Comparison of Experimental and Calculated Hydrogen Bonding Properties of Some Urea and Triazine Inhibitors of Photosystem II

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Previous studies of structure/activity relationships of photosystem II inhibitors, including comparisons of their inhibitory power in herbicide-resistant and susceptible chloroplasts, have led to predict the role of hydrogen bonding, associated to hydrophobicity, in the binding onto the Q_B site. The crystallographic structures of bacterial reaction centers now allow these bonds to be identified. In order to be able to understand the binding of various herbicides and the effects of resistance mutations within the Q_B site, a reliable estimation of hydrogen bonding strengths is needed. We show here, by calculating interactions with model compounds, controlled by physicochemical measurements, that the hydrogen bonding properties of the C=X nucleophilic moiety present in most PS II inhibitors are different for triazines as compared to urea or amide derivatives. Semiempirical methods (AM 1) fail to reproduce the energies of hydrogen bonds between a triazine ring nitrogen and a phenolic proton. An empirical method (SIBFA), designed to reproduce interaction energies, has been adapted with the aim of calculating the binding energies of various herbicides with models of the Q_B site.

A great number of powerful inhibitors of photosynthetic electron flow, discovered in the last forty years, act by binding onto the Q_B pocket at the reducing side of photosystem II. This has been propicious to the development of Quantitative Structure/Activity Relationships (QSAR) studies, using essentially the Hansch approach [1] within series of homologous compounds. Only qualitative comparisons can be done between various diuron-like inhibitors belonging to structurally different chemical families. The main feature which emerged from such comparisons [1] was the presence of a common –NH–CX– moiety in most of the PS II inhibitors, such as ureas, triazines, uracils, but with the exception of phenolics. An electrophilic NH and a nucleophilic C=O/–C=N– moieties, present in urea-like and triazine-like inhibitors respectively, have been proposed to act as hydrogen bond donor and acceptor respectively [2–4]. However, the presence of an unsubstituted NH is not an absolute requisite for binding and a minimum model for diuron-like inhibitors has been proposed [5], consisting in an essential nucleophilic $C=X^{\delta-}$ (with X = O, N or S) and a neighbouring + charge on a nitrogen atom linked to an aromatic ring.

Information about the interactions involved in the binding of various herbicides to the Q_B site can be deduced from the decrease in the inhibitory activities in herbicide-resistant chloroplasts, in which a component of the site is mutated. The possibility of two or several overlapping sites has been proposed to explain the contrasting behaviors, in term of inhibitory power, of many inhibitors in triazine-resistant chloroplasts [6, 7]. By comparing the inhibitory activities of several derivatives of the –NH–CO– amide basal structure, essentially phenylureas, in triazine-resistant and -susceptible chloroplasts (R/S ratios), it was observed that the inhibitory power of some urea/amide inhibitors is substantially reduced (up to 18-fold) by triazine resistance [8, 9], a level of agricultural significance [9]. These variations of R/S ratios in homologous series could be linked to particular molecular properties [8]:

- An hydrophilic substituent (phenyl or cyclooctyl) is antagonizing the effect of the triazine resistance mutation, pointing out a stabilization by an hydrophobic interaction unaltered by the

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mutation. This substituent is absent in triazines (R/S > 200) and uracils (R/S = 50–100). Indeed, the addition of a benzyl to one NH of a triazine resulted in a reduction of R/S to around 10 [10], suggesting that this Φ-NH is homologous to that of phenylureas.

- By comparing R/S ratios in couples of phenylurea with similar hydrophobicity, it appeared that the negative charge δ^- on C=O was also antagonizing the effect of the resistance mutation. This led to the conclusion that resistance was impairing an interaction formed within the Q_B site by the aromatic-linked NH [8]. It is now known that the triazine resistance mutation in higher plants is due to a Ser 264 Gly substitution [11] and that the binding of triazines on *R. viridis* site [12] involves two hydrogen bonds, one of which is formed between a triazine NH and the Serine 223 (homologous of the Serine 264 in higher plants). However, the mechanism of the Φ-NH interaction remains unclear as regards the binding of urea/amide compounds.

Hence, these QSAR studies showed that an hydrophobic ring plays an important role in the binding of compounds endowing it, while compounds lacking it have their binding strength more strongly dependent on hydrogen bonds. This scheme remained compatible with the postulated homology between the –NH–CO– of phenylureas and the –NH–CN– of triazine-like compounds [1]. However, crystallography has shown that triazines form a double hydrogen bond with the –NH–CN– moiety in the *cis* conformation [12], as previously proposed from computer studies of double hydrogen bonding between triazines and dipeptides [13]. Calculations demonstrated that this *cis* conformation was also possible for diuron, but very unlikely, since its energy stands 4 kcal/mol above that of the *trans* conformation [14]. Spectroscopic studies also suggested that diuron binds through its carbonyl to a proton of His 215 [15, 16] and recent crystallographic data (Sinning and Michel, this issue) favor this pattern.

