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Review Report
on PhD thesis of Ms Anna Grzybkowska entitled:
„Mechanisms of enzymatic and uncatalyzed s-triazine hydrolysis"

S-triazines represent a widespread class of compounds exhibiting a wide spectrum of biological activities, e.g. anti-cancer, antiviral, fungicidal, bactericidal and herbicidal. The s-triazine herbicides such as atrazine were first developed and introduced in 1958 by J.R. Geigy and over 1 billion kilograms have been applied globally ever since. The extensive usage of s-triazines as herbicides in agriculture and forestry causes serious environmental and health concerns and there is no surprise that in recent years, studies on a multitude of herbicides, especially their fate and degradation, have accelerated. For example, it has been found that metabolism of atrazines is initiated by an enzymatic hydrolytic dechlorination reaction leading to dehalogenated product hydroxyatrazine. Hydroxylated analogs of the pesticides reveal no toxicity anymore and faster biodegradation making this hydrolytic pathway an interesting object to investigate. However, despite a large body of biochemical, structural and analytical data our understanding of both abiotic and enzyme-catalyzed degradation mechanisms of s-triazine compounds is still elusive. On the other hand, computational chemistry tools can play an important role in resolving and rationalizing an experimental data and more importantly giving insight into molecular details of reaction mechanisms in vacuo and those being catalyzed by enzymes. When mechanistic insight into the reaction is needed

kinetic isotope effects (KIEs) are also very useful as they provide uniquely sensitive probe of transition state structures (which may happen to be ambiguous from computations when the two transition-state structures are of the same energy) and valuable information about the rate determining step of the reaction. Combination of computational tools and KIEs data (from experiment and calculations) seems to be very potent means to investigate in details the mechanism of the hydrolysis reaction in s-triazines as it was done in the reviewed thesis.

Chapter 1 of the thesis presents an interesting and well written introduction to s-triazines world and their abiotic degradation as well as biodegradation paying special attention to review the literature findings in this aspect. Also, in the same chapter the author provides basic knowledge on the computational methodology (MD and QM/MM), kinetic isotope effects (KIE), and compound specific isotope analysis (CSIA). Finally, the goals of the study are outlined. Introduction is followed by two chapters devoted to abiotic and biotic hydrolysis of s-triazines, respectively. Both chapters start by describing methodological background followed by meticulous analyses of the obtained results and conclusions. The thesis ends up highlighting the major findings of the conducted research and pinpointing the weak points of the method utilized in this study.

The main objective of this thesis was to investigate the mechanism of s-triazine compounds degradation uncatalyzed and catalyzed enzymatically in terms of multiscale computer modelling approaches and experimentally determined KIEs (only for triazine). Specifically, the role of the enzyme, the effect of the single mutation in the active site, the importance of the aromatic ring activation, as well as the replacement of the substituent (ametryn instead of atrazine) in the hydrolysis reaction were explored.

Modelling of the uncatalyzed hydrolysis of various s-triazines compounds under acidic, neutral and alkaline conditions in vacuo and in aqueous solution involved density functional theory (DFT) calculations of reaction energetics and structural changes of the geometries of the stationary points along the reaction coordinate. Additionally, chlorine KIE was derived experimentally for atrazine along with carbon, chlorine, sulfur and average nitrogen (over all five positions in the ring) KIEs obtained computationally for atrazine, terbuthylazine and ametryn. The results reveal that regardless of reaction pH abiotic hydrolysis of chloro-substituted s-triazine derivatives occurs through a concerted mechanism comprising the nucleophilic attack and the chlorine elimination with its concomitant protonation. On the contrary, ametryn hydrolysis in both acidic and alkaline pH proceeds according to the

step-wise mechanism (when sulfur is protonated a concerted mechanism is found) in which after the nucleophilic attack the Meisenheimer-like complex is formed and the thiomethyl group is eliminated and protonated. Interestingly, the final product of the hydrolysis of chloro-s-triazines is in enol form whereas in case of ametryn in keto form. The present work supports previous findings that aromatic ring in atrazine is protonated prior to the nucleophilic attack for the reaction under acidic conditions.

