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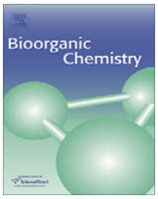

Intrinsic reactivity of uric acid with dioxygen: Towards the elucidation of the catalytic mechanism of urate oxidase

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ABSTRACT

Urate oxidase catalyzes the transformation of uric acid in 5-hydroxyisourate, an unstable compound which is latter decomposed into allantoïn. Crystallographic data have shown that urate oxidase binds a dianion urate species deprotonated in N3 and N7, while kinetics experiments have highlighted the existence of several intermediates during catalysis. We have employed a quantum mechanical approach to analyze why urate oxidase is selective for one particular dianion and to explore all possible reaction pathways for the oxidation of one uric acid species by molecular dioxygen in presence of water. Our results indicate the urate dianion deprotonated in N3 and N7 is among all urate species that can coexist in solution it is the compound which will lose the most easiestly one electron in presence of molecular dioxygen. In addition, the transformation of this dianion in 5-hydroxyisourate is thermodynamically the most favorable reaction. Finally, several reaction pathways can be drawn, each starting with the spontaneous transfer of one electron from the urate dianion to molecular dioxygen. During that period, the existence of a 5-hydroperoxyisourate intermediate, which has been proposed elsewhere, does not seem mandatory.

1. Introduction

Urate oxidase (EC 1.7.3.3) is an enzyme which catalyzes the oxidation of uric acid into 5-hydroxyisourate, while reducing dioxygen into hydrogen peroxide [1] (see Fig. 1). The metastable 5-hydroxyisourate species is latter transformed into S-allantoön either naturally or with the help of other enzymes [2,3]. Urate Oxidase, or uricase (UOx), is present in many living organisms with the notable exception of humans and higher primates [4]. It belongs to the purine degradation pathway and it prevents the accumulation of uric acid in blood. The absence of a UOx expression in human has both advantages and disadvantages: because uric acid is a potent antioxidant it helps reducing the presence of free radicals in cells, but in high concentration it can accumulate in plasma and induce high pathologies that may be fatal. Sanofi-Aventis produces and sells Rasburicase ™ whom active pharmaceutical ingredient is an urate oxidase bearing the primary structure of *Aspergillus flavus* urate oxidase. This drug helps to prevent precipitation of uric acid in kidneys occurring during tumor lysis syndrome, a life-threatening potential complication of cancer therapy [5,6].

Urate oxidase reaches its maximum activity between pH 8 and pH 10, but it is still largely active at physiological pH [7,8]. Isotopic studies by Bentley and Neuberger have shown that molecular dioxygen is at the origin of the formation of hydrogen peroxide [9]. Kahn et al. have also found by isotope labellings that the oxygen of the water molecule needed in the overall reaction equation is the oxygen bonded in position 5 in 5-hydroxyisourate [1]. In aqueous solution, uric acid possesses two pKas at around 5.5 and 10.5 [1,10,11]. This implies at physiological pH it is not uric acid as depicted Fig. 1, but a urate monoanion that reacts with O_2 inside urate oxidase. NMR spectra as well as theoretical calculations [1,12–14] have demonstrated: (i) the first major ionization site of uric acid is N3 and the second major ionization site is N9; (ii) solution of uric acid can be composed of mixed anionic species. At pH 7, the N3 deprotonated urate species (UAN_3^-) is not the only deprotonated species: the N9 deprotonated urate species (UAN_9^-) is also present albeit in a smaller amount. At pH above 10–11, the urate species deprotonated in N3 and N9 ($\mathrm{UAN}_3^-\mathrm{N}_9^-$) is the major species in solution, but other dianion species like $\mathrm{UAN}_3^-\mathrm{N}_7^-$ or $\mathrm{UAN}_1^-\mathrm{N}_9^-$ can be present although in a rather smaller amount.

A first crystallographic structure from Colloc'h et al. [15] indicated UOx bind uric acid in the $\mathrm{UAN}_3^-\mathrm{N}_9^-$ dianionic form. However, this was later corrected by subsequent crystallographic structures [16–18] that demonstrated UOx only binds a urate form deprotonated in N3 and N7, the $\mathrm{UAN}_3^-\mathrm{N}_7^-$ dianion or any other form bearing no proton on N3 and N7. Activation of the UAN_3^- monoanion at pH 7 is postulated to occur through a catalytic diad formed by Lys10 and Thr57 [19]. The detailed mechanism of the UOx catalysis is rather

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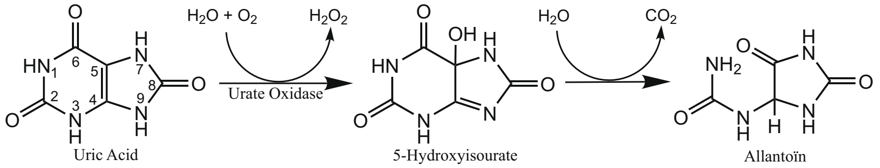

Fig. 1. Urate oxidase catalyses the first step of the conversion of uric acid into allantoön: the production of the 5-hydroxyiosurate intermediate.

unknown. Kahn et al. have performed stopped flow absorbance and fluorescence spectrophotometry that demonstrated the formation of two intermediates during the reaction [20]. They have been interpreted as the activated dianion species and a 5-hydroperoxyisourate intermediate by analogy with the reaction mechanisms of pterins and some flavins with dioxygen. However, there is no direct evidence the structure of the second intermediate occurring during the UOx catalysis is indeed a 5-hydroperoxyisourate.

Therefore, before trying to model the oxidation reaction of uric acid by dioxygen inside the active site of UOx, it would be interesting to first elucidate the *intrinsic* reactivity of this system, i.e., to enumerate all the possible mechanisms involving one uric acid molecule, one dioxygen, and one water molecule, and forming one hydroxyisourate molecule with one hydroperoxide molecule. Kahn has performed such calculations but only considering the UAN_3^- monoanion as it is the most found anion at physiological pH [21]. With the publications of new UOx crystallographic structures [16,22,18], many questions arise: why does UOx bind the minor $UAN_3^-N_7^-$ dianion instead of a major anionic species: either UAN_3^- monoanion or $UAN_3^-N_9^-$ dianion? Is the mechanism postulated by Kahn with the UAN_3^- monoanion still valid with the $UAN_3^-N_7^-$ dianion? Are there any other possible mechanisms? Are they more energetically favored?

In this article, we present a detailed theoretical study of all possible reaction mechanisms involving one uric acid, one dioxygen, and one water molecule to form one hydroxyisourate and one hydroperoxide molecule. After describing how our computations were made, we discriminate among all possible initial reaction pathways, and enumerate in detail all the reaction pathways involving a neutral uric acid. Then, we focus on the energetics involved in the reaction of two specific reactants: the UAN_3^- urate monoanion and the $UAN_3^-N_7^-$ urate dianion. All mechanisms and energetics involving uric acid, a urate monoanion different from UAN_3^-, or a urate dianion different from $UAN_3^-N_7^-$ are gathered in Supplementary materials.

