IIIB. The filled symbols indicate the linear epitopes of 13105100 and 9201 monoclonal antibodies. Positively charged amino acids are marked by the symbol (+). Photosensitive side chains are numbered.

according to the equilibrium (Dairou et al., 2002),

$$2M \leftrightarrows D \tag{1}$$

The concentrations of the monomer [M] and the dimer [D] are related by the equilibrium constant, K_d:

$$K_d = \frac{[D]}{[M]^2} \tag{2}$$

The total concentration of the porphyrin being equal to

$$C = [M] + 2[D] \tag{3}$$

straightforward calculations lead to

$$[M] = \frac{\{-1 + (1 + 8K_dC)^{1/2}\}}{4K_d} \tag{4}$$

$$[D] = \frac{\{1 + 4K_dC - (1 + 8K_dC)^{1/2}\}}{8K_d} \tag{5}$$

The dimerization constants for the various porphyrins have been determined previously in PBS (Dairou et al., 2002). They are given in Fig. 1. The relative amounts of monomer and dimer for the typical concentrations used in the present study have been calculated according to Eqs. (4) and (5). They are plotted as a function of the total porphyrin concentration in Fig. 4.

The typical absorption spectra of the monomer and dimer forms are given in Fig. 5. Full monomerization can be achieved for DS at the lowest concentrations and at very low ionic strength in buffer (Dairou et al., 2002). All the porphyrins exist as monomers in methanol. A sharp absorption band with a maximum at 400 nm (the Soret band) characterizes the monomer. The dimer displays a marked shift to the blue of the Soret band with a maximum at 384 nm.

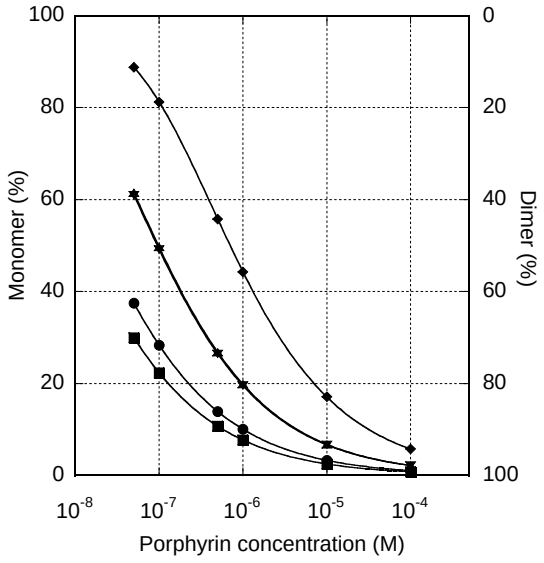


Fig. 4. Relative contributions (%) of monomer and dimer forms of porphyrins as a function of their concentrations in phosphate buffer solutions: (◆) DS; (▼) DS-MMa; (▲) DS-MMb; (●) DS-DM; (■) DS-DE. Curves for DS-MMa and DS-MMb overlap.

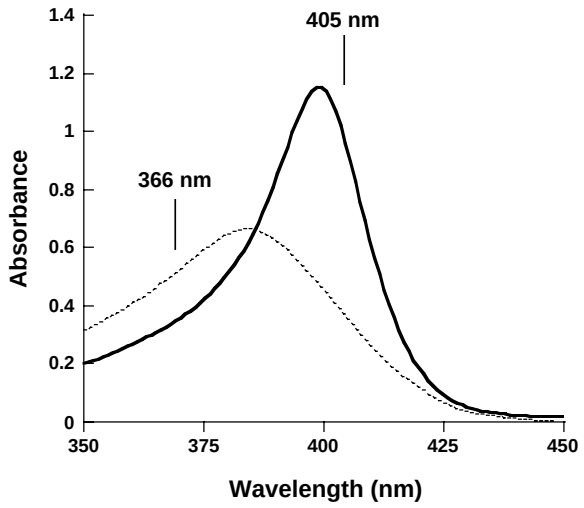


Fig. 5. Absorption spectra of 5×10^{-6} M solutions of DS-DM: (- - -) dimer in PBS; (—) monomer in methanol. The wavelengths used for irradiation experiments are indicated.