Recently developed computational methods allow the geometric and electronic structures of inhibitors to be reliably determined. However, different stable configurations are possible for a particular inhibitor, some of which may be favored by interactions with the site. This often makes it necessary to take into account the strength of electrostatic interactions. We examine here how the hydrogen bonding properties of urea/amide and triazine inhibitors can be evaluated by a semi-empirical and a new empirical computation methods and by physicochemical measurements (essentially IR spectroscopy in organic solvents).

Materials and Methods

IR spectroscopy

Hydrogen bonds with model compounds were studied in spectroscopic grade carbon tetrachloride in quartz infrasil cells with an optical path of 1 to 5 cm from 3800 to 3200 cm^{-1}. Concentrations were sufficiently low (around 1 mM) to almost prevent autoassociations. Cells were maintained at 8 °C.

Phenols of increasing OH acidities were *p*-octylphenol, *p*-chlorophenol, 3,4,5-trichlorophenol.

Calculations

AM 1 calculations [17] were done using either the MOPAC package or the GAUSSIAN 88 [18] interface implemented in IBM 3090-600 J/VF, with AM 1-88 parameters including chlorine [19]. Geometry optimization used Berny optimization with conjugated gradient.

SIBFA calculations [20, 21] were done using SIBFA program on IBM 3090-600 J/VF. SIBFA stands for Sum of Interactions Between Fragments *ab initio* computed. It is an empirical procedure relying on analytical formulas to compute the separate terms of the interaction energy. These formulas were further improved by *ab initio* calculations of water dimers, in order to reproduce as accurately as possible hydrogen bond interactions [22]. Molecular entities are built out of constitutive molecular fragments separated by single bonds, for which *ab initio* SCF computations were performed beforehand. One of the particularities of the method is that the electrostatic interaction energy is computed by means of multipolar expansion derived from the *ab initio* SCF molecular wave function. *Ab initio* SCF calculations relevant for this paper were done using Dunning's D95 basis set in GAUSSIAN 88.

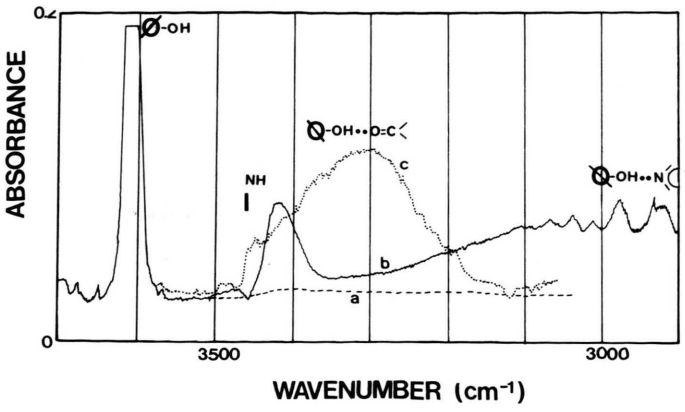


Fig. 1. Infra-red spectra in carbon tetrachloride of hydrogen bonds of the *p*-chlorophenol proton with the carbonyl of neburon or with a ring nitrogen of ametryne. a: 2 mM *p*-chlorophenol against CCl_4. b: 2 mM *p*-chlorophenol + 2 mM neburon against 2 mM neburon. c: 2 mM *p*-chlorophenol + 2 mM ametryne against 2 mM *p*-chlorophenol.

Results

Unassociated phenols exhibit on OH longitudinal vibration near 3610 cm^{-1}. In the presence of urea/amide or triazine inhibitors, the amplitude of this free vibration band decreases (allowing an association constant to be calculated) and a broader band appears at lower frequencies (Fig. 1), due to an hydrogen bond of the phenolic proton with the carbonyl of ureas/amides or with the ring nitrogen of triazines.

For ureas, the frequency shift Δv OH was related to the H-bonding strength in a series of homologous compounds. We checked that the frequency shift was greater when the acidity of phenol is increased by chlorine substituents. Deuteriation shifted the free OH vibration to lower frequencies and the Δv DH was lower. The frequency shift is increased when the N-phenyl of urea is substituted by electron repelling groups (the *p*-isopropyl of isoproturon) as compared to electron attracting (the 3,4-dichlorophenyl of diuron). This effect can be detected on the inhibitory activity for two compounds of equivalent Log P, such as chlortoluron and isoproturon [8, 14]. The semi-empirical methods AM1, while qualitatively reproducing this variation, gave much lower differences in the charges of carbonyl and in the computed H-bonds energies within the phenylureas [23].