2. Computational details

To assess the different possible mechanisms of urate oxidase, energetic differences among all putative intermediates and the reactants have been computed using either the Gaussian03 [23] software for *ab initio* and DFT calculations, or our own GEOMOP software [24] for semiempirical calculations. Energetic balances given, hereafter, always represent the difference between the sum of the intermediate species energies and the sum of the reactant species energies.

Semiempirical calculations have been performed at the PM3 [25] level; *ab initio* calculations used the combination of Hartree-Fock level with a 6-31G** basis set; DFT calculations employed B3LYP exchange-correlation potential [26–28] with a 6-31G** or 6-31+G** basis set. All intermediates were optimized in each of these quantum computation levels and frequency calculations were performed each time to verify whether these intermediates were true minima (i.e., they did not possess any imaginary frequency). MP2 single point energies have been computed on optimized B3LYP geometries. Where indicated, thermal corrections have been performed at 298.15 K and free energy quantities have been calculated under a 1 atm pressure.

Notations are used as follows: HF/6-31G** for example means a full geometry optimization without any constraints or any symmetry restrictions using a Hartree Fock theory level combined with a 6-31G(d, p) basis set; MP2//B3LYP/6-31G** means a single point energy calculation at the MP2 level using a 6-31G** basis set performed on the geometry obtained after a B3LYP/6-31G** optimization.

Solvent effects have been considered with the help of the distributed multipole reaction field model [29] implemented with the Gaussian98 packaged [30]. The modeling of the water solvent is considered in this study through the use of a 78.54 dielectric constant. Solvent effect calculations are referred with the self consistent reaction field (SCRF) notation.

3. Results

3.1. Initiation of the reaction

The oxidation of uric acid (U0A) by dioxygen (O_2) leads to the formation of 5-hydroxyisourate (5-HIU) and hydrogen peroxide (H_2O_2). Experimentally, it has been found that the hydrogen peroxide is composed of the two oxygens coming from the dioxygen molecule; while in 5-HIU, the hydroxyl group attached in position 5 is formed from the water molecule [1,9]. From the two half redox equations which can be drawn to describe urate oxidase action:

$$\mathrm{UA} + \mathrm{HOH} \rightarrow 5 - \mathrm{HIU} + 2\mathrm{H}^+ + 2e^- \tag{1}$$

$$\mathrm{O}_2 + 2e^- + 2\mathrm{H}^+ \rightarrow \mathrm{H}_2\mathrm{O}_2 \tag{2}$$

It is a reasonable assumption to postulate that from the two protons which help forming hydrogen peroxide, one proton comes from U0A while the other is originated from the water molecule. It is very unlikely that the proton originated from the water molecule will first transfer to dioxygen. In addition, while it has been found that some residues could act as a general base in UOx [19], the equilibrium of the reaction will not change: whether the proton is first abstracted by UOx or directly transferred from uric acid to molecular dioxygen, the final balance of the reaction will still consist of the loss of one proton for uric acid. Thus, we consider that the initial reaction between U0A and O_2 should imply a proton transfer between the two species with or without electron transfer. Four hypotheses depicted in Fig. 2 can be envisaged as an initial

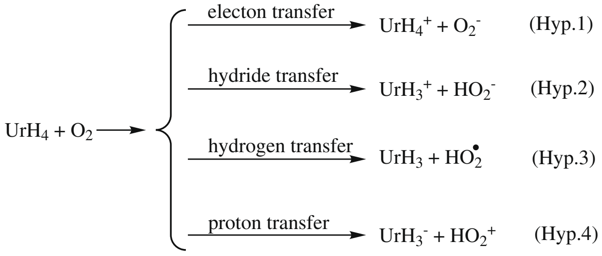

Fig. 2. Four hypotheses related to the initiation reaction of the oxidation of uric acid by dioxygen.

reaction step between uric acid (or urate) and molecular dioxygen: a direct electron transfer (Hyp. 1), a hydride transfer (Hyp. 2), a hydrogen transfer (Hyp. 3), or a proton transfer (Hyp. 4).

The ΔE associated with the possible initial reactions for acid uric (U0A), the $\mathrm{UAN_3^-}$ monoanion obtained by deprotonation of the $\mathrm{N_3{-}H}$ site of U0A, and the $\mathrm{UAN_3^-N_7^-}$ dianion obtained by deprotonations of the $\mathrm{N_3{-}H}$ and $\mathrm{N_7{-}H}$ sites of U0A are reported in Table 1. For hypotheses 2–4, only the abstraction of a proton in position 9 of the urate species has been here considered.

For uric acid (U0A), our results indicate the initial reaction step with O_2 should be a hydrogen transfer (Hyp. 3). Considering for example B3LYP/6-31G** calculations, the Hyp. 1 intermediate consisting of a superoxide molecule infinitely separated from a uric acid cation should be 190.1 kcal/mol higher in energy than uric acid with molecular dioxygen. In the same manner, the formation of a hydroperoxide anion (Hyp. 2) leads to intermediates 219.3 kcal/mol above the reactants, and the protonation of O_2 is more energetically not favorable by forming intermediates 257.1 kcal/mol above the reactants. Other gas-phase calculations using various methods like PM3, HF/6-31G**, or MP2//B3LYP/6-31G** gives similar results: a hydrogen transfer is the initial step starting with uric acid that leads to the less destabilized intermediates. The use of a dielectric continuum to simulate solvent effects leads to the same conclusions although the relative reaction energies are much smaller for Hyp. 1, 2 and 4. Overall, in the case of uric acid which mainly exists in low pH conditions, the formation of a perhydroxyl radical seems to correspond to the only initial possible reaction which could further leads to the formation of 5-hydroxyisourate.

For $\mathrm{UAN_3^-}$ which corresponds to the main existing species at neutral pH, similar conclusions than with U0A can be drawn. Using gas phase B3LYP/6-31G** calculations, the first intermediates according to our four hypotheses are, respectively, 73.3, 100.0, 31.6, and 356.2 kcal/mol higher in energy than the reactants. It is again the hydrogen transfer that leads to the most stable intermediate. While solvent effects stabilize the products compared to the reactants, this conclusion still holds. The main difference between U0A and $\mathrm{UAN_3^-}$ reactivity is that, in the case of the monoanion, Hyp. 1 gives intermediate not so far away in terms of energy than Hyp 3: the hydrogen transfer between the reactants is still preferred than a direct electron transfer, but the gap between both hypothesis is shrunk.

Table 1
Net reaction energies (ΔE) for the four possible initial reactions (hypotheses 1–4) of O_2 with U0A (uric acid), $\mathrm{UAN_3^-}$ (urate monoanion), and $\mathrm{UAN_3^-N_7^-}$ (urate dianion), respectively (ΔE in kcal/mol).