3.2. Protocols for measurements of antibody binding inhibition

The inhibition of anti-gp120 mAb binding by porphyrins may arise from various mechanisms. In the dark, the porphyrin can compete with the antibody for binding to its epitope. This process is expected to be reversible to some extent. Under light, amino acids of gp120 can be damaged by reactive species produced through the excitation of the porphyrin. However, photochemical modification of the porphyrin itself cannot be excluded. Then, possible contribution of photoproducts must be checked. Four protocols have been designed, accordingly. As shown in Fig. 3, the porphyrin was either left, or not, in contact with the anti-gp120 antibody. The two protocols used in the dark experiments were designed with two objectives. The elimination of porphyrin in protocol D_{II} allowed us to check possible interaction of the porphyrin with the anti-gp120 antibodies. It was also possible to estimate the reversibility of the attachment of

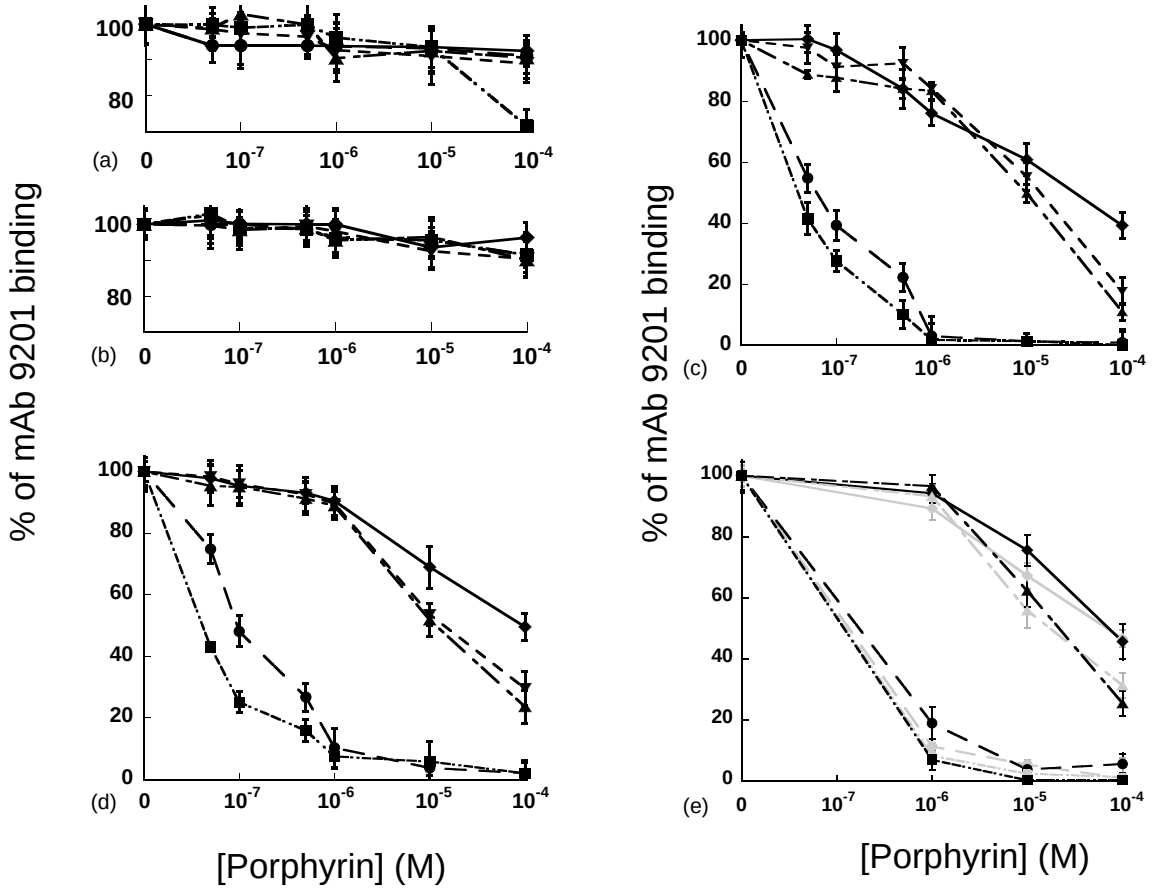


Fig. 6. Inhibition of mAb 9201 binding by porphyrins: (a and b) experiments carried out in the dark according to protocols D_I and D_{II}, respectively; (c and d) light irradiation experiments (10-min irradiation at 405 nm) according to protocols L_I and L_{II}, respectively; (e) light irradiation experiments according to protocols L_{II} including three washes with PBS-Tween after light irradiation but before 9201 addition. (◆) DS; (▼) DS-MMa; (▲) DS-MMb; (●) DS-DM; (■) DS-DE. In panel e, results for experiments carried out according to protocol L_{II} but without additional washing are depicted as grey lines for the sake of comparison. Error bars represent standard deviations on the means.

