doi: 10.4149/gpb_2015003

# Structure and binding efficiency relations of $Q_B$ site inhibitors of photosynthetic reaction centres*

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**Abstract.** Many herbicides employed in agriculture and also some antibiotics bind to a specific site of the reaction centre protein (RC) blocking the photosynthetic electron transport. Crystal structures showed that all these compounds bind at the secondary ubiquinone ($Q_B$) site albeit to slightly different places. Different herbicide molecules have different binding affinities (evaluated as inhibition constants, $K_I$, and binding enthalpy values, $\Delta H_{bind}$). The action of inhibitors depends on the following parameters: (i) herbicide molecular structure; (ii) interactions between herbicide and quinone binding site; (iii) protein environment. In our investigations $K_I$ and $\Delta H_{bind}$ were determined for several inhibitors. Bound herbicide structures were optimized and their intramolecular charge distributions were calculated. Experimental and calculated data were compared to those available from databank crystal structures. We can state that the herbicide inhibition efficiency depends on steric and electronic factors, i.e. geometry of binding with the protein and molecular charge distribution, respectively. Apolar bulky groups on N-7 atom of the inhibitor molecule (like t-buthyl in terbutryn) are preferable for establishing stronger interactions with $Q_B$ site, while such substituents are not recommended on N-8. The N-4,7,8 nitrogen atoms maintain a larger electron density so that more effective H-bonds are formed between the inhibitor and the surrounding amino acids of the protein.

**Key words:** Herbicides — Bacterial reaction centers — Photosynthesis — Molecular modeling

## Introduction

Specific classes of chemicals, known as herbicides, are widely used in agriculture as weed killers. Some of them are classified as "photosynthetic herbicides": indeed they block the photosynthesis of plants, as well as that of many algae and bacteria (Wraight 1981; Paddock et al. 1988), due to the high structural and functional similarities of photosynthetic apparatus in these organisms (Wraight and Stein 1983; Stein et al. 1984; Michel and Deisenhofer 1988; Barber 1993; Cardona et al. 2012). Although it has already been well studied for a couple of decades, the mode of action of these compounds still remains in the focus of many publications and research interests, either from academic groups or industries. The reasons for attracting such an attention are summarized below.

Herbicides represent useful probes for understanding the structural and functional details of charge separation and stabilization processes within the photosynthetic machinery of many organisms. As an example, atrazine and terbutryn (typical representatives of photosynthetic herbicides), were cocrystallized after binding to the secondary electron acceptor side of the photosynthetic reaction center protein (RC) (Brookhaven Protein Data

*This article was presented at the Regional Biophysics Conference, organized by The Slovak Biophysical Society in Smolenice, May 15–20, 2014.

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Bank: 2PRC – RC/ubiquinone, 1DXR – RC/terbutryn, 5PRC – RC/atrazine complexes) (Lancaster and Michel 1999; Lancaster et al. 2000). Indeed, the bacterial RC hosts fundamental photosynthetic events taking place after light excitation, such as primary charge separation and stabilization (Sebban et al. 1995; Allen and Williams 1998; Okamura et al. 2000; Paddock et al. 2003). Consequently, any information we may gain about those processes improve our knowledge in the direction of possible green technology applications (e.g. food production, environmental monitoring and ecology, bio-hybrid systems in integrated optoelectronics, as detailed e.g. by Cogdell and Lindsay 1998; Kruse et al. 2005; Jones 2009; Nagy et al. 2010, 2014; Cogdell et al. 2013).

Moreover, triazine herbicides such as atrazine and terbutryn were proved to be harmful for human health (Marchini et al. 1988; Tchounwou et al. 2000). They can accumulate in agricultural products (like fresh fruits, vegetables and milk), in water sources like rivers, lakes, reservoirs and in ground water (Kuang et al. 2003; Quaghebeur et al. 2004), remaining their chemical structures stable for long time (Lányi and Dinya 2003). Herbicides are also referred as able to intercalate into DNA strands resulting in genetic modifications (Riahi et al. 2010). Despite the fact that people are more and more reluctant to the use of chemicals in agriculture (contrary to the attitude of 1960s and 1970s) and that many turned to green technologies, industries still have much interest in designing new and more effective type of chemical structures, including photosynthetic herbicides.

Furthermore, now researchers have got a quite clear picture of the kinetic and thermodynamic stabilization processes related to the electron transport within the RC (Sebban et al. 1995; Allen and Williams 1998; Okamura et al. 2000; Paddock et al. 2003). However, due to the explosive development of computer and information technologies, molecular biology, optoelectronics, photonics and nanotechnologies, this picture is under continuous updating. It is very likely that our general knowledge about the primary electron transfer steps will be again modified, allowing to design new generations of applications in many fields such as optoelectronics (e.g. integrated biohybrid systems (e.g. Dimonte et al. 2012; Hajdu et al. 2012; Nagy et al. 2014a, 2014b), biosensors (Mallardi et al. 2007; Nagy et al. 2014; Swainsbury et al. 2014) and artificial photosynthesis, e.g. photovoltaics (Kamran et al. 2014) or photosynthesis inspired/mimicking systems (e.g. Maaza et al. 2012; Mongwaketsi et al. 2012)).

Moreover, more and more sensitive risk assessments and safety regulations recently stimulated strong efforts towards the specific detection of herbicides in the environment with the minimum possible analytical threshold limit (Giardi et al. 2009). For this purpose, besides classical chemical techniques (e.g. high performance liquid chromatography), alternative biological sensing elements towards herbicides are of special interest. These latter devices exploit the specific interaction between analytes (i.e. different types of herbicides) and sensing elements (e.g. whole cells of photosynthetic organisms, RC proteins, etc.) (Ventrella et al. 2010, 2011; Husu et al. 2013; Scognamiglio et al. 2013; Nagy et al. 2014 and references therein). In particular, bio-nanohybrid systems are greatly promising since they fulfill all the requirements for possible efficient biosensor applications. Indeed, a suitable biosensor should optimize most of the following features: complexity and costs of the method itself (e.g. the required instrumentation, like in high performance chromatography); size of the device (e.g. for portability, etc.); necessary amount of sample; rapidity and reproducibility of response; possibility of on-line, real time detection; sensitivity and selectivity towards target analytes. In this view, our results provide new structural and functional details about the kinetics and energetics of the interaction between several triazine herbicides and RCs isolated from *Rb. sphaeroides*. In particular, experimental data obtained by titration of the RC photochemistry in detergent micelles and artificial lipid vesicles will be compared with data obtained by computer simulation. Therefore our results may represent a valuable contribution to the design of a new generation of herbicide detection biosensors such as sensitive and selective electrochemical and optoelectronic devices.

## Materials and Methods

### *Materials*

Carotenoid-less *Rb. sphaeroides* R-26 cells were grown photoheterotrophically under anaerobic conditions in medium supplemented with potassium succinate (Ormerod et al. 1961). Chromatophores and RCs were prepared as described earlier (Tandori et al. 1995). RCs were solubilized by DDAO (*N*,*N*-dimethyldodecylamine *N*-oxide; Fluka) and purified by ammonium sulfate precipitation followed by DEAE-Sephacel (Sigma) anion-exchange chromatography.

In order to obtain RCs with full and known $Q_B$ site quinone activity for herbicide titration experiments, proteins were prepared by deprivation of native ubiquinone-10 (UQ-10) and subsequent reconstitution through addition of the desired amount of UQ-10. The quinone depletion was carried out according to the method of Okamura (Okamura et al. 1975).

Deprived RCs were concentrated by centrifuge filter (Whatman VectaSpin 3, 10 kDa exclusion) to 80–100 μM. The fractions with OD280/OD803 ratio between 1.27 and

1.50 were collected and used for further experiments. For optical spectroscopy, the RC suspension was diluted to 1 μM in detergent suspension (10 mM TRIS, 100 mM NaCl, 0.01% DDAO, pH 8.0 buffer). The $Q_B$ site of RCs was reconstituted with native UQ-10 addition just before herbicide titration experiments (see below).