Methods	Hyp. 1	Hyp. 2	Hyp. 3	Hyp. 4
U0A				
PM3	177.1	198.5	24.0	225.9
HF/6-31G**	195.9	254.9	28.7	262.9
B3LYP/6-31G**	190.1	219.3	30.9	246.3
MP2//B3LYP/6-31G**	203.0	218.4	51.7	257.1
PM3 (SCRF)	52.5	73.4	23.8	94.0
B3LYP/6-31G** (SCRF)	70.0	101.3	29.9	132.6
$\mathit{UAN_3^-}$				
PM3	65.8	87.9	25.9	312.3
HF/6-31G**	75.9	111.2	32.8	372.1
B3LYP/6-31G**	73.2	100.0	31.6	356.2
MP2//B3LYP/6-31G**	87.3	97.9	48.0	352.3
PM3 (SCRF)	33.2	53.9	22.5	105.7
B3LYP/6-31G** (SCRF)	41.9	66.8	26.3	152.6
$\mathit{UAN_3^-N_7^-}$				
PM3	−45.0	−33.2	12.5	416.2
HF/6-31G**	−43.5	−17.3	17.3	483.2
B3LYP/6-31G**	−47.7	−26.5	19.0	468.1
MP2//B3LYP/6-31G**	−36.9	−24.7	36.7	462.7
PM3 (SCRF)	11.8	20.6	13.2	120.9
B3LYP/6-31G** (SCRF)	5.8	25.2	15.2	176.2

In the case of $\mathrm{UAN_3^-N_7^-}$ dianion which corresponds to the species bound to the active site of urate oxidase at high pH, gas phase computations indicate hypotheses 1 and 2 can give intermediates with lower combined energies than the reactants. According to B3LYP/6-31G** calculations: (i) a direct electron transfer from urate dianion to form a superoxide molecule should be very easy and leads to stabilized intermediates (−47.7 kcal/mol below the reactants); (ii) hydride transfer which would correspond to the simultaneous transfer of one proton and two electron from $\mathrm{UAN_3^-N_7^-}$ to O_2 also leads to stable intermediates (−26.5 kcal/mol below the reactants); and (iii) similarly to reactions with U0A and $\mathrm{UAN_3^-}$, the hydrogen transfer hypothesis leads to intermediates slightly above the reactants in terms of energy (19.0 kcal/mol). Using a dielectric continuum, DFT computations show the intermediates bound to the formation of a superoxide are 5.8 kcal/mol higher in energy than the reactants (Hyp. 1), the intermediates related to the formation of a perhydroxyl radical (Hyp. 3) are 15.2 kcal/mol higher in energy, and the intermediates related to the formation of a hydroperoxide anion (Hyp. 2) are 25.2 kcal/mol higher in energy. Overall, it seems the initial reaction of urate dianion together with dioxygen leads first to an electron transfer and the formation of a superoxide. This superoxide then can induce either a proton transfer (Hyp. 3) or a hydrogen transfer (Hyp. 2) to form either a perhydroxyl radical or a hydroperoxide anion, respectively. From our DFT and PM3 computations, it seems the formation of a perhydroxyl radical should be the preferred intermediate that will later lead to the formation of 5-hydroxyisourate.

Overall, the three considered urate species (uric acid U0A, urate 3-monoanion $\mathrm{UAN_3^-}$, and urate 3-7-dianion $\mathrm{UAN_3^-N_7^-}$) seem to share the same initial reactivity towards dioxygen O_2: they react with the latter to form a perhydroxyl radical (Hyp. 3) that can be, in high pH conditions, furthermore, transformed into a hydroperoxide anion (Hyp. 2). This reaction can be initiated in one or several steps: a first electron transfer between the urate species and the dioxygen (Hyp. 1), a proton transfer from the urate species to the superoxide (Hyp. 1 → Hyp. 3), and possibly a second electron transfer from the urate species to the perhydroxyl radical to form a hydroperoxide anion (Hyp. 3 → Hyp. 2). The last envisaged step would, therefore, be the decomposition of a solvent water molecule to form a 5-hydroxyisourate (5-HIU) together with hydrogen peroxide (H_2O_2).

A first explanation why urate oxidase binds the urate 3-7-dianion $\mathrm{UAN_3^-N_7^-}$ can be drawn from these preliminary calculations: the first electron transfer between this dianion and O_2 is direct in gas phase (i.e., with no barrier) and relatively easy in solution while the urate 3-monoanion $\mathrm{UAN_3^-}$ and uric acid cannot easily transfer one electron to dioyxgen. This is due to the difference in ionization potentials (IPs) of uric acid and its anions: in gas phase urate dianions have a large negative IP while urate monoanions and uric acid have a positive IP [13,21]. Furthermore, it has been demonstrated that from the three dianion species that can be naturally present in solution, $\mathrm{UAN_3^-N_7^-}$ bears the largest negative IP, which means it is the most antioxidant urate species [13,14,21,31].

3.2. General mechanism of uric acid with dioxygen

After or concomitantly to the transfer of a first electron from uric acid to dioxygen to form a superoxide, a proton must be transferred from urate radical species to this superoxide (Hyp. 2 from above). Two protons from urate radical species are possible candidates: the proton H-9 bound to the N-9 nitrogen or the proton H-7 bound to N-7 nitrogen. The former will lead to the formation of 5-hydroxyisourate using Hypothesis [H-9 → 5-OH], while the latter will lead to the formation of 4-hydroxyisourate according to Hypothesis [H-7 → 4-OH] (see Fig. 3). We do not consider here the possible depro-