The H-bonded vibration of OH in phenol-triazine complexes was much broader than that formed by amide or urea compounds (compare Fig. 1, spectra b and c), which precluded the measurement of a frequency shift. This corresponds to particular properties of the =N−···HO− bonds, in which the bonded proton is supposed to be delocalized [24], giving an absorption continuum. The =N−···HO− bond also induced a frequency shift of the triazinic NHs, much greater than that observed in ureas. This corresponds to an acidification of NH due to an inductive effect through the aromatic ring.

The association constants determined by IR spectrometry were used to control the results obtained by different computation methods. Taking the hydroxyl of *p*-chlorophenol as a model of proton donor, the interaction energies calculated by AM1 (Fig. 2) were much lower for hydrogen bonds formed with a triazine ring nitrogen than for those formed by an urea/amide carbonyl. In

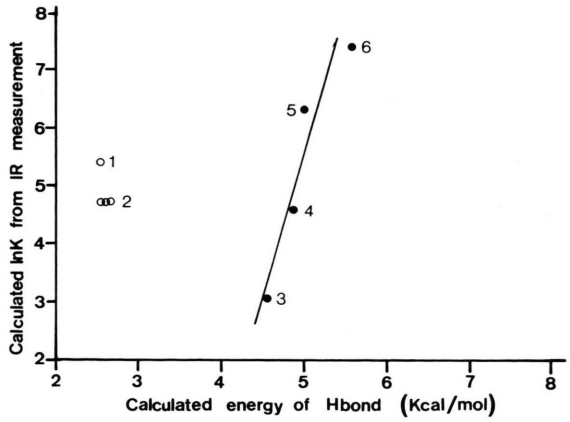


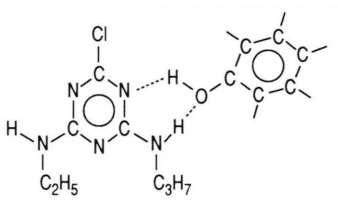
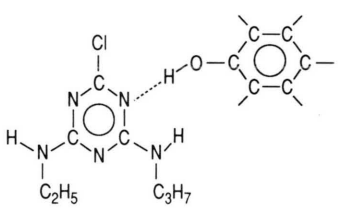
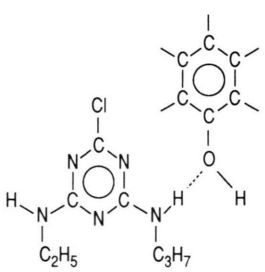
Fig. 2. Comparison between the association constant K for herbicide/*p*-chlorophenol complexes measured by infra-red and the bonding energies obtained by AM1 calculations. K was determined from absorbance of the unbounded hydroxyle longitudinal vibration at 3612 cm^{-1}. 1, ametryne; 2, atrazine; 3, swep; 4, diuron; 5, isoproturon; 6, cycluron.

sharp contrast, the IR-determined association constants were of similar magnitudes.

In order to investigate this discrepancy between calculation and experimental data, the empirical method SIBFA was compared to AM 1. Table I shows that the energies of hydrogen bonds formed by the phenol hydroxyl with atrazine (II) and with fenuron were much closer when obtained with SIBFA (5.87 and 8.81 kcal/mol respectively, ratio: 1.5) rather than with AM 1 (1.92 and 4.89 kcal/mol respectively, ratio: 2.55). Hence, results from SIBFA are in better agreement with the IR measurements.

In order to detect a cooperative effect between C=N and NH hydrogen bonds, as suggested by IR studies, an energy was also calculated for a single hydrogen bond between a triazine NH and the oxygen of the phenol hydroxyl, then the sum of energies of the two single bonds was compared to that of the double-bonded complex (Table I). Although this double interaction was geometrically disfavored, AM 1 was able to detect a positive cooperative effect (+0.44 kcal/mol), whereas a negative effect was found with SIBFA, which cannot represent long-range inductive effects in conjugated systems. The interaction energy values from SIBFA (which include induction effects) are higher than those obtained by AM 1. These energies correspond to *in vacuo* conditions and calculations have to be done in the presence of a solvent (CCl_4) to allow for a control of the calculated absolute values by IR spectroscopy. However, they can be used in a relative way, for comparison of inhibitor interaction properties.

Table I: Comparison of the energies (kcal/mol) of hydrogen bonds *in vacuo* between phenol and atrazine or fenuron calculated by SIBFA and AM 1.