*Proteoliposome preparation*

RC-containing vesicles (proteoliposomes) were prepared according to Giustini et al. (2005). Depending on the desired lipid in the sample, pure phosphatidylcholine (PC, 4.1 mg), phosphatidylglycerol (PG, 4.0 mg) or cardiolipin (CL, 4.0 mg) (all of maximum purity grade from Sigma) were dissolved in chloroform and dried under $N_2$ flow. Then the lipid film was resuspended in 5 mM KCl, 5 mM phosphate pH 7.0 buffer and sodium cholate 4% solution in the same buffer enough to reach a minimum ~5 cholate/phospholipid molar ratio (in order to obtain uniform monodispersed mixed lipid-detergent micelles). After short sonication until clarity, RCs (dispersed in 10 mM TRIS, 100 mM NaCl, 0.01% DDAO, pH 8.0) were added to the suspension. Subsequent detergent removal by elution through a Sephadex G-50 (Pharmacia) column with 5 mM KCl, 5 mM phosphate pH 7.0 buffer led to RC-containing small unilamellar vesicles. Both the presence and concentration of RCs in the liposomes were checked by measurement of the 802 nm absorption band: in every experiment the RC concentration was constant in the (2.2 ± 0.2) μM range. Finally, the amount of UQ-10 necessary to reconstitute the $Q_B$ site at a minimum 0.90 occupancy fraction was added as aqueous solution in the presence of 30% TX-100, always checking for the slow phase fraction in the analysis of charge recombination measurements.

*Kinetic spectrophotometry*

In order to reach an UQ-10/RC 2÷4 molar ratio and to reconstitute the $Q_B$ site at a minimum 0.90 occupancy free UQ-10/Triton X-100 30% was added to the RCs solubilized in aqueous DDAO micelles (at 1.0 ± 0.1 μM constant protein concentration in 10 mM TRIS, 100 mM NaCl, 0.01% LDAO, pH 8.0 buffer). The $Q_B$ site occupancy was estimated from the amplitude of the slow phase at low detergent concentration, by means of biexponential fit analysis of kinetic traces, reported hereafter as unity fraction or 100% of $Q_B$ site activity, or rather full functionality (Shinkarev and Wraight 1997). DDAO detergent concentrations higher than its critical micelle concentration (CMC) value (~0.025%, i.e. 1.09 mM) would make impossible a 90% recovery of the $Q_B$ site functionality (Agostiano et al. 1999). UQ-10 and Triton X-100 were both used as powders from Sigma at maximum purity grade (> 99%). Indeed any further addition of UQ-10 did not lead to significant increments of occupancy (Wraight and Shopes 1989; Nagy et al. 2008).

Herbicide titrations were carried out as follows: progressive additions of small volumes of herbicide suitable stock solutions in ethanol (50 mM maximum solubility for all inhibitors except atrazine) to RC/UQ-10/DDAO micelle samples were performed, until a maximum of 2% ethanol on the total sample volume was reached. The herbicides used (terbutryn, terbumeton, ametryn, prometon, prometryn, atrazine) were all in form of powders from Fluka at maximum purity grade (99%), a generous gift of Prof. Endre Lehoczki (University of Szeged). Herbicide titrations with terbutryn on proteoliposomes were carried out as in RC/DDAO micelle samples.

Flash-induced absorption changes on the RC micelle and liposome samples (1.5 ml volume) were measured routinely by a single-beam kinetic spectrophotometer of local design (Tandori et al. 1995). The $P/P^+$ redox changes of the bacteriochlorophyll dimer (primary donor of the photoinduced electron transfer, either in reduced P or oxidized $P^+$ form) were detected by monitoring the absorption change at 860 nm after flash excitation, during the back reaction (known also as charge recombination) from the oxidized $P^+$ to ground P state. The charge separated state may be alternatively $P^+Q_A^-Q_B$, $P^+Q_AQ_B^-$ or rather $P^+Q_A^-I$ (I denotes the inhibitor molecule) depending on whether: the $Q_B$ site was occupied by a native ubiquinone and direct protein electron transfer from $Q_A$ to $Q_B$ has not proceeded yet ($P^+Q_A^-Q_B$); $Q_B$ site was occupied and the $e^-$-transfer has already reached $Q_B$ ($P^+Q_AQ_B^-$); the $Q_B$ site was occupied by an inhibitor molecule blocking the electron transfer beyond $Q_A$ ($P^+Q_A^-I$).

*Mulliken population analysis*

After construction of the inhibitor chemical structure, semiempirical geometry optimization was carried out (AM1). Further calculations were carried out on the optimized structures at the HF/6-31G(d,p)//HF/6-31G(d,p) of theory to determine the atomic charges of molecules (Creuzet and Langlet 1993). The calculations were done using the GAMESS US software.

*Modeling the crystal structure*

For the calculations on RC-inhibitor complexes (RCs from *Blastochloris viridis*), 2PRC, 1DXR and 5PRC structures were downloaded from the Brookhaven Protein Data Bank (www.rcsb.org). Graphs were constructed and the binding distances were calculated by HyperChem 7.0. Furthermore, the proteins were prepared using the Protein Preparation Wizard implemented in Maestro 9.3.5 (Schrödinger, LLC; 2012). After this process ligand interaction diagrams were created using the Maestro 9.3.5.