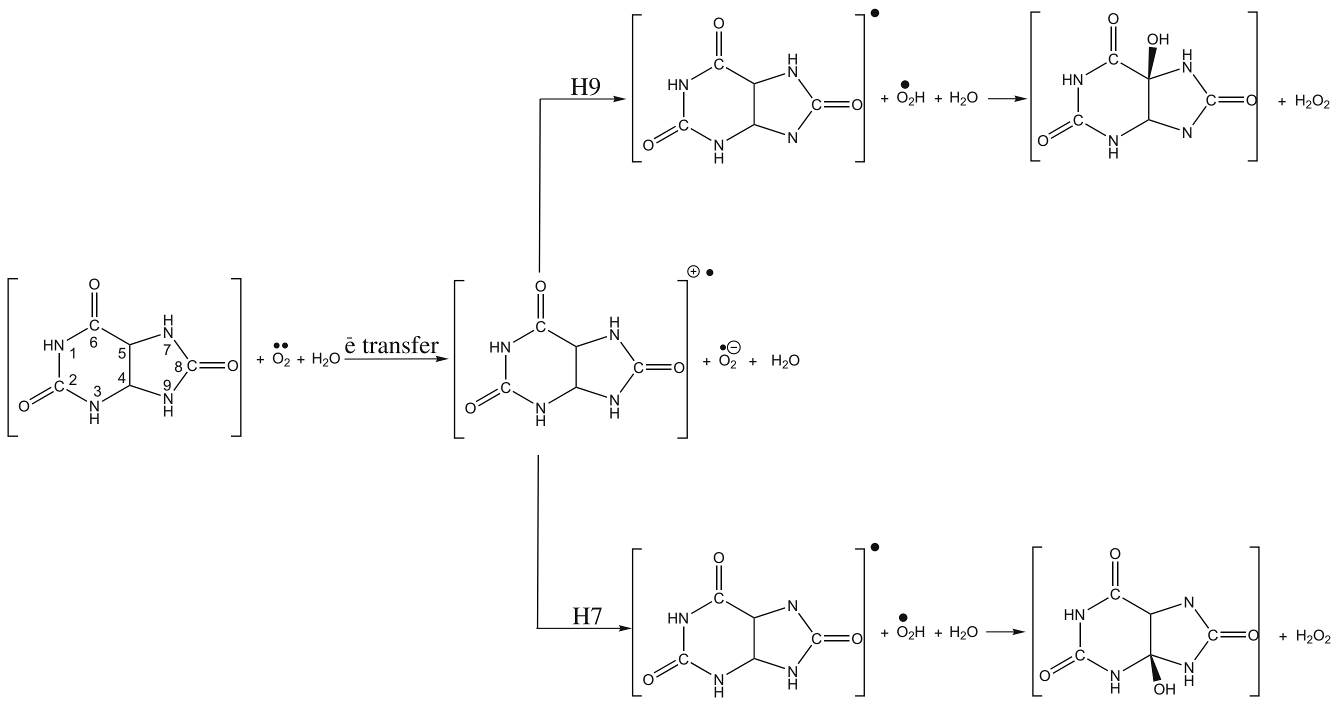

Fig. 3. Two possible proton transfers from uric acid to dioxygen.

tonation of one of the two other existing protons in uric acid (H-1 and H-3), because urate oxidase crystallographic structures clearly show the bound nitrogens N-1 and N-3, respectively, are involved in hydrogen bonding with UOx amino acids (N-1 is a hydrogen bond donor while N-3 is a hydrogen bond acceptor).

The second proton needed to fulfill the reaction can then be originated from either the urate species or a water molecule. Several possible reaction mechanisms can, therefore, be envisaged for uric acid and its anionic forms.

3.2.1. Firs possibility: proton transfer from N9 to O2 (Hyp. [H-9 → 5-OH])

From the Hypothesis [H-9 → 5-OH] of Fig. 3 which consists of a transfer of the proton bound to the N-9 nitrogen of uric acid to the superoxide, three possible reactivities can be guessed (see Fig. 4).

From the reactants (I^0), the first reaction consists of transferring an electron from uric acid to $O_2(II^0)$, then to transfer the H-9 proton to the superoxide. This forms a perhydroxyl radical together with a urate radical (III^0). This two doublet spin species can, furthermore, react in different ways: (i) the OOH radical directly adds up to the C-5 carbon of urate radical (V^0) to form a singlet spin 5-hydroperoxyisourate (IX^0) which after elimination of hydrogen peroxide (XII^0) can lead to the 5-hydroisourate product (XV^0); (ii) or the water molecule can react with the OOH radical to form the hydrogen peroxide together with a hydroxyl radical (VI^0) that can add up to the radical urate to create a triplet spin 5-hydroxyisourate ($XIII^0$) which can finally reach its final singlet spin state (XV^0); (iii) or the H-7 hydrogen can be transferred to form the hydrogen peroxide together with a triplet spin dehydrourate (X^0) which can then transform themselves into intermediates XII^0 or intermediates $XIII^0$.

In a previous computational study, Kahn [21] considered only the first reaction path presented here.

3.2.2. Second possibility: proton transfer from N7 to O2 (Hyp. [H-7 → 4-OH])

Another global mechanism can be envisaged when transferring the H-7 proton from the radical urate species to the superoxide (Hyp. [H-7 → 4-OH]). The same kinds of reactivity can then be considered, except the final product can only be 4-hydroxyisourate instead of 5-hydroxyisourate (see Fig. 5), because the addition of a OH or OOH group can only be done via the C-4 carbon of uric acid.

Given all the reaction hypothesis presented here, one can envisage up to six different intrinsic reaction mechanisms for uric acid, as depicted in Figs. 4 and 5. We have studied all total energy variations along all these reactions paths for uric acid, its four derived monoanions, and its 5 different dianions (7,9-dianion cannot react according to our mechanisms). Here, we present in detail only the intrinsic reactivity of 3-monoanion and 3,7-dianion, because they are the most interesting species related to urate oxidase reactivity. The results concerning the other possible urate species are gathered in Supplementary materials.

3.3. Intrinsic reactivity of the urate 3-monoanion (UAN_3^-)

3.3.1. Relative energies

The relative reaction energies calculated using different quantum methods are reported in Table 2 for Hypothesis [H-9 → 5-OH] (Fig. 6), and Table 3 for Hypothesis [H-7 → 4-OH] (Fig. 7). From Table 2, the first step of the reaction begins with an initial transfer of an electron from urate 3-monoanion to dioxygen (see Fig. 6). This step gives intermediate (II^-) with an energy of 73.2 kcal/mol using B3LYP/6-31G**, and 87.3 kcal/mol using MP2//B3LYP/6-31G** quantum methods, respectively. Then a proton transfer can be initiated from N-9 to form a OOH radical. This radical intermediate (III^-) is located 31.6 kcal/mol above reactants (I^-) using B3LYP/6-31G**, or 48.0 kcal/mol using MP2//B3LYP/6-31G** quantum methods, respectively. To reach the final products, three possible reaction pathways can be considered. They are each presented by a different row per method in Table 2.

(1) The direct attachment of the OOH molecule on the C-5 of urate (V^-) is associated to an energy above the reactants of 58.5 kcal/mol according to DFT calculations, and 72.6 kcal/mol according to MP2 level. The change in spin state, from