the porphyrin to gp120. Protocol L_{II} was designed to check possible role of porphyrin photoproducts.

3.3. Inhibition of antibody binding in the dark

As shown in Fig. 6a and b, the binding of mAb 9201 was not significantly inhibited by the various disulfonated porphyrins in the dark. Only a very small effect can be noted with the highest concentration of DS-DE in protocol D_I. On the other hand, as shown in Fig. 7a, a significant inhibition of mAb 13105100 binding was observed with protocol D_I. The inhibition was a function of porphyrin concentration. The efficiency was found to depend on the porphyrin structure in the order DS-MMb ∼ DS ∼ DS-MMa < DS-DM < DS-DE. Although it was slightly reduced, inhibition was maintained in protocol D_{II} (Fig. 7b), indicating that direct interaction of the porphyrins with the antibody was not responsible for the observed effect. Extensive washing of the

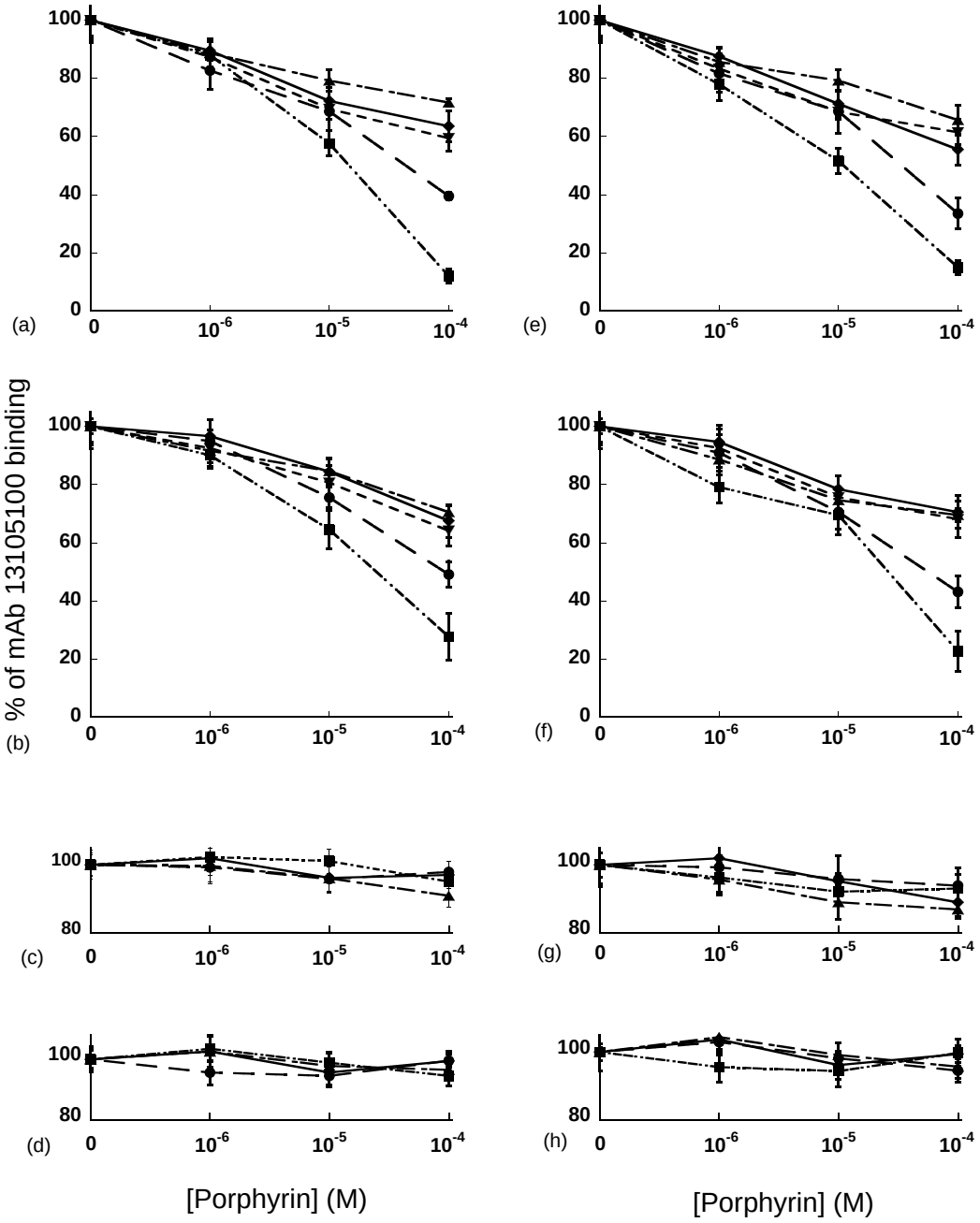


Fig. 7. Inhibition of mAb 13105100 binding by porphyrins: (a–d) experiments carried out in the dark according to protocols D_I, D_{II}, including three additional washes with PBS-Tween before mAb 13105100 addition or with excess of mAb 13105100 (10 μg/ml), respectively; (e–h) light irradiation experiments (10-min irradiation at 405 nm) according to protocols L_I, L_{II}, including three additional washes with PBS-Tween before mAb 13105100 addition or with excess of mAb 13105100 (10 μg/ml), respectively. (◆) DS; (▼) DS-MMa; (▲) DS-MMb; (●) DS-DM; (■) DS-DE. Error bars represent standard deviations on the means.

plates following removal of the porphyrin solution restored a full capacity of mAb 13105100 to bind gp120 (see Fig. 7c). Moreover, when the mAb 13105100 concentration was increased, the level of the peroxidase assay was progressively restored, indicating competition between the porphyrin and mAb 13105100. Results with excess antibody (10 μg/ml) are shown in Fig. 7d.

3.4. Inhibition of antibody binding after light irradiation