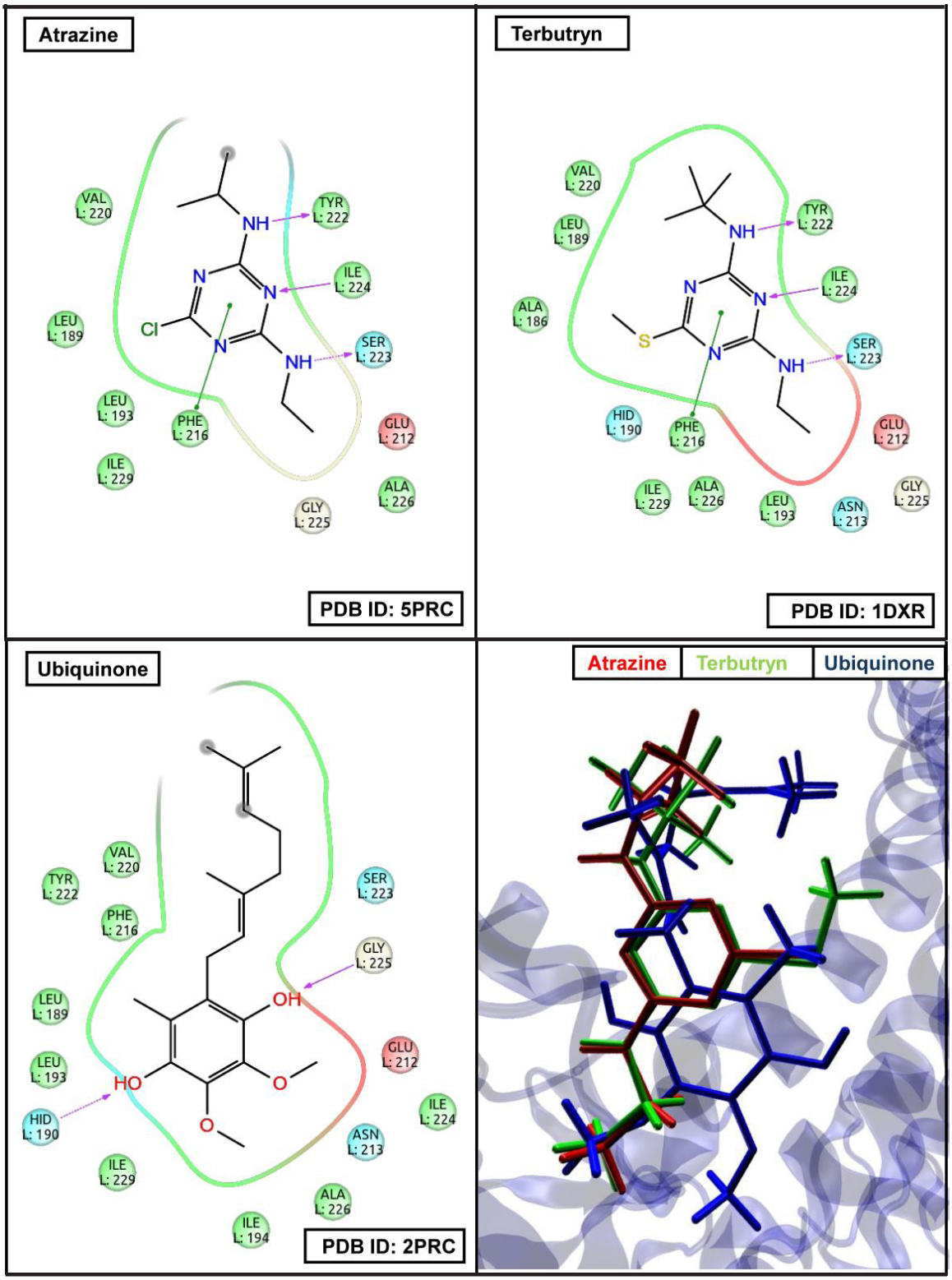



**Figure 1.** Ligand interaction diagrams of atrazine, terbutryn and ubiquinone (with involved amino acid residues) at the $Q_B$ site pocket of the Reaction Centre from *Blastochloris* (formerly *Rhodopseudomonas*) *viridis*, as indicated. The crystal structures of the complexes (5PRC, 1DXR and 2PRC) were downloaded from the Protein Data Bank and prepared by Protein Preparation Wizard implemented in Maestro 9.3.5. The figure itself was prepared by using Maestro 9.3.5 and VMD 1.9.1.

**Results**

*Titration of RCs by inhibitors*

It is known that additions of triazine herbicides used in agriculture (e.g. atrazine, terbutryn, prometryn, ametryn, etc.) to a reaction mixture containing RC-DDAO micelles progressively inhibits, and finally blocks, the electron transfer between the $Q_A$ (corresponding to a $P^+Q_A^-Q_B$ charge separated state) and $Q_B$ quinone (corresponding to a $P^+Q_AQ_B^-$ charge separated state). Once populated the excited state by short flash pulses (see Materials and Methods), such inhibition leads to a progressive shift from $P^+Q_AQ_B^-$ to $P^+Q_A^-Q_B$ (or $P^+Q_A^-I$) in the charge recombination process to $PQ_AQ_B$ (or $PQ_AI$) ground state (Okamura 1984; Halmschlager et al. 2002). Therefore, measuring the residual fraction of back electron transfer from the $P^+Q_AQ_B^-$ state on the total recombination at increasing herbicide concentrations is a direct way to monitor the effects of inhibition. Crystal structures revealed that these compounds bind at the secondary quinone ($Q_B$) site, albeit to slightly different places (Demeter et al. 1985; Lancaster et al. 2000). Fig. 1 shows the relative orientation of terbutryn and atrazine molecules modeled within the $Q_B$ site of the *Bl. viridis* RC crystal: they competitively inhibit the ubiquinone binding at the same site. It is reasonable to hypothesize that the mode of action of the inhibitor depends essentially on the herbicide chemical structure and the protein environment, both influencing the nature of the interactions established at the binding site. The chemical structure of the compounds employed in our study and numbering of their atoms are summarized in Figs. 2 and 3.

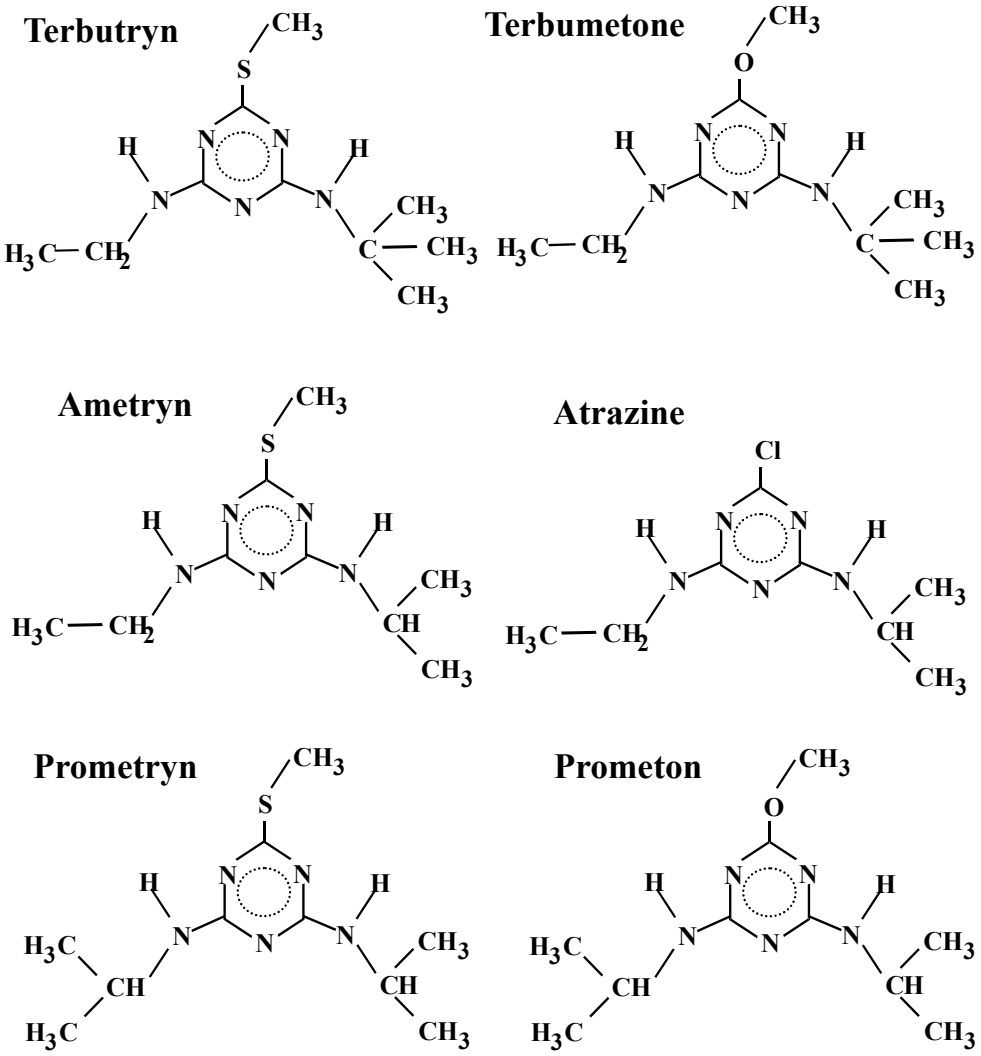



**Figure 2.** Chemical structures of the triazine herbicides used in our experiments.

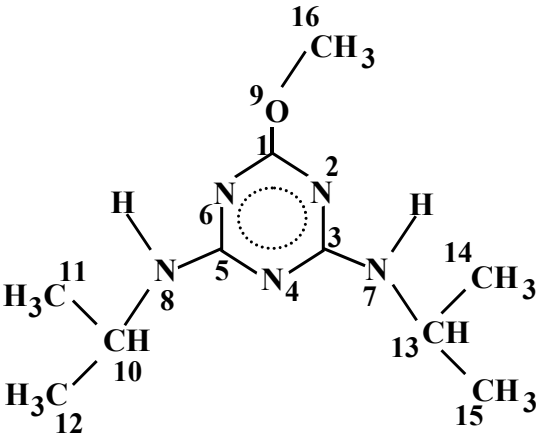



**Figure 3.** Numbering of atoms and characteristic groups of the herbicide molecules employed in our studies. Here the example of prometon is reported.