Atrazine-phenol		AM 1	SIBFA
	**I**	3.97	6.89
	**II**	1.92	5.87
	**III**	1.56	3.19
Difference of energies (**I**)−(**II**)+(**III**)		+0.44	−2.17
Fenuron-phenol C=O···HO		4.89	8.81

The calculation of a dissociation constant of a herbicide with the Q_B site with SIBFA is not yet feasible, and *in situ* spectroscopic control would be useful. The Q_A^- S_2 recombination, corresponding to the Q thermoluminescence band, is influenced by the nature of the herbicide in the Q_B site [25], and compounds such as diuron may influence the properties of Q_A^- by binding to His215 [15, 16] through its nucleophilic carbonyl. We observed small variations of the Q band in the presence of different ureas or amides, but they were not strong enough to establish a correlation with the H-bonding properties of the carbonyl.

Discussion

The calculation of hydrogen bonding energies is still an unsolved problem and results may vary widely (Table I) according to computational methods. These results have to be controlled by physico-chemical measurements. Whereas the semi-empirical quantum method (AM 1) provides a satisfactory prediction of the geometry of PS II inhibitors, as compared to their cristal coordinates, it fails to give a good representation of the hydrogen bonding properties of triazines. This difficulty can be solved by using empirical methods such as SIBFA, which are designed to reproduce interaction energies, while also allowing computations to be performed on large structures. Valuable calculations of herbicide-site interaction can often be performed using parametric point charges, but they are likely to become inaccurate for com-

pounds like triazines, in which induction effects (polarization and charge transfer) represent an important part of the electrostatic interactions.

Recent crystallographic data obtained with diuron-sensitive mutants of *R. viridis* (Sinning and Michel, this issue) bring informations on the binding of urea/amide inhibitors. The phenyl ring is parallel to the Phe255, which corresponds to the hydrophobic interaction deduced from QSAR studies. The carbonyl would bind to His, as also suggested by spectroscopic studies, a feature which agrees with the antagonizing role of this carbonyle towards the Ser264Gly triazine-resistance mutation, located at the opposite part of the Q_B site. QSAR studies also suggest the role of a Φ-NH interaction contributing to binding and this could explain the positive cross-resistance of most urea and amide herbicide towards the Ser264Gly mutation. However, from the crystal structure, NH of ureas seems too far away from the hydroxyle of Serine264 to allow an hydrogen bond to be formed. In order to explain this discrepancy, several possibilities have to be considered. (i) The Q_B pocket has no exactly the same geometry in PS II and in bacterial reaction centers and a NH-Serine 264 hydrogen bond does exists, as for triazines, in higher plants, algae and cyanobacteria. (ii) The urea NH interacts with a part of the site which is modified by a local influence of the Ser264Gly mutation. This could be either an hydrogen bond with another aminoacid, an electrostatic interaction of the positively charged N [5] or a decrease of an electronic contribution (charge transfer) of the N-phenyl in its stacking with Phe255. (iii) Even though the Ser264Gly mutation does not create a steric constraint, such a small change would be sufficient to decrease the binding affinity of every diuron-like inhibitor.

The PS II herbicide site is one of those able to tightly bind thousands of inhibitors of widely different structures. Although stringent steric constraints have been evidenced for some of them [26–28], the overvall picture is that of an hydrophobic interaction reinforced by strong hydrogen bonds, which seem to contribute at a greater extent to the binding of triazine-like compounds than to that of phenyl-ureas and amides. This might explain a lack of stereospecificity of the Q_B site which has prevented an accurate topological mapping to be established from QSAR results, before the fairly definitive evidence provided by the crystallography of the bacterial reaction centers. It becomes now possible to appraise the relevance of the informations deduced from QSAR studies, which are still needed in the investigation of new pesticide sites for which crystal structure are not available. The now established role of hydrogen bonding at the PS II site has been early recognized from structural features. However, visual inspection also suggested an homology between the NH–CO of ureas and the NH–CN of triazines, which turns out to be unlikely when energies of conformation were calculated, as further evidenced by crystallography. Semi-empirical (AM1) and empirical (*e.g.* SYBYL) methods are now able to calculate the geometry and charge distribution of an herbicide molecule and, in contrast with the classical QSAR methods, allow unrelated chemical structures to be compared. The quantitative assessment of the electrostatic interactions of a herbicide with site models is a more difficult problem, but has to be considered when sufficient information about the site structure is available. Otherwise, structure/activity comparisons, supported by molecular calculations, can provide useful conjectures about the herbicide-site interactions, the reality of which may be demonstrated essentially by applying QSAR methods to the study of resistant mutants.

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