Irradiation by light of the gp120-coated plates incubated with the porphyrins was performed before the addition of antibodies as indicated in Fig. 3. The porphyrins were excited in the Soret region at 405 nm. As illustrated in Fig. 6c, a significant inhibition of mAb 9201 binding was observed. The effect was porphyrin dose dependent. The efficiency of the porphyrins was significantly improved by esterification. Both the number of carboxylic chains esterified and the length of the ester chain appeared to be important as shown by the order of efficiency: DS < DS-MMa ∼ DS-MMb < DS-DM < DS-DE. The efficiency of DS-DE was remarkable with 50% inhibition of mAb 9201 binding at a concentration of 40 nM.

The results obtained with protocols L_I and L_{II} were fairly similar, with only a small reduction of activity upon removal of the porphyrin (see Fig. 6d). This excludes a direct effect of the porphyrin on the mAb itself. In the same way, contribution of stable photoproducts eventually formed from porphyrin can be ruled out. Extensive washing of the plates with PBS-Tween before light irradiation restored full capacity of mAb 9201 binding (data not shown). This indicates that the porphyrin must be in contact with gp120 in order to be active, but the binding appears to be reversible. On the other hand, extensive washing performed after light irradiation did not reduce the efficacy of the porphyrin, which indicates that permanent damages are produced by irradiation (see Fig. 6e).

The role of activation by light in promoting the activity of the photosensitizers is well demonstrated by changing either the irradiation time or the intensity of the flux of incident photons, as shown in Fig. 8.

The results obtained with mAb 13105100 were very different. As shown in Fig. 7e and f, the inhibition of mAb 13105100 binding was not amplified after irradiation. As shown in Fig. 7g and h, extensive washing after irradiation or the use of mAb 13105100 in excess did not reveal any permanent damage.