The herbicide efficiency on inhibiting the RC photochemistry, expressed in terms of binding free energy to the $Q_B$ site ($\Delta G$), and the relative order of inhibition ability were determined by measuring the recovery process from flash induced absorption changes at 860 nm in the presence of inhibitors (Wraight and Shopes 1989; Tandori et al. 1995). Fig. 4 shows the residual photochemical activity of the $Q_B$ site quinones in RCs suspended in detergent micelles, referred as the fraction of $Q_B$ site occupancy (Fr_$Q_B$, corresponding to the slow phase charge recombination from $P^+Q_AQ_B^-$ state) as a function of the inhibitor [Inh] to RC molar concentration ratio [Inh]/[RC]. Of course, the more herbicide was added, the higher inhibition of $Q_B$ site activity was obtained, together with inhibition of the $Q_A$ to $Q_B$ electron transfer (Wraight and Stein 1983). Dividing [Inh] by [RC] results in dimensionless ratio values in the abscissa that allows an easier comparison between experiments with different herbicides and slightly variable RC concentrations (see caption of Figure 4).

The measured fraction of the residual $Q_B$ site activity was fitted by the following equation (Okamura 1984; Paddock et al. 1988; Halmschlager et al. 2002):

$$\mathrm{Fr\_Q_B} = 1 - \left( \frac{1}{1 + \frac{K_I}{[\mathrm{Inh}]/[\mathrm{RC}]}} \right) \quad (1)$$

The fit allowed to determine the inhibition constant value ($K_I$ sometimes referred also as $I_{50}$ (Tandori et al. 1995)) for each herbicide, defined as the herbicide concentration required to induce 50 % inhibition of the above mentioned $Q_B$ site activity.

The resulting values are summarized in the second column of Table 1. At least in terms of magnitude order, our results are in reasonable agreement with literature data from isolated *Rb. sphaeroides* RCs (third column), although often obtained by means of different experimental methods (Brown et al. 1984; Okamura 1984; Stein et al. 1984; Tandori et al. 1991). The efficiency of the herbicide inhibition was found to follow the order, according to the $K_I$ values: terbutryn > terbumeton > ametryn > prometon > prometryn > atrazine.

The reasons and explanation of this order will be provided in the Discussion section, together with presentation of further data supporting our interpretation.

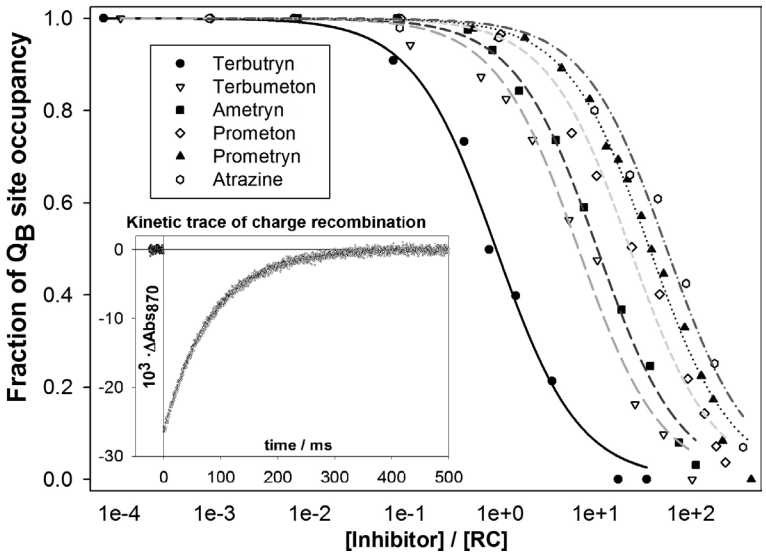



**Figure 4.** The residual ubiquinone binding activity (expressed as fraction of $Q_B$ site occupancy by quinone molecules) is reported versus the inhibitor/RC molar concentration ratio for different herbicides towards RCs suspended in DDAO detergent micelles. In every experiment the RC concentration was kept at a constant value within the $(0.9–1.1) \times 10^{-6}$ M range. Experimental herbicide titration data (represented as different geometrical shape markers) were fitted by a standard inhibition equation derived from the Michaelis Menten kinetic model (see text) in order to obtain the inhibition constants ($K_I$) for each herbicide used (black or gray, solid, dashed or dotted overlying curves). Inset: typical kinetic trace acquired by the described experimental apparatus (see Materials and Methods).

*Model calculations*

Thanks to the wealth of available information based on molecular biology studies, recent crystallographic data and *ab initio* calculations, it is possible to compare molecular properties with biological activities. In our studies we investigated the relationships between the calculated electronic and geometry parameters of triazine derivatives and their inhibition mode on bacterial reaction centers. Data are summarized in Table 2.

The main role in the herbicide-RC interaction is assumed by the –(6)N-(1)C-(9)X-(2)N- and –(7)HN-(3)C-(4)N-(5)C-(8)NH– molecular regions of the inhibitor (see Fig. 3). (7)N and (8)N nitrogen atoms, bearing both large negative charge accumulation, are on opposite sides of the molecule anchoring different substituents and interacting with different environment of the protein. Due to the possibility of establishing hydrogen bonds the role of nitrogen atoms appears more important. Nevertheless it is interesting to note the role of charges on ring carbon atoms. It is surprising that, although the (1)C-substituent is important from the point of view of the binding, the charge on this atom is not as relevant. On the other hand, the partial charge on (3)C has, instead, a definite effect on the binding efficiency.

The dependence of the binding energy on the negative charges of the (7)N and (8)N nitrogen atoms is affected by electronic effects of the (1)C-substituent. Indeed, introducing an electron withdrawing (–Cl or $–OCH_3$) group in place of

**Table 1.** Experimental inhibition constants ($K_I$), calculated inhibitor dissociation constants ($K_I^D$) and binding free energy changes ($\Delta G_{bind,I}°$) for several herbicides towards RCs in DDAO micelles, in comparison with the corresponding literature values for ubiquinone-10

| Herbicide | $K_I$ (μM) | | $K_I^D$(μM) | | $\Delta G_{bind,I}°$ (kJ/mol) | $K_Q^D$ (μM) literature |
|---|---|---|---|---|---|---|
| | calculated values | literature | calculated values | literature | | |
| Terbutryn | 1.3 ± 0.1 | 3[a] ÷ 6[b]<br>0.7 ± 0.2[d1]<br>0.13[d2]<br>0.8[e]<br>5[f] ÷ 9[g]<br>1.1[h]<br>0.1[i]<br>0.7[l]<br>2[n] | 0.34 ± 0.03 | 0.8 ± 0.2[b]<br>0.05[i]<br>0.18[j] | –18 ± 2 | 2[b]<br>2.0 ± 0.5[m]<br>1.7 ± 0.4[k]<br>4.5[j] |
| Terbumeton | 7 ± 1 | - | 3.1 ± 0.4 | | –12 ± 1 | |
| Ametryn | 15 ± 1 | 20[a] ÷ 30[b,c]<br>2.2 ± 0.5[d1] | 5.8 ± 0.4 | 0.4[j] | –11 ± 1 | –14 ± 1<br>($\Delta G_{bind,Q}°$ (kJ/mol), calculated) |
| Prometon | 25 ± 2 | > 400[a]<br>25 ± 5 [d1] | 11 ± 1 | | –9 ± 1 | |
| Prometryn | 43 ± 2 | 6 ± 1 [d1] | 18 ± 1 | | –8 ± 1 | |
| Atrazine | 63 ± 6 | 90[c] ÷120[a]<br>20 ± 5[d1]<br>5.2[d2]<br>20[g] ÷160[f]<br>150[l] | 27 ± 3 | 2.5[j] | –7 ± 1 | –9.0 ± 1.3[m]<br>($\Delta G_{bind,Q}°$ (kJ/mol) literature) |