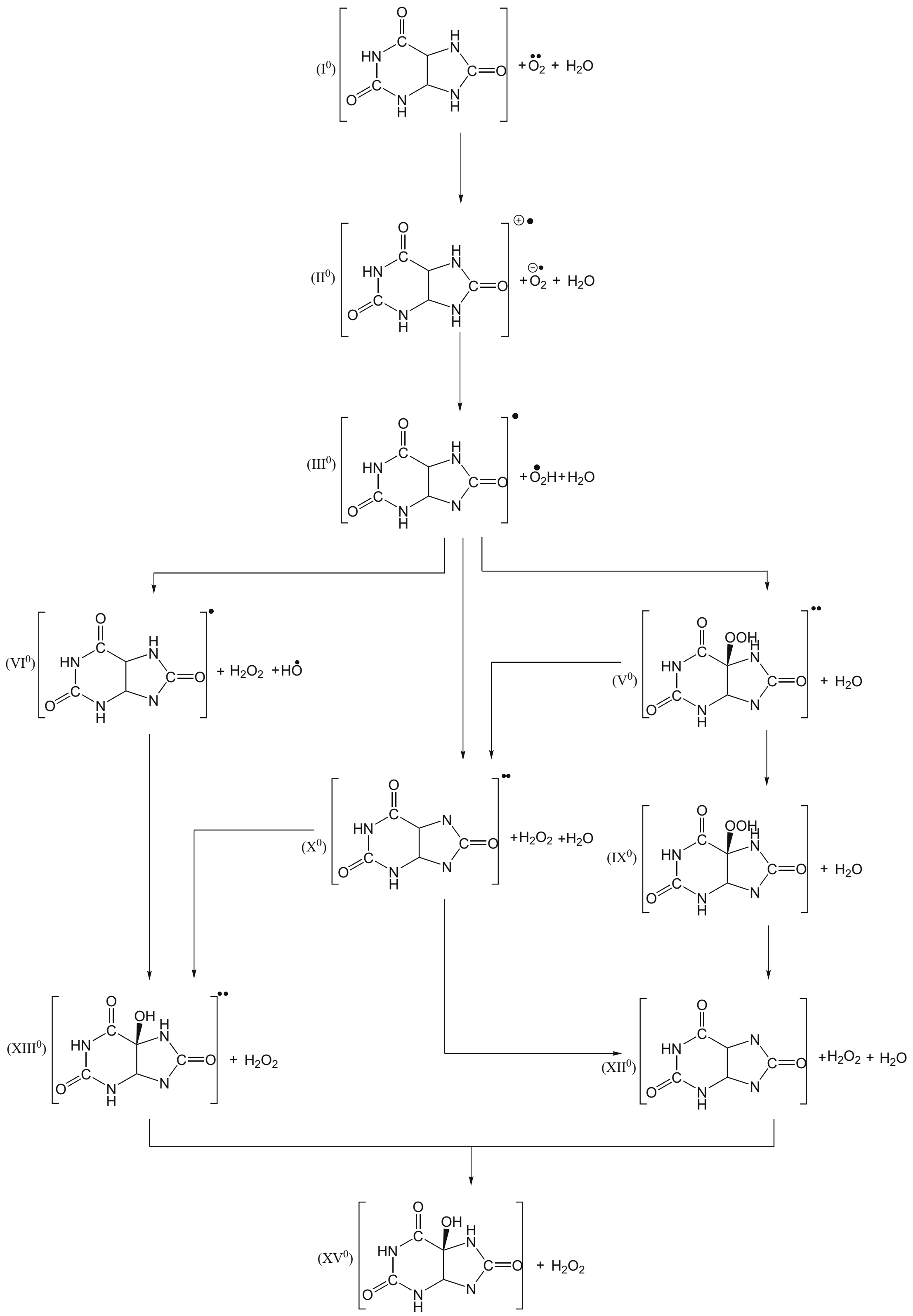

Fig. 4. Hypothesis [H-9 → 5-OH]: oxidation of uric acid by dioxygen initiated by an electron transfer and a H-9 proton transfer to O_2. (For convenience, the Lewis representations of the derived species of uric acid here do not contain proper bond orders.)

triplet to singlet, gives a 5-hydroxyisourate (IX^-) with an energy of −7.0 or −12.6 kcal/mol using DFT or MP2 methods, respectively. The elimination of a hydrogen peroxide molecule to form the dehydrourate intermediate (XII^-) is performed with a loss in potential energy of 23.3 or 27.6 kcal/mol using DFT or MP2 methods, respectively, compared to intermediate (IX^-). Last, a final hydration of the latter compound enables the formation of 5-hydroxyisourate

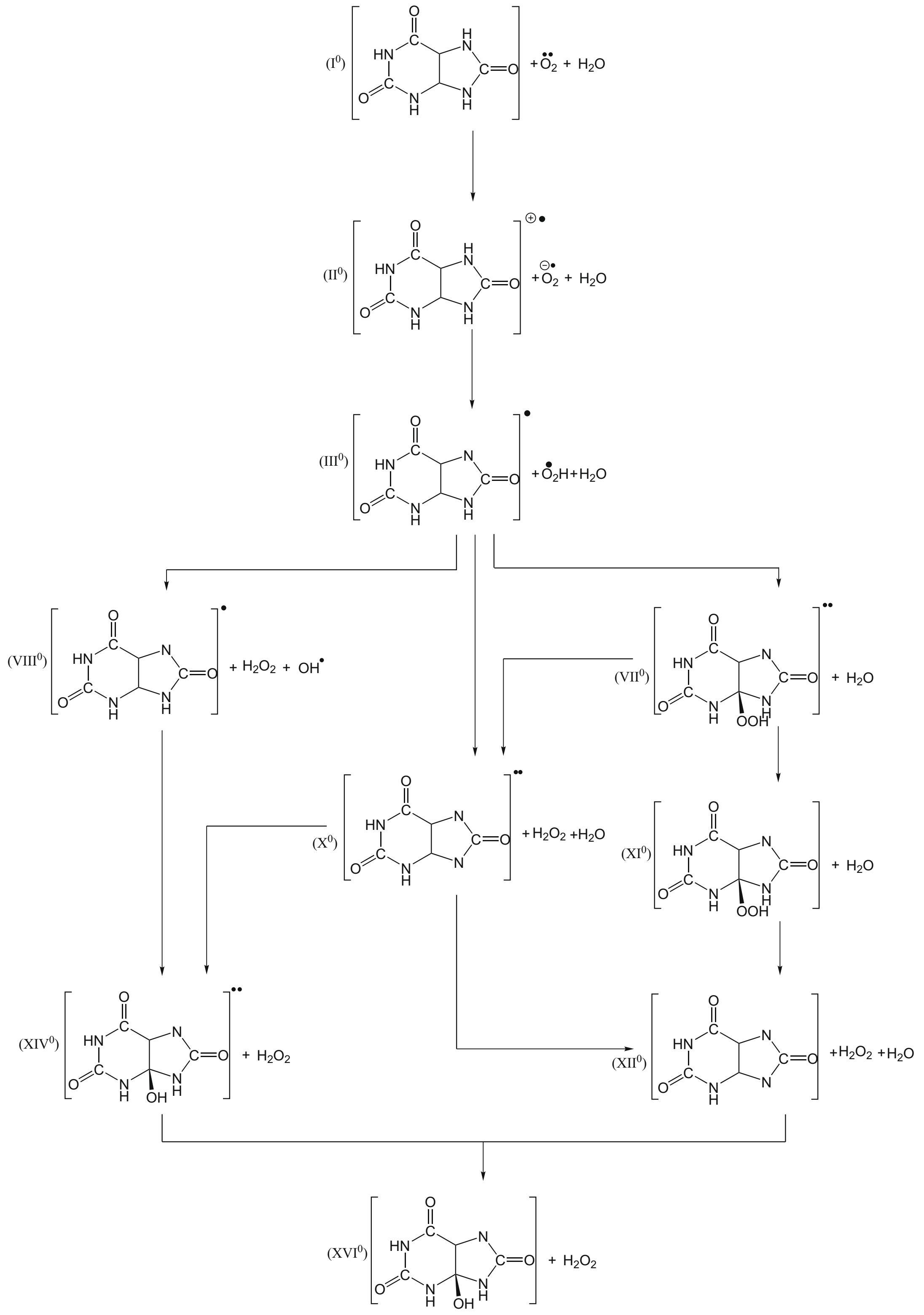

Fig. 5. Hypothesis [H-7 → 4-OH]: oxidation of uric acid by dioxygen initiated by an electron transfer and a H-7 proton transfer to O_2.

which lies 0.9 or 3.4 kcal/mol using DFT or MP2 methods, respectively, below the initial reactants (I^-).