3.5. Identification of the active photosensitizer form

As outlined above, the disulfonated porphyrins dimerized in aqueous solution. Then, the following question arises: is the dimer or the monomer form responsible for the observed effect? As the absorption spectra of the dimer and monomer forms differ significantly (see Fig. 5), the answer can be found by changing the wavelength of the irradiation light. It

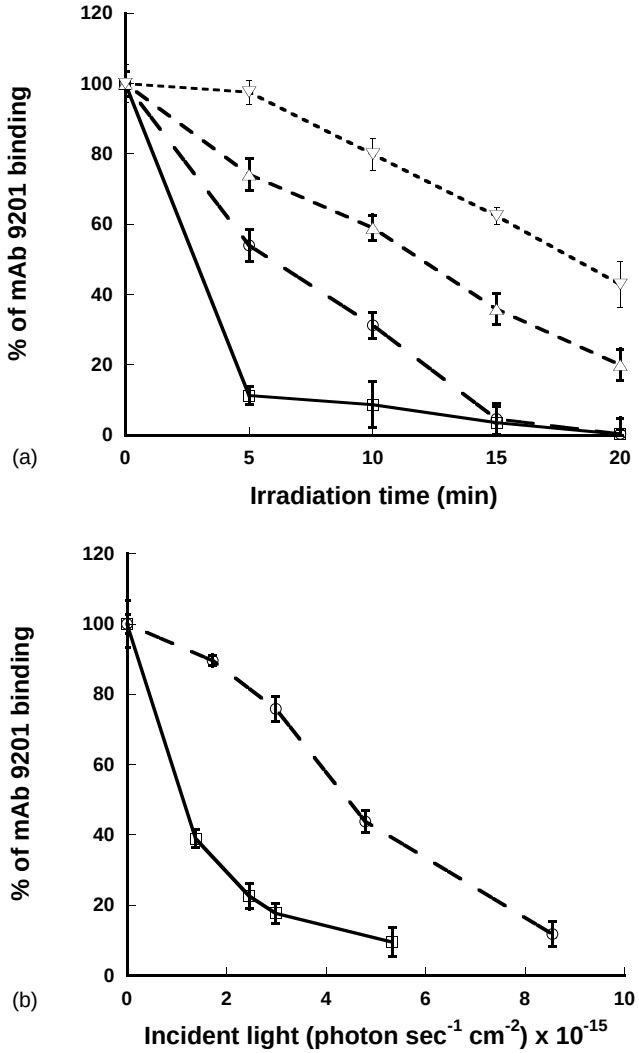


Fig. 8. (a) Inhibition of mAb 9201 binding to gp120 by DS-DM vs. the light irradiation time; porphyrin concentrations: (▽) 5×10^{-8} M; (△) 1×10^{-7} M; (○) 5×10^{-7} M; (□) 1×10^{-6} M. (b) Inhibition of mAb 9201 binding by DS-DM vs. incident light intensity: (○) 366 nm; (□) 405 nm. Protocol L_{II} was used in panels a and b.

can be assumed that for each wavelength, λ, the number of photons absorbed by the photosensitizer targeted to gp120 $N_{abs}(\lambda)$ is proportional to the number of incident photons per time unit, $N_{inc/t}(\lambda)$, to the irradiation time, t, and to the porphyrin molecular absorption coefficient, $\varepsilon(\lambda)$. This assumption is valid until no screen effect arise from the porphyrin solution contained in the well (Cantor and Schimmel, 1980), which is true with the volume of solution used in the present study, at least for concentrations up to 10^{-5} M. Then,

$$N_{abs}(\lambda) = \alpha N_{inc/t}(\lambda) t \varepsilon(\lambda) \quad (6)$$

where, α is a proportionality factor independent of the wavelength.

We selected two wavelengths, 405 and 366 nm, corresponding to intense emission mercury lines of our arc lamp. As shown in Fig. 5, these wavelengths correspond