[a] Stein et al. 1984 (determined from the extent of *cyt c* photooxidation, DDAO 0.14 % = 6.1 mM); [b] Stein et al. 1984 (determined from the fraction of fast charge recombination phase, DDAO 0.14%); [c] Stein et al. 1984 (determined from the extent of $Q_B$ reduction, DDAO 0.14%); [d1] Okamura 1984 (determined from the extent of rapid *cyt c* oxidation, DDAO 0.025% = 1.09 mM); [d2] Okamura 1984 (determined by the extent of *cyt c* oxidation under multiple turnover conditions, DDAO 0.025%); [e] Tandori et al. 1991 (determined from the damping of the semiquinone oscillation, Triton X-100 0.1% in the buffer); [f] Brown et al. 1984 (determined from fluorescence induction measurements); [g] Brown et al. 1984 (determined from flask kinetic analysis of charge recombination); [h] Diner et al. 1984 (determined from flask kinetic analysis of charge recombination, DDAO 1% = 43.6 mM); [i] Paddock et al. 1988 (determined from the extent of *cyt c* photooxidation, DDAO 0.025%); [j] Wraight and Shopes 1989 (determined by charge recombination measurements, but in the presence of Triton X-100 0.1% instead of DDAO); [k] McComb et al. 1990 (determined by direct assay of $Q_A^-$ to $Q_B$ electron transfer, charge recombination measurements and assay of RC turnover; DDAO 0.1% = 4.36 mM); [l] Jockers R. et al. 1993 (determined by stationary photobleaching); [m] Shinkarev and Wraigh 1997 (determined by charge recombination measurements); [n] Halmschlager et al. 2002 (determined by charge recombination measurements); * Our estimation based on (1.7 ± 0.4) μM value (ref. k) and literature method (see text).

**Table 2.** The atomic charges of ubiquinone-0 (UQ-0) and the inhibitors investigated calculated with Mulliken population analysis as described in Materials and Methods

| UQ-0 | | | Atrazine | | Terbutryn | | Terbumeton | | Ametryn | | Prometon | | Promatryn | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Atom | Knapp* | Partial charge | Atom | Partial charge | Atom | Partial charge | Atom | Partial charge | Atom | Partial charge | Atom | Partial charge | Atom | Partial charge |
| 1C | 0.38 | 0.512 | 1C | 0.396 | 1C | 0.387 | 1C | 0.923 | 1C | 0.386 | 1C | 0.915 | 1C | 0.376 |
| 2C | 0.26 | 0.310 | 2N | −0.629 | 2N | −0.682 | 2N | −0.737 | 2N | −0.678 | 2N | −0.747 | 2N | −0.669 |
| 3C | −0.15 | 0.311 | 3C | 0.895 | 3C | 0.914 | 3C | 0.918 | 3C | 0.900 | 3C | 0.911 | 3C | 0.895 |
| 4C | 0.65 | 0.524 | 4N | −0.712 | 4N | −0.724 | 4N | −0.721 | 4N | −0.722 | 4N | −0.730 | 4N | −0.713 |
| 5C | −0.42 | −0.299 | 5C | 0.895 | 5C | 0.893 | 5C | 0.895 | 5C | 0.893 | 5C | 0.901 | 5C | 0.893 |
| 6C | 0.15 | 0.016 | 6N | −0.629 | 6N | −0.674 | 6N | −0.685 | 6N | −0.673 | 6N | −0.701 | 6N | −0.666 |
| 7C | 0.12 | −0.179 | 7N | −0.844 | 7N | −0.863 | 7N | −0.882 | 7N | −0.841 | 7N | −0.839 | 7N | −0.856 |
| 8C | −0.02 | −0.182 | 8N | −0.840 | 8N | −0.841 | 8N | −0.859 | 8N | −0.840 | 8N | −0.839 | 8N | −0.860 |
| 9C | −0.30 | −0.501 | 9Cl | 0.036 | 9S | 0.206 | 9O | −0.645 | 9S | 0.206 | 9O | −0.616 | 9S | 0.213 |
| 10O | −0.45 | −0.525 | 10C | −0.066 | 10C | −0.063 | 10C | −0.065 | 10C | −0.062 | 10C | 0.058 | 10C | 0.072 |
| 11O | −0.33 | −0.674 | 11C | −0.521 | 11C | −0.520 | 11C | −0.528 | 11C | −0.520 | 11C | −0.473 | 11C | −0.493 |
| 12O | −0.27 | −0.674 | 12C | 0.069 | 12C | −0.642 | 12C | −0.169 | 12C | −0.642 | 12C | −0.491 | 12C | −0.496 |
| 13O | −0.47 | −0.536 | 13C | −0.485 | 13C | 0.220 | 13C | 0.221 | 13C | 0.073 | 13C | 0.059 | 13C | 0.070 |
| | | | 14C | −0.485 | 14C | −0.475 | 14C | −0.484 | 14C | −0.483 | 14C | −0.490 | 14C | 0.492 |
| | | | | | 15C | −0.475 | 15C | −0.484 | 15C | −0.483 | 15C | −0.473 | 15C | −0.497 |
| | | | | | 16C | −0.503 | 16C | −0.512 | 16C | | 16C | −0.172 | 16C | −0.659 |

* UQ-0 values are compared with the ones found in the literature (Rabenstein et al. 1998).

$-SCH_3$ reduces the correlation between the binding energy and a negative charge on (4)N. The higher negative charge on (2)N atom, instead, increases the binding efficiency.

## Discussion

In every experiment two important conditions were always satisfied: (i) there was a direct competition to the binding site between the inhibitor and the quinone; (ii) an equilibrium was reached on the time scale of the electron transfer to the $P^+Q_AQ_B^-$ state, i.e. we could write $P^+Q_A^-Q_B \leftrightarrow P^+Q_AQ_B^- = P^+(Q_AQ_B)^-$ (Paddock et al. 1988). As a consequence, the inhibitor dissociation constants ($K_I^D$) could be calculated from the $K_I$ values (Table 1, fourth column) on the basis of experimentally measured data together with literature results. By using the known concentration of the free added ubiquinone ((Q) as RC enzyme substrate in competition with inhibitors) and the quinone dissociation constant ($K_Q^D$, literature estimations on the right hand side column of Table 1) (Stein et al. 1984; Paddock et al. 1988; McComb et al. 1990) $K_I^D$ holds:

$$K_I^D = \frac{1}{1+\frac{[Q]}{K_Q^D}} \quad (2)$$

In every experiment [Q] had a known constant value in the 1.9÷4.4 μM range (corresponding to a 2÷4 molar ratio between free quinones and RCs). For calculating $K_Q^D$ one of the most recognized literature values (1.7 ± 0.4 μM) was selected[1], which was measured at lower DDAO concentrations (4.36 mM) (McComb et al. 1990). Also, this value falls much closer to our experimental conditions (see Materials and Methods) than other available literature data (Stein at al. 1984). Similarly to $K_I$, a comparison between our calculated $K_I^D$ for terbutryn and literature values reveals an acceptable agreement, keeping into account possible variability sources due to the use of a different detergents (e.g. "I" of Table 1) or to the use of the same detergent in different concentrations.