This is this reaction path that has been studied by Kahn [21] and that showed the formation of a 5-hydroperoxyisourate intermediate lower in energy than the 5-hydroisourate product.

(2) A hydrogen can be transferred from the water molecule to the OOH radical to form H_2O_2 and OH radical (VI^-). This reaction step gives intermediate at 64.2 or 75.7 kcal/mol using DFT or MP2 methods, respectively, above the reactants. Then the hydroxyl radical adds up to the C-5 carbon to form a 5-hydroxyisourate in its triplet state ($XIII^-$) with

Table 2
Relative reaction energies for the oxidation mechanism of urate 3-monoanion using Hypothesis [H-9 → 5-OH] according to different quantum methods (ΔE in kcal/mol).

UAN_3^-	PM3			HF/6-31G**			B3LYP/6-31G**			MP2//DFT/6-31G**		
I$^-$	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0
II$^-$	65.8	65.8	65.8	75.9	75.9	75.9	73.2	73.2	73.2	87.3	87.3	87.3
III$^-$	25.9	25.9	25.9	32.8	32.8	32.8	31.6	31.6	31.6	48.0	48.0	48.0
V$^-$	–	–	30.5	–	–	81.5	–	–	58.5	–	–	72.6
VI$^-$	44.1	–	–	54.7	–	–	64.2	–	–	75.7	–	–
IX$^-$	–	–	−4.0	–	–	10.0	–	–	−7.0	–	–	−12.6
X$^-$	–	17.8	–	–	74.2	–	–	59.9	–	–	83.4	–
XII$^-$	–	5.0	5.0	–	31.3	31.3	–	16.3	16.3	–	15.0	15.0
XIII$^-$	24.5	–	–	73.1	–	–	65.7	–	–	81.6	–	–
XV$^-$	−10.9	−10.9	−10.9	13.1	13.1	13.1	−0.9	−0.9	−0.9	−3.4	−3.4	−3.4

energies at 65.7 and 81.6 using DFT or MP2 methods, respectively. Finally, the transformation of the latter system to a singlet state leads to the final products (XV$^-$).

(3) A proton transfer of H-7 to the OOH radical can form H_2O_2 associated with a triplet state dehydrourate intermediate (X$^-$). The energy then rises to 59.9 or 83.4 kcal/mol according to our DFT and MP2 calculations, respectively. From this state the reaction pathway can finally bifurcate to intermediate (XIII$^-$) or (XII$^-$) identified earlier.

From Table 2, the oxidation of urate 3-monoanion by dioxygen leads to the formation of a 5-hydroxyisourate product according to our Hypothesis [H-9 → 5-OH]. This reaction is thermodynamically favored as the products are set at −0.9 kcal/mol from DFT calculations and −3.4 kcal/mol from MP2 calculations, respectively. PM3 and HF/6-31G** computations share similar conclusions: the formation of the 5-hydroxyisourate from urate 3-monoanion is thermodynamically favored as compared to the reactants.

The comparison between the three hypothetical reaction pathways shows the first reaction pathway (i.e., the third row in Table 2) goes through the formation of an intermediate (IX$^-$) more stable than the reactants and the products. The higher energies of the intermediates in the two other reaction pathways can be easily interpreted using the fact that, in most cases, these are triplet state energies higher than their singlet state counterparts (intermediates (V$^-$) vs. (IX$^-$), (X$^-$) vs. (XII$^-$), and (XIII$^-$) vs. (XV$^-$)).

Table 3 gathers the different relative energies of the considered intermediates existing along the different hypothetical mechanisms of Hyp. [H-7 → 4-OH] (Fig. 7). From these results, the first part of the reaction consisting in an electron transfer between urate and dioxygen to form the superoxide (II$^-$) is the same as in Hyp. [H-9 → 5-OH]. Then, a proton transfer is initiated between N-7 and O_2 giving intermediate (III$^-$) with energies of 24.3 and 39.8 kcal/mol above the reactants using B3LYP and MP2 methods, respectively. Comparing the proton transfer energies between H-7 and H-9, one can see the proton transfer from the N-7 position of urate 3-monoanion (II$^-$ → IV$^-$) is more favorable than the proton transfer from the N-9 position (II$^-$ → III$^-$).

After this proton transfer, three different reaction mechanisms are possible in Hyp. [H-7 → 4-OH] as in Hyp. [H-9 → 5-OH]. Along this reaction pathways, the fixation of the OOH or OH groups are done via the C-4 carbon of urate. Comparing both hypothesis, the fixation of the OOH and OH groups on C-4 in a triplet state gives more stable intermediates (VII$^-$ and XIV$^-$ *vs.* V$^-$ and XIII$^-$, respectively). However, the changes to a singlet state with the fixation on C-4 gives intermediates less stable than their C-5 counterparts (XI$^-$ and XVI$^-$ *vs.* IX$^-$ and XV$^-$, respectively). Finally, when comparing the energies of the final products in Hyp. [H-9 → 5-OH] and Hyp. [H-7 → 4-OH], one can note the 5-hydroxyisourate product is more stable than the 4-hydroxyisourate one by 6.9 and 16.1 kcal/mol according to DFT and MP2 calculations, respectively.

3.3.2. Thermodynamical analysis

We have computed in the cases of Hyp. [H-9 → 5-OH] and Hyp. [H-7 → 4-OH] thermodynamical data representing the different considered mechanisms. They are reported Tables 4 and 5. We have calculated the Zero Point Energy corrections (ZPE), the relative enthalpies, and the relative free energies along each reaction pathway. From these tables, one can note the ZPE corrections have a negligible influence on the relative energy values. The relative enthalpies relate similar energy changes as the relative internal energies (ΔE) and the relative free energies are in good agreement with the relative internal energies. Thus, the computational results we have obtained show the free energy changes along all the considered reaction pathways confirm the conclusions made from the internal energies: the 5-hydroxyisourate is thermodynamically more stable than the 4-hydroxyisourate.

Similar thermodynamical data have also been computed for each reaction pathway and for each urate derived species (i.e., uric acid, its mono and dianions). For the clarity of this article and because they do not change the main conclusions presented here as with urate 3-monoanion, they are reported in Supplementary materials.