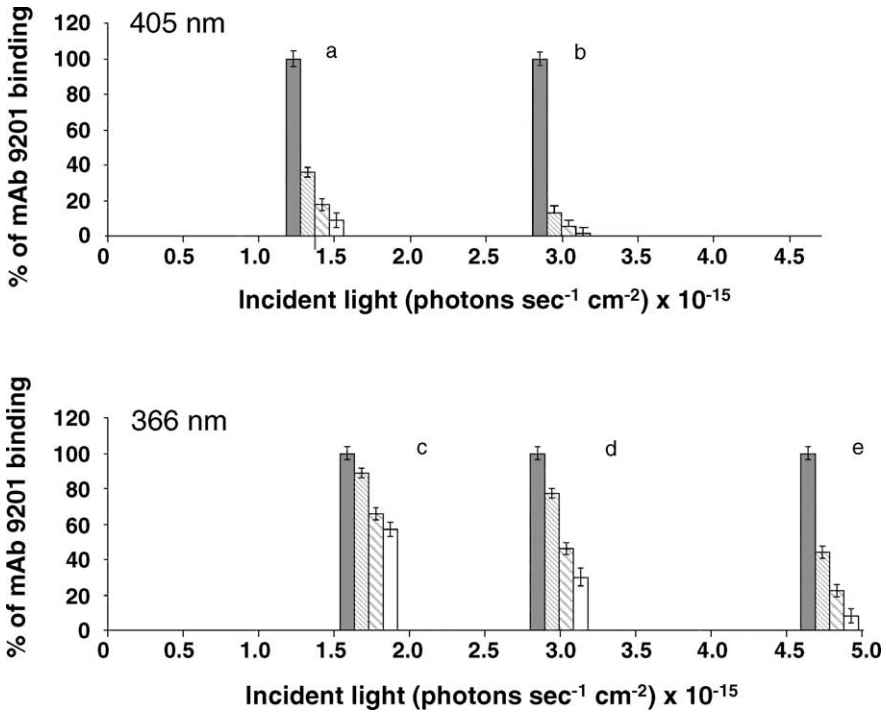


Fig. 9. Inhibition of mAb 9201 binding to gp120 by DS-DM after light irradiation at two wavelengths for various fluxes of incident photons. Top: 405 nm; bottom: 366 nm. The porphyrin concentrations are: 0, filled bars; 1×10^{-6} M, finely hatched bars; 1×10^{-5} M, broadly hatched bars; 1×10^{-4} M, open bars. The meaning of symbols a–e is given in the text. For each bar quadruplet, the same flux was used. The exact value is given by the center of the quadruplet. Protocol L_{II} was used.

to a better absorption of either the monomer or the dimer form. In a first series of experiments, the light fluxes at the two wavelengths were adjusted to the same value by using appropriate neutral filters. The irradiation time was set at 10 min. In these conditions, the ϵ value of the active form that determines the number of absorbed photons would control the observed effect. The results for DS-DM are shown as profiles "b" and "d" in Fig. 9. Whatever was the porphyrin concentration, the irradiation at 405 nm was more efficient. This suggests that the monomer form was involved despite the fact that the incubation solution mostly contained dimers.

A second series of experiments was designed. The purpose was to measure the relative effect of the two wavelengths and to compare the results to what would be predicted if either the monomer or the dimer would be involved. In order to check these two hypotheses without interference with other parameters, the light fluxes were adjusted so that the same number of photons would be absorbed by the postulated active species at each wavelength. According to relation (6), the values of both the incident light flux, $N_{\mathrm{inc}/t}(\lambda)$, and the molar extinction coefficient $\varepsilon(\lambda)$ must be taken into account. In the case of the monomer, the molar extinction coefficient at 405 nm is higher (see spectra shown in Fig. 5). The value of the ratio $R_{\mathrm{mon}} = \varepsilon(405)/\varepsilon(366)$ is 3.60. Then, in order to test the monomer hypothesis, two series of samples were irradiated. One was irradiated with an incident flux of photons at 405 nm of 1.38×10^{15} photons s^{-1} cm^{-2}, the other was irradiated at 366 nm with 4.97×10^{15} photons s^{-1} cm^{-2}. By using the ratio of molar extinction coefficient, R_{mon}, it can be easily assessed that the number of absorbed photons will be the same at the two wavelengths. As a consequence, if the monomer hypothesis is true, the profile of mAb binding inhibition at the two wavelengths should be identical. The same reasoning was used for the dimer hypothesis. In this case, $R_{\mathrm{dim}} = \varepsilon(405)/\varepsilon(366)$ was 0.58. The values of the incident light fluxes leading to the same quantity of absorbed photons would be 2.99×10^{15} photons s^{-1} cm^{-2} and 1.72×10^{15} photons s^{-1} cm^{-2} at 405 and 366 nm, respectively.