Moreover, the sixth column from left hand side of Table 1 shows our calculated thermodynamical free energy change values for the binding of herbicides to the $Q_B$ site of RCs in standard conditions ($\Delta G_{bind,I}°$, at 298 K and 1 bar pressure). Also, the measured $Q_B$ site occupancy by quinones and the fraction of the back reaction from $P^+Q_A^-Q_B$ or $P^+Q_AQ_B^-$ states (Wraight and Stein 1983), $K_I^D$ and $\Delta G_{bind,I}°$ values are strongly dependent on the DDAO concentration in mixed protein-detergent micelles. For this reason these values have been calculated according to (McComb et al. 1990). Indeed,

[1] Within experimental uncertainty this value may be easily converted to another recognized literature result (Shinkarev and Wraight 1997): $K_Q^D$ divided by [Q] (free quinone concentration in our samples, see text above) provides the dimensionless (0.4÷0.9) range, which in turn transformed into a dimensionless association constant for the quinone binding at $Q_B$ site gives $K_Q$ (using the same symbol of the cited ref.) = (1÷2), very close to the (0.6÷1.6) range reported in the above mentioned reference.

for this purpose, a mole fraction equivalent of $K_I^D$ ($\chi_I^{det}$) was defined as $K_I^D$/(DDAO), with detergent concentration expressed in molar units (DDAO 0.03% = 0.436 mM). Then, $\Delta G_{bind,I}°$ was calculated as:

$$\Delta G^{O}_{bind,I} = RT \ln(\chi_I^{det}) \quad (3)$$

The aim of such calculations is to easily compare the binding strength of different herbicides and native ubiquinone cofactor (UQ-10) to the $Q_B$ site of the RCs. The quinone binding free energy at the $Q_B$ site ($\Delta G_{bind,Q}°$) was calculated from the literature $K_Q^D$ value in the same way already explained for $\Delta G_{bind,I}°$ ($\Delta G_{bind,Q}° = -14 \pm 1$ kJ/mol, right hand side column of Table 1, indicated as "calculated"). Another similar value, taken directly from literature, is also reported ($\Delta G_{bind,Q}° = -9.0 \pm 1.3$ kJ/mol, indicated as "literature", same column and same Table as previous data.).

The $\Delta G_{bind}$ values listed in Table 1 for several triazine herbicides and ubiquinone cofactor reveal an acceptable agreement in their order of magnitude with literature values for RC-DDAO micelles (Table 1) and proteoliposomes (Table 3). The similarity between the terbutryn and ubiquinone binding free energies confirms that the competition between the quinone and inhibitor is essentially determined by classic thermodynamical factors, such as their relative concentrations and that of the detergent, influencing the binding/unbinding dynamics in the RC/DDAO micelles.

The experimental arrangements we employed for measuring the binding equilibrium of quinone and herbicides, i.e. aqueous detergent micelles or liposomes solutions, both try to mimic the *in vivo* conditions. The reactant UQ-10 and herbicide molecules (all insoluble or very scarcely soluble in water), had a very high tendency to migrate and to stay into the hydrophobic phase of the micelles and vesicle bilayer instead of remaining within the aqueous phase. Furthermore, just like *in vivo*, the $Q_B$ site of the RCs is accessible only to the quinone pool molecules from the bulk internal phase of mixed micelles or from the membrane bilayer.

Therefore, the logarithm of the octanol/water partition coefficient (log $K_{ow}$) can be extremely useful parameters to investigate and carry out reliable estimations about the dynamics of the competition between ubiquinone and inhibitors in the hydrophobic phase of the RC-containing micelles and liposomes. Log $K_{ow}$ values were found as high as 17.16 for UQ-10 (http://www.chemicalize.org/structure/#!mol=Ubiquinone-10), 2.61 for atrazine, 2.97 for prometon, 3.72 for terbutryn (Hansch et al. 1995; Paschke et al. 2004), and estimated as 4.67 (http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?sid=24865072#x27) for DDAO.

Moreover, calculations based on molar volumes of PC and CL apolar tails (0.634 l/mol, Angelico et al. 2000)) showed that the fraction of hydrophobic phase available for UQ-10 and herbicide molecules in liposomes, should be around $2 \times 10^{-3}$, thus increasing their effective availability towards the RCs by ~2000 times compared to their analytical nominal concentration in the sample. In this view, the topological discontinuity of the bilayer hydrophobic domain compared to the continuous aqueous phase (similarly to the discontinuity of the hydrophobic domain of the RC-DDAO micelles (Shinkarev and Wraight 1997)) accounts for the significant role in the ubiquinone distribution in proteoliposomes (Palazzo et al. 2000). Low UQ-10 to RC molar ratios would exacerbate the critical role of possible inhomogeneous distributions, and make very likely the existence of vesicles rich in RCs but poor in quinone molecules. In order to take

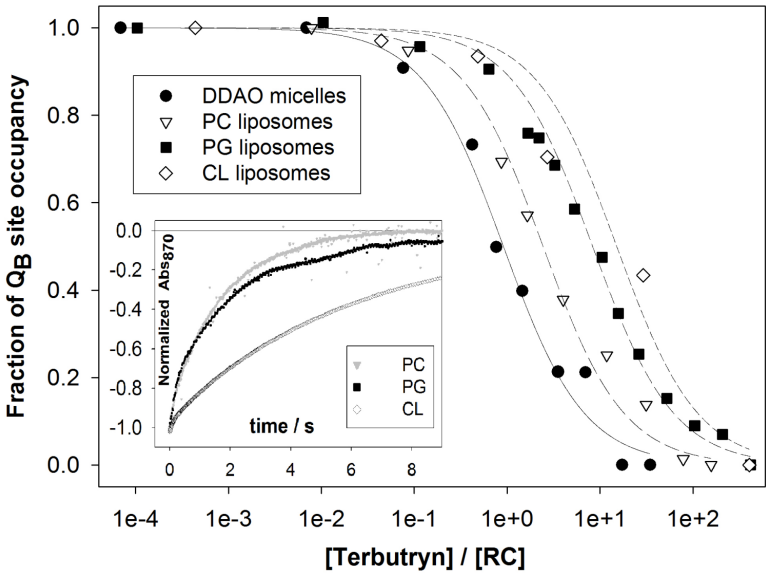



**Figure 5.** Fraction of the slow phase charge recombination as a function of the inhibitor to RC molar concentration ratio in different RC matrices, such as RC-DDAO micelles (already shown in Figure 4) and RC-liposome aqueous suspensions (vesicles made of PC (phosphatidylcholine), PG (phosphatidylglycerol) and CL (cardiolipin), with different symbols are used as indicated). Experimental data were fitted by the same equation 1 used for herbicide titrations carried out on RC-DDAO micelles, obtaining in this latter case full and dashed curves. *Inset*: Experimental kinetic normalized traces for PC, PG and CL liposomes: only the first 9 seconds are shown. The decay of the $P^+Q_AQ_B^-$ state became increasingly slower in the same order as the stability of the state increased: PC < PG < CL.

special care of reconstituting the $Q_B$ site with a minimum occupancy fraction of 0.90 before each herbicide titration experiment we adopted UQ-10 concentrations higher than those of RC for, at least, one order of magnitude (see Materials and Methods).

Figure 5 shows the residual photochemical activity of $Q_B$ site quinones (i.e. fraction of $Q_B$ site occupancy) as a function of the terbutryn/RC molar concentration ratio for proteoliposomes made of PC, PG and CL. Data were compared to those from RC-DDAO aqueous micelle samples (c.f. with Figure 4).