In my view, the author shall discuss possible reasons why carbon KIE value (contrary to chlorine and average nitrogen KIEs) predicted based on the BP86-optimized structure of atrazine under acidic condition (Table 2.3) does not comply neither with the experimental (within the experimental uncertainty) nor with the MPW1K-based value. For ametryn the predicted carbon KIEs (for two different protonation patterns) for acidic reaction are much too high as compared to the experimental value. Again, any attempt to enlighten this discrepancy is missing. Also, it is not clear from the methodology sections whether the same DFT functional was used for geometry optimization and vibrational frequency calculations needed to derive KIEs.

In chapter 3 the results of MD and subsequent QM/MM MD, as well as static QM/MM with the semi-empirical Hamiltonian, modelling of atrazine and ametryn hydrolysis catalyzed by chlorohydrolase (TrzN) and its E241Q mutant are presented. One- and two-dimensional free energy profiles along the defined reaction coordinate were computed by the QM/MM MD method for various models including protonated and unprotonated substrate bound to either the native enzyme or its mutant. Also, constructed potential energy surfaces (PES) were used to locate preliminary transition state structures and eventually full reaction pathways ranging from reactant to product (through intrinsic reaction coordinate (IRC) calculations). These derived structures were subject to geometry optimization and vibrational frequency calculations at the QM/MM level of theory. The potential energy profiles were refined using MPW1K functional instead of semi-empirical QM method within QM/MM scheme. Finally, chlorine, sulfur, carbon, and nitrogen KIEs were predicted. In my view, this part of the research focusing on description of molecular details of possible pathways and corresponding potential energy profiles of s-triazines enzymatic hydrolysis reaction is the most interesting albeit most demanding task conducted by Ms Grzybkowska, both conceptually and computationally.

The analysis of the results leads to very interesting observations that in case of atrazine

and ametryn substrates bound to wild type TrzN their ring protonation is due to multiple proton transfer, which is governed, however, by different mechanisms. In the former substrate, E241 is directly involved while in the latter one water molecule plays a crucial role in this process. After the ring protonation, nucleophilic attack occurs leading to the formation of the Meisenheimer-like complex followed by leaving group departure and its protonation, either directly (ametryn) or through H274 (atrazine). The rate determining step for the catalyzed hydrolysis of ametryn is the leaving group protonation while for atrazine is the nucleophilic attack. When atrazine is bound to TrzN mutant, the aromatic ring is directly protonated via one proton transfered from the nucleophile prior to the nucleophilic attack and this step seems to determine the overall rate of the reaction.

Having KIEs values proved very useful in cases where more than one plausible mechanism was considered, as in ametryn bound to wild type TrzN, in which the question was whether sulfur is directly protonated with the proton from OH group or it involves the contribution of H274. Comparison of the measured and predicted (for those two mechanisms) sulfur KIEs supported the former scenario. Moreover, similar comparison of Cl-KIEs allowed to reject the chloride departure as the rate determining step in the investigated reaction in atrazine-wild type TrzN and atrazine-E241Q mutant complexes.

Notwithstanding the insightful results summarized above, I have the following points to consider:

- Despite stable and low values of RMSD of backbone and active site of the protein the total simulation time (classical MD) is 4 ns may be too short to get the system well equilibrated. Since the equilibrium phase space is not sufficiently explored the system may equilibrate into a basin that is not the native basin of the TrzN enzyme. As a consequence determination of quantities that are highly sensitive to protein structure, e.g. Gibbs free energies or KIEs would be biased.

- With regard to the static QM/MM simulations of potential energy surfaces it would be interesting to perform DFT "cluster" calculations in which a much larger portion of the enzyme would be represented by the QM method than it happens in the QM/MM scheme. What differences or similarities would you expect in reaction mechanisms when explored by these two computational schemes?

- The author is well aware of obvious shortcomings of semi-empirical QM/MM sim-

ulations. It would be very insightful, even for just one model, to re-optimize the PM3ZnB/MM-based geometries using DFT/MM level of theory as one would be able to evaluate a methodology error in calculations of potential energy profiles due to the usage of non-QM stationary points.

In closing, all the objectives of the thesis were successfully achieved. Ms Grzybkowska needs to be credited for her valuable contribution to the body of knowledge regarding mechanisms of s-triazines degradation which has been delivered to the scientific community through two original articles published in high-quality journals: *Physical Chemistry Chemical Physics* and *Environmental Science & Technology*.

Overall, the doctoral dissertation submitted by Ms Grzybkowska represents high level scientific work. It is well written with small number of typographical errors and in my opinion it meets all the requirements laid down for a PhD degree. As a results, I would like to propose to award Ms Grzybkowska the PhD degree after successful defense.

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