The profiles corresponding to the monomer hypothesis are identified by "a" and "e" in Fig. 9. The irradiation conditions marked "b" and "c" were used to check the dimer hypothesis. The monomer hypothesis appears to be right. In order to further sustain this conclusion, we used the Kolmogorov–Smirnov statistic test to compare the couples of inhibition profiles for the two wavelengths (Siegel and Castellan, 1988). This test determines if samples come from a single population or from two different populations. The test showed that the two samples corresponding to the dimer hypothesis are different with a reliable interval of 99%. On the other hand, the two samples corresponding to the monomer hypothesis were not significantly different. Thus, the monomer form of the porphyrin is responsible for the light-dependent inhibition of mAb 9201 binding to gp120.

4. Discussion

Continuous efforts are developed to find new strategies that could complement the existing anti-HIV drug cocktails and/or would be suitable for prophylactic approaches. In line with these objectives, we investigated the feasibility of targeting the glycoprotein gp120 by anionic photosensitizers. These molecules can operate via two mechanisms. An effect in the dark arising from the reversible attachment of the molecule to glycoprotein sites is expected to alter its function. This mechanism is common to other drugs. A second effect can be triggered upon activation of the photosensitizer by light. This induces the formation of very active species able to damage some amino acids irreversibly.

A series of disulfonated deuteroporphyrins with different overall negative charges were selected. Two monoclonal antibodies were used. Their interactions with gp120 in the dark or after photoactivation of the porphyrin was monitored by ELISA. One antibody (mAb 13105100) was directed against the V3 loop, a major determinant of the association of the virus with co-receptors. The other (mAb 9201) was raised against the C5 region that is critically involved in the association of gp120 with gp41. It is worth noting that the epitopes of the two antibodies comprise several positively charged residues making possible electrostatic interactions with the porphyrins.

Despite the presence of positive charges on both epitopes, inhibition of antibody binding by the disulfonated porphyrins is observed only for the V3 loop in the dark. Similar results were described for a tetracarboxylic porphyrin that reacted selectively with a V3 mimicking peptide among a series of peptides mapping gp120 (Neurath et al., 1993). The V3 loop is also the major determinant of interaction of gp120 with other polyanions (Moulard et al., 2000). The affinity of these polyanions for gp120 was not modified by deletion of the C5 region (Moulard et al., 2000). A molecular model indicated that the charge of the V3 loop, but not its precise structure, dominates the electrostatic potential of the conserved co-receptor binding region. It was suggested that the interaction with polyanions mostly depends on the charge density rather than a particular motif (Kwong et al., 2000). Careful analysis of interactions of series of porphyrins with gp120 did confirm the presence of electrostatic bonds. However, three negative chains on the porphyrin macrocycle were sufficient to provide the best efficacy (Debnath et al., 1994). Large evidence for interactions with hydrophobic residues was also provided (Neurath et al., 1995).

The efficiency of our disulfonated porphyrins to inhibit the binding of the anti-V3 antibody in the dark increased upon reduction of their negative charge to two. Also, esterification of the carboxylic groups by the more hydrophobic ethyl chains was favorable. This reinforces the hypothesis that interactions of porphyrins with the V3 loop is optimized by the presence of both anionic and hydrophobic groups (Debnath et al., 1994). However, the propensity of the porphyrins to self-associate follows the same order. The increase of charge density that accompanies dimerization might also play a role.

It should be pointed out that the interaction of the porphyrins with the V3 loop is reversible. Indeed, the inhibition of mAb binding is abolished through the use of antibody in excess or by extensive washing of the microplates before antibody addition (see Section 3).

In contrast to results obtained for the V3 loop, the interaction between the disulfonated porphyrins and the C5 region was found to be extremely weak.

Irradiation of photosensitizers by light in presence of molecular oxygen induces the formation of reactive oxygen species with deleterious effects on biological systems, a phenomenon known as photodynamic action (Spikes, 1982). The photosensitizer raised to the triplet state can either react by electron transfer with surrounding molecules to yield radical species (type I mechanism) or transfer its energy to molecular oxygen to produce singlet oxygen (type II mechanism) (Foote, 1968). Disulfonated porphyrins have been shown to be good singlet oxygen generators, even when they are dimerized (Dairou et al., 2002). Among the various amino acids, only five, namely cysteine, histidine, methionine, tryptophan and tyrosine are vulnerable to sensitized photo-oxidation (Smith, 1989; Bensasson et al., 1993). Chemical alteration involves the side chains of these amino acids. The disulfide bridges and peptide links in proteins are usually not broken upon photo-oxidation. In type I mechanism, the photosensitizer and the amino acid chain to be altered must be within a few angstroms unless electron transfer could proceed by jumps involving several side chains arranged adequately. During its lifetime, around 0.1 μs in biological systems, singlet oxygen can travel over a distance of a few hundreds of angstroms (Moan and Berg, 1991; Niedre et al., 2002).