The inhibition constants ($K_I$) values obtained from the fit of the experimental points according to Eq. 1 are reported in the second column of Table 3. Due to the higher $K_I$ values for proteoliposomes compared to micelles it is evident that the presence of a lipid vesicle environment makes less efficient the herbicide inhibition process with respect to an aqueous detergent micelle suspension. This effect becomes progressively higher changing the zwitterionic lipid PC to PG, which bears a single negative charge, and finally to the double negatively charged CL.

The $K_I$ values were transformed into the calculated inhibitor dissociation constants of terbutryn ($K_I^D$as demonstrated also in Table 1) from the $Q_B$ site (third column in Table 3). This data treatment kept into account the free UQ-10 overall concentration (not referred only to the hydrophobic phase, since UQ-10 was solubilized in aqueous phase by Triton X-100 detergent) and the quinone dissociation constant measured in PC proteoliposomes ($K_Q^D$ = 0.55 μM (Milano et al. 2007)), according to Eq. 2. After conversion to a mole fraction equivalent of $K_I^D$ (indicated as $\chi_I^{det}$, where the detergent was DDAO, soluble both in the vesicle bilayer and in aqueous phase, Eq. 3), binding free energy values for terbutryn added to RC liposomes in standard conditions ($\Delta G_{bind,TBT}°$, fourth column of Table 3) were calculated. The magnitude order of these latter data is well in agreement with literature results reported for PC proteoliposomes (Palazzo et al. 2000) (added to Table 3).

To our knowledge and opinion, there might be at least two possible reasons for the diminished efficiency of the herbicide inhibition towards the quinone binding to $Q_B$.

First, zwitterionic phospholipids such as PC and, anionic lipids such as PG and CL, in larger contribution, show the specific effect of stabilizing the $P^+Q_AQ_B^-$ excited state (see. also inset of Figure 5) leading to an increment of the RC population with reconstituted $Q_B$ site (as measured by the increased contribution of the slow phase charge recombination) (Agostiano et al. 2005). Since $Q_B^-$ is a strongly bound quinol form, it is very difficult for terbutryn to compete for the binding site with this species. Although it has already been reported in the literature (Nagy et al. 2004; Rinyu et al. 2004), this proof of the lipid influence on the RC photochemistry has not yet been explained fully and quantitatively.

The second explanation might involve the differential accessibility of the secondary quinone site between the hydrophobic pool of the membrane bilayer and the hydrophilic cytoplasmic surface. As already reported, the volume available for the competitive binding between ubiquinone and herbicide molecules in proteoliposome samples was not the whole aqueous phase but only about 0.2 % of the total, corresponding to the hydrophobic lipid bilayer. Therefore, in response to a higher concentration of both species due to a reduced bilayer volume, the $Q_B$ site should be more sensitive to a much more hydrophobic UQ-10 (with much higher log $K_{ow}$), having a long isoprenoid tail, than to terbutryn, (with minor log $K_{ow}$), resulting in an easier saturation of $Q_B$ site with quinones.

Such hypothesis might also be supported by kinetic considerations. In RC-DDAO micelle and proteoliposome samples the exchange dynamics of quinones between the bulk solution and $Q_B$ site is two-fold (Shinkarev and Wraight 1997). One component is a slow exchange between the heterogeneous distribution of mixed RC-detergent and RC-free detergent micelles. This component exists also in liposome suspensions, as an exchange between different vesicles with heterogeneous distributions of quinones and herbicides. The

**Table 3.** Experimental inhibition constants ($K_I$) and calculated inhibitor dissociation constants ($K_I^D$ ) and binding free energy changes ($\Delta G_{bind,TBT}°$) for terbutryne towards RC in liposomes made of different phospholipids, compared to the corresponding values of ubiquinone-10 ($\Delta G_{bind,Q}°$)

| RC samples towards terbutryne titration | $K_I$ (μM) terbutryne | $K_I^D$(μM) terbutryne | $\Delta G_{bind,TBT}°$ (kJ/mol) | $\Delta G_{bind,Q}°$ (kJ/mol) |
|---|---|---|---|---|
| RC-DDAO micelles | 1.3 ± 0.1 | 0.34 ± 0.03 | –18 ± 2 | – |
| PC liposomes | 3.1 ± 0.3 | 0.54 ± 0.05 | –17 ± 2 | – |
| PG liposomes | 8 ± 1 | 0.74 ± 0.07 | –16 ± 2 | – |
| CL liposomes | 30 ± 10 | 1.5 ± 0.2 | –14 ± 3 | –14.6 ± 0.6* |

The liposomes were prepared from PC (phosphatidylcholine), PG (phosphatidylglycerol) and CL (cardiolipin), as indicated. * Palazzo et al. 2000 (obtained for RCs in PC vesicles).

other component is a fast exchange between the detergent phase and the $Q_B$ binding site of the protein within the same micelle or vesicle. This component becomes even faster and less critical in liposomes due to a better reconstituted lipid environment for the RC membrane protein. Therefore, the much higher hydrophobicity of UQ-10 compared to terbutryn might lead also to an acceleration of the above mentioned first exchange type mechanism between vesicles (e.g. represented by higher on-rate and off-rate kinetic constants). The more hydrophilic chemical structure of the herbicide would not be able to guarantee the same acceleration alone.

Literature data on RC-containing liposome membranes fully confirm this interpretation, at least from the kinetic point of view. While UQ-10 on-rate ($k_{Q,on}$) and off-rate ($k_{Q,off}$) constants were estimated as $7 \times 10^7$ $M^{-1} \cdot s^{-1}$ and $40$ $^1s^{-1}$, respectively (Milano et al. 2003), the same parameters for terbutryn were $(1 \div 8) \times 10^4$ $M^{-1} \cdot s^{-1}$ and about $0.02$ $s^{-1}$, with a difference of three orders of magnitude (Baker and Percival 1991). Future developments of these studies will be oriented also towards the construction of a suitable kinetic simulation model of the RC-quinone-herbicide dynamics from experimental data, in order to obtain reliable on-rate and off-rate parameters (see Future Perspectives section).

Finally, preliminary dynamic light scattering (DLS) measurements on proteoliposome sizes (not shown) contribute to explain the minor terbutryn inhibition efficiency found in CL and PG vesicles with respect to PC as a consequence of an higher heterogeneity of the quinone and terbutryn distribution in those liposomes. Indeed, keeping constant lipid amount (~4 mg), and added ubiquinone/Triton-X 100 and available hydrophobic volume (CL has about double molar mass than PC and PG, but also double apolar molar volume of its tails), significantly larger CL vesicles (80 ± 10 nm size, 0.20 polydispersity index) than PG and PC (50 ± 10 nm size, 0.20 polydispersity index) likely gave rise to a more variable distribution of quinone and herbicide molecules among liposomes. This, in turn, would be reflected in a less efficient inhibition by terbutryn towards RCs reconstituted in PG and CL liposome suspensions. Further investigations are, however, necessary to describe the possible correlations between vesicle size and size distribution, the nature of lipids employed and the inhibition efficiency more accurately.

## Conclusions

Final and conclusive explanations are summarized as electronic (i.e. inductive) and steric effects of the herbicide molecular substituent groups (cf. with Fig. 2 and Table 2).

*Electronic effects*

Steric and electronic interactions, as well as hydrogen bond connections, play important role in the herbicide binding to the protein, which should be determined by the unique structures of the molecules as specified in the following considerations. Inductive (electron withdrawing –I or releasing +I) and resonance effects (–R and +R) of substituents are essential in determining the partial charge distribution on the herbicide ring atoms, whose most important ones are the nitrogen and the (1)C carbon atoms. The presence of (+I) or (–I) groups on (1)C, indeed, has a critical role in allowing or not, respectively, electronic charges to be available for hydrogen bonds between the (7)N and (8)N nitrogen atoms and the suitable amino acid residues in the protein $Q_B$ pocket. Model calculations about molecular charge distributions in fact revealed a correlation between the presence of negative charges on (7)N and (8)N atoms and electronic effects of the (1)C substituents.