3.3.3. Influence of diffuse functions

Because we mainly here study the properties of anions by means of quantum mechanics, we have analyzed the possible influence of diffuse functions in the used basis set through a B3LYP/6-31+G** study. Tables 6 and 7 present the results of our computations performed in this basis set. For the first step of the reaction (I$^-$ → II$^-$) the use of diffuse functions decreases the relative energies from 73.2 to 60.9 kcal/mol. In contrast, for the rest of the reaction pathways, the diffuse functions have nearly no effect on the relative energies. These results are confirmed by the computations of ZPE corrections, relative enthalpies, and relative free energies as reported in Tables 6 and 7.

3.4. Intrinsic reactivity of the urate 3,7-dianion

In the case of urate 3,7-dianion, only one proton transfer can be performed between urate and dioxygen: the transfer of the proton attached to N-9 because, to the opposite of uric acid or urate 3-monoanion, the H-7 proton is not present. The Hypothesis [H-7 → 4-OH] considered for the two previous species is, therefore, irrelevant. However, there are still two possible mechanisms: Hypothesis [H-9 → 5-OH] and Hypothesis [H-9 → 4-OH], respectively, depicted in Figs. 8 and 9. Similarly, Hypothesis [H-9 → 5-OH] consider a final position of the OH group attached to C5, while Hypothesis [H-9 → 4-OH] ends with the formation of a 4-hydroxyisourate. In each hypothesis there is still three possible reaction pathways as mentioned earlier: one through the formation of a hydroperoxyisourate, one through the formation of a dehydrourate, and one through the formation of a hydroxyl radical. The relative reaction energies calculated for the intrinsic reactivity of

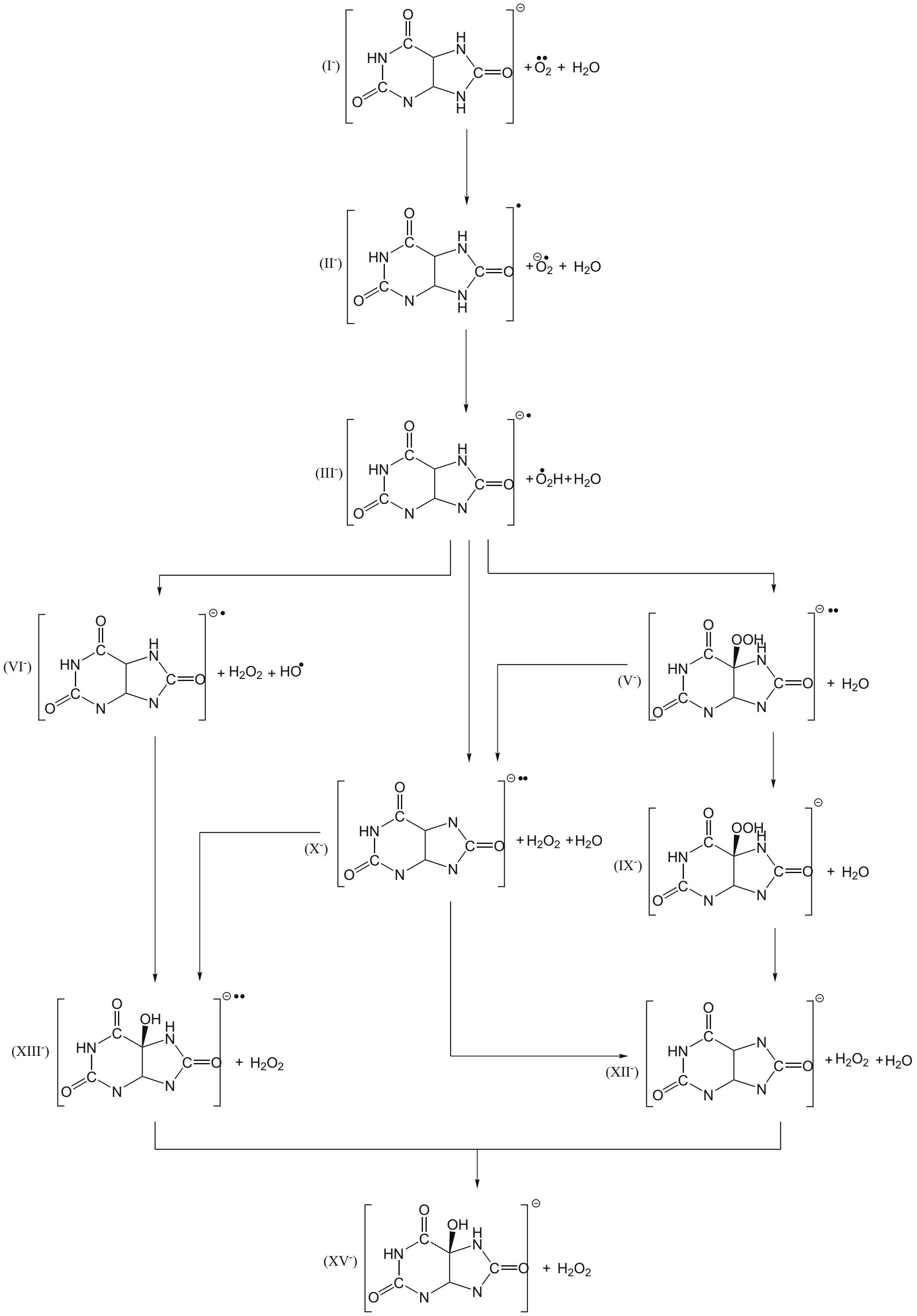

Fig. 6. Reactivity of the 3-monoanion derived from uric acid with dioxygen according to Hypothesis [H-9 → 5-OH].

urate 3,7-dianion using different quantum methods are reported in Table 8 for Hypothesis [H-9 → 5-OH], and Table 9 for Hypothesis [H-9 → 4-OH].

The first part of the reaction is identical for all six conceivable mechanisms: it consists of the reactants (I^{2-}) in transferring an electron from urate 3-7 dianion to dioxygen (II^{2-}). This is accomplished with an energy gain of, respectively, −47.7 and −36.9 kcal/mol according to our B3LYP/6-31G** and MP2//B3LYP/6-31G** computations. Then, the proton transfer from N-9 to form perhydroxyl radical (III^{2-}) leads to an energy of 19.0 kcal/mol or 36.7 kcal/mol above the reactants using DFT or MP2 calculations, respectively.

Table 3
Relative reaction energies for the oxidation mechanism of urate 3-monoanion using Hypothesis [H-7 → 4-OH] according to different quantum methods (ΔE in kcal/mol).