Light irradiation had no effect on the inhibition of anti-V3 antibody binding, as shown in Fig. 7e and f. Furthermore, extensive washing of the microplates after exposure to light restored full capacity of the antibody to recognize its epitope (see Fig. 7g) with no evidence for permanent damages. In fact, the V3 loop of gp120 IIIB does not contain photosensitive amino acids (see Fig. 3). The presence of photosensitive residues in the C5 epitope makes it more susceptible to attack by reactive oxygen species (see Fig. 3). Irreversible damages are produced within this epitope, accordingly. It should be noted that these photosensitive residues are highly conserved (Kwong et al., 1998).

The monomer forms of the disulfonated porphyrins were clearly identified as the photo-active species from irradiation experiments performed at two wavelengths. The observed photodamages cannot arise from reactive species produced in solution. Indeed, as shown in Fig. 4, the porphyrins in the incubation solution exist predominantly as dimers, especially for the most active compounds. It can be concluded that a site with a high affinity for porphyrin monomers exists on gp120. The curves shown in Fig. 2 indicate that the affinity of the two antibodies for their target is similar. The

dissociation constant of mAb 9201 is even somewhat higher. Then, the absence of an effect in the dark of porphyrins on the binding of mAb 9201 cannot be due to an unfavorable competition. As a consequence, it is unlikely that the high affinity site for porphyrins belongs to the C5 region. It must be pointed out that the effects in the dark on the V3 loop and light-induced damages to the C5 epitope are quite similar if we consider the porphyrin efficacy. This might suggest that the same site is involved. It could be hypothesized that reactive species such as singlet oxygen produced by porphyrins bound to the V3 loop could react with amino acids distant by several dozen of angstroms. This process could involve diffusion of singlet oxygen near the surface of the glycoprotein or through the protein matrix. Indeed, various studies have shown that proteins do not impose a significant barrier for the diffusion of small molecules such as oxygen (Coppey et al., 1981; Papp et al., 1991). Conformational fluctuations of the polypeptide chain on nanosecond to microsecond timescales can open channels through which small molecules migrate to other sites (Ostermann et al., 2000). It can be assumed that the same would be true for singlet oxygen until it finds a photosensitive residue.

In conclusion, disulfonated porphyrins bind competitively to the V3 loop of glycoprotein gp120. Most likely, they are able to induce photodamages to remote sites on the protein. Here, we give an example of light-induced damages to the non-exposed C5 region. Moreover, these damages are likely to involve highly conserved photosensitive residues (Kwong et al., 1998). Then, in addition to usual effects arising from reversible attachment of these molecules to accessible sites, damages might be produced to remote sites. Protein areas not accessible to usual drugs could be altered. Hence, owing to diffusion of the active species produced by the photoactive drugs, non-exposed regions with critical functions but less subjected to mutation might be attained. Obviously, screening of the damages on various functionally important sites on gp120 is necessary to fully support this attractive concept. The photoinactivation of HIV-1 virions by these anionic porphyrins remains to be checked. These points will be addressed in further studies. Photodecontamination of cellular blood components has received increasing attention in the past years. Psoralens that make adducts with nucleic acids upon ultraviolet light activation have been used in randomized clinical trials (van Rhenen et al., 2003). The production by porphyrins of active species able to diffuse at short distance offers an alternative mechanism. Targeting pathogen proteins is expected to improve the efficacy of the treatment, while preserving functions of the blood components. Also, this class of photosensitizers would be devoid of any mutagenic potential, a concern with drugs interacting with nucleic acids (Rapp and Kemeny, 1977). Besides, porphyrins and related compounds can be excited by red light, an advantage to treat red blood cells. All these features make porphyrins and related compounds attractive candidates for photodynamic treatment in prophylactic approaches of viral infections.

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