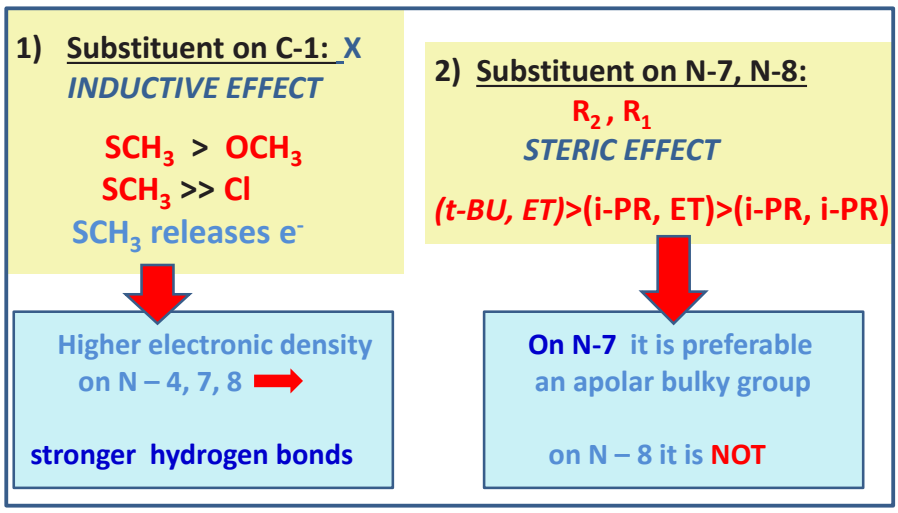
Moreover, due to (+I, +R) effects, the thiomethyl group ($-SCH_3$) gives rise to a higher binding efficiency (terbutryn, $K_I = 1.3 \pm 0.1$ μM) compared to the methoxy group ($-OCH_3$, terbumeton, $K_I = 7 \pm 1$ μM). Similarly, the (–I > +R) effect of the –Cl substituent on the (1)C atom makes the binding of atrazine ($K_I = 63 \pm 6$ μM, substituent: –Cl) weaker than that of ametryn ($K_I = 15 \pm 2$ μM, substituent: $-SCH_3$) (Table 1). These results stressed out that only (+I) substituents on the (1)C atom lead to more intense hydrogen bond interactions between the herbicide molecule and RC, and thus to a more efficient inhibition of the protein photosynthetic activity (Scheme 1).

*Steric effects*

From the point of view of the interactions between the alkyl groups bound to the (7)N and (8)N atoms and the protein $Q_B$ site, steric effects are very significant. For steric reasons an apolar bulky group on the (7)N atom of the molecule (like t-buthyl in terbutryn) is preferable, while it is not recommended on (8)N (like isopropyl in prometryn). Indeed, after the binding of the inhibitor the alkyl group on (7)N is located in a more apolar protein region, which surrounds the isoprenoid tail of the ubiquinone in native conditions. In the opposed way, the alkyl group on (8)N is located in a more polar protein environment, so that an apolar bulky group is not suitable for that position. The summary of electronic and steric effects is given in Scheme 1.

*Future perspectives*

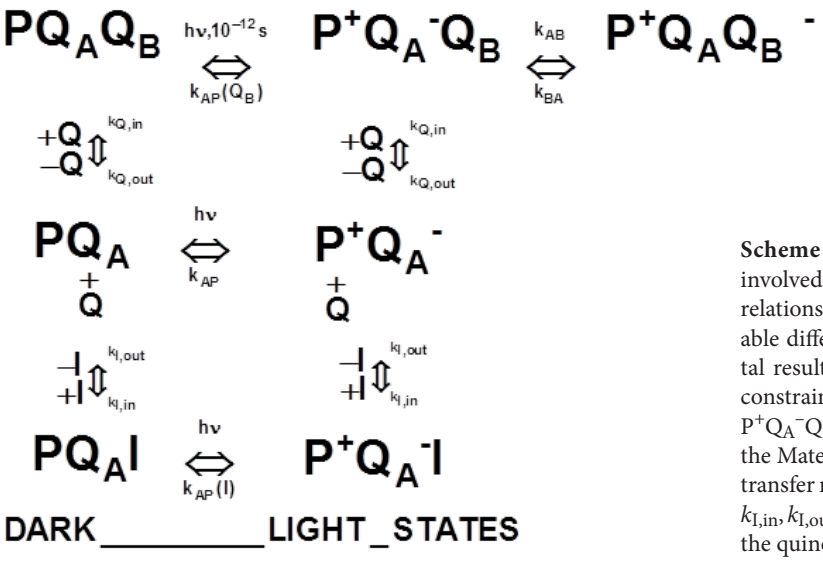
The main target of our future studies is to provide detailed description of the relationships between the structures of the inhibitor (herbicide) molecules and the thermodynamic




**Scheme 1.** Summary of effects of herbicide structure substituents on the inhibition efficiency. Abbreviations mean the following chemical groups: t-BU, tertiary buthyl; i-PR, isopropyl; ET, ethyl.

(binding energy) and kinetic ($k_{on}$ and $k_{off}$) parameters of the herbicide-protein interaction. This can be done through sets of kinetic equations modeling the charge transfer kinetics within the RC protein and the binding/unbinding equilibrium dynamics of the quinone and inhibitor molecules. Indeed, although similar investigations have already been carried out so far, with reasonable results, these provide only limited theoretical interpretation to a restricted set of experimental results, without particular attention to the presence of herbicide inhibitors (Halmschlager et al. 2002; Milano et al. 2003). Nevertheless, this information may be significant for several applications.

The ambitious objective related to the setup of a simulation model is obtaining reliable on-rate and off-rate kinetic parameters for ubiquinone and herbicides, supported by experimental results and calculations. Moreover, such a wide research development should necessarily take into account both (i) the 40–50 years (from 1960s on) of experimental and theoretical progresses in the study of photosynthesis and RC structure and functions and (ii) recent computer/informatics facilities and molecular biology results. As a basis for setting up the model, Scheme 2 summarizes all the cofactor-state species involved in the functional dynamics of the RC-ubiquinone-herbicide system.




**Scheme 2.** Summary of the RC cofactor-state species involved in the kinetic simulation model. Mathematical relations between state species will be represented by suitable differential equations exploiting reliable experimental results and literature values as initial conditions and constraints. The RC population states ($PQ_AQ_B$, $P^+Q_A^-Q_B$ $P^+Q_A^-Q_B^-$, $PQ_A$, $P^+Q_A^-$, $PQ_AI$, $P^+Q_A^-I$) are explained in the Materials and Methods. $k_{AP}$, $k_{AB}$, $k_{BA}$ are the electron transfer rate constants indicated on the figure. $k_{Q,in}$, $k_{Q,out}$, $k_{I,in}$, $k_{I,out}$ are the binding and unbinding rate constants for the quinones and the inhibitors, respectively.

**Acknowledgements.** This work was supported by the grants from Switzerland through the Swiss Contribution (SH/7/2/20), by the project named "TÁMOP--4.2.2.A-11/1/KONV-2012-0060 – Creating the Center of Excellence at the University of Szeged", which is supported by the European Union and co-financed by the European Social Fund. Thanks are due to the helpful discussions with members of European Cooperation in Science and Technology network, PHOTOTECH COST TD1102. BF and EGB thank the financial support from the FP7 Marie Curie Actions of the European Commission *via* the ITN ECHONET Network (MCITN-2012-316379) and from the University of the Basque Country/UPV-EHU. Authors thank the helpful contribution to the measurements and discussions with ESTRATO Research and Development Ltd (Budapest, Hungary).

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Received: September 22, 2014
Final version accepted: January 8, 2015
First published online: March 2, 2015