UAN_3^-	PM3			HF/6-31G**			B3LYP/6-31G**			MP2//DFT/6-31G**		
I^-	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0
II^-	65.8	65.8	65.8	75.9	75.9	75.9	73.2	73.2	73.2	87.3	87.3	87.3
IV^-	20.5	20.5	20.5	25.6	25.6	25.6	24.3	24.3	24.3	39.8	39.8	39.8
VII^-	–	–	40.7	–	–	55.1	–	–	48.4	–	–	58.1
$VIII^-$	38.7	–	–	47.5	–	–	57.0	–	–	67.4	–	–
X^-	–	17.8	–	–	74.2	–	–	59.9	–	–	83.4	–
XI^-	–	–	2.6	–	–	19.3	–	–	9.5	–	–	2.5
XII^-	–	5.0	5.0	–	31.3	31.3	–	16.3	16.3	–	15.0	15.0
XIV^-	35.9	–	–	58.2	–	–	54.1	–	–	66.0	–	–
XVI^-	−1.7	−1.7	−1.7	32.1	32.1	32.1	6.0	6.0	6.0	12.7	12.7	12.7

In Hypothesis [H-9 → 5-OH], the direct addition of a OOH molecule to the state (III^{2-}) leads to state (IV^{2-}): a triplet 5-hydroperoxyisourate lying 51.4 kcal/mol above the reactants (I^{2-}) according to B3LYP/6-31G**. The corresponding singlet state (VII^{2-}) has a relative energy of −17.7 kcal/mol using the same DFT method. The departure of a OOH^- molecule to form a dehydrourate (X^{2-}) forms again a stable intermediate −26.5 kcal/mol below the initial reactants. A proton can then be transferred between H_2O and OOH^- to form a hydroxyl anion together with hydrogen peroxide (intermediate XI relatively located at +0.2 kcal/mol). Finally, the addition of the hydroxyl anion to the dehydrourate intermediate leads to the formation of the final product lying at only −1.1 kcal/mol relatively to the reactants.

The second reaction pathway envisaged in Hypothesis [H-9 → 5-OH] starts with an electron transfer from the doublet dehydrourate (III^{2-}) to the perhydroxyl radical to form triplet dehydrourate together with a singlet hydroperoxide anion ($VIII^{2-}$). This state is lying at 17.2 kcal/mol above the reactants using B3LYP/6-31G**. The corresponding singlet state is (X^{2-}) and the reaction can continue following the pathway described in the previous paragraph.

The third reaction pathway envisaged in Hypothesis [H-9 → 5-OH] implies the formation of a hydroxyl radical through a hydrogen transfer from the water molecule to perhydroxyl radical (V^{2-}). This state is lying 51.6 kcal/mol above the reactants according to B3LYP/6-31G** computations. The addition of the hydroxyl radical to doublet dianion dehydrourate leads to a triplet 5-hydroxyisourate 70.7 kcal/mol above (I^{2-}). The corresponding singlet state is the final product (XIV^{2-}).

Analog results to Hypothesis [H-9 → 5-OH] have been obtained for Hypothesis [H-9 → 4-OH] as shown in Table 9. The only major difference is the final product formed is the 4-hydroxyisourate. This final state (XV^{2-}) has a relative energy of +12.5 kcal mol and +8.4 kcal/mol using B3LYP/6-31G** and MP2//B3LYP/6-31G** computations, respectively.

4. Discussion

Our calculations on the possible initiations of the reaction show the best substrate reactant for dioxygen is a urate dianion because all urate dianions have negative ionization potentials. They then tend to transfer spontaneously one of their electrons to dioxygen (Step I^{2-} to Step II^{2-}), while neutral uric acid and urate monoanions do not. Because uric acid has two pKas in solution (around 5.5 and 10.5), urate dianion forms are mainly present in solution at very high pH. This can explain in part why the optimum pH for urate oxidase activity is above 8 [7,8]. However, this optimum pH is not as high as one could expect if urate oxidase was to directly bind urate dianions (i.e., only ~1% of urate ions at pH 8 are urate dianions). At the optimum pH or even more at physiological pH, one of the roles of the enzyme will be to activate the most prominent urate 3-monoanion in urate 3,7-dianion. Experimentally, Imhoff et al. has recently performed site-directed mutagenesis on urate oxidase and suggested the presence of a catalytic diad formed by an active site of theorine and a neighbouring lysine [19]. These two residues could act together as a general base to abstract the hydrogen in position 7 of urate 3-monoanion.

Crystallographic data show that urate oxidase only binds the urate 3,7-dianion although urate 3,9-dianion is the major species in solution [13]. By comparing the difference in ionization potentials between $UAN_3^-N_7^-$ and $UAN_3^-N_9^-$ ($\Delta IP = -9.6$ kcal/mol according to B3LYP/6-31G**) and the relative difference in energy between these two compounds ($\Delta E = 2.3$ kcal/mol according to B3LYP/6-31G**), the energetic balance for losing one electron is in favor of urate 3,7-dianion. From our calculations, this is still true regardless of the quantum methodology used (see [13] and Supplementary materials). Thus, urate oxidase only binds the urate 3,7-dianion because it is the substrate that will enable the easiest initiation of the reaction: a spontaneous transfer of one electron to dioxygen. Biologically, this implies UOx gene has evolved to bind the most reducing available urate species.

Comparing Tables 8 and 9, it appears that the 5-hydroxyisourate product is favored compared to 4-hydroxyisourate, because it is the most thermodynamically stabilized product. This result is valid for all starting urate species (see Supplementary materials). Thus, the biochemical reaction catalyzed by urate oxidase is the most thermodynamically favored reaction starting from a uric acid species and giving a hydroxyisourate species. By "choosing" a urate 3,7-dianion as the reaction substrate and a 5-hydroxyisourate as the reaction product, urate oxidase allows for a first fast initiation of the reaction and a thermodynamically favored product. This can be regarded as one of the catalytic effects of urate oxidase.

From the three possible reaction pathways hypothesized in Hypothesis [H-9 → 5-OH], one can note all triplet spin intermediate states are always much higher in energy than their singlet counterparts. This happens for IV^{2-} vs. VII^{2-}, for $VIII^{2-}$ vs. X^{2-}, and for XII^{2-} vs. XIV^{2-} states. The initial reactant is a triplet state system because of the presence of triplet dioxygen, while the final product is a singlet system (XIV^{2-}). This change in spin state can be easily understood by noting the first step of the mechanism in which a spontaneous transfer of one electron between urate 3,7-dianion and triplet dioxygen occurs. This forms a still global triplet spin state intermediate, which is the sum of two doublet molecules. Because in the enzyme the two lone electrons will be located on quite separated molecular moieties, this triplet state will be degenerated in energy with the corresponding singlet state system (two electron with +1/2 spin *vs.* one electron with +1/2 spin and one electron with −1/2 spin). Thus, intermediates II^{2-} and III^{2-} can be viewed equally as triplet or singlet intermediates having the same energies. Therefore, the whole reaction mechanism from intermediate II^{2-} (after the spontaneous electron transfer) to the final product XIV^{2-} can happen in singlet state only, without requiring any triplet intermediates. Accordingly, our three hypothesized mechanisms can be considered without the triplet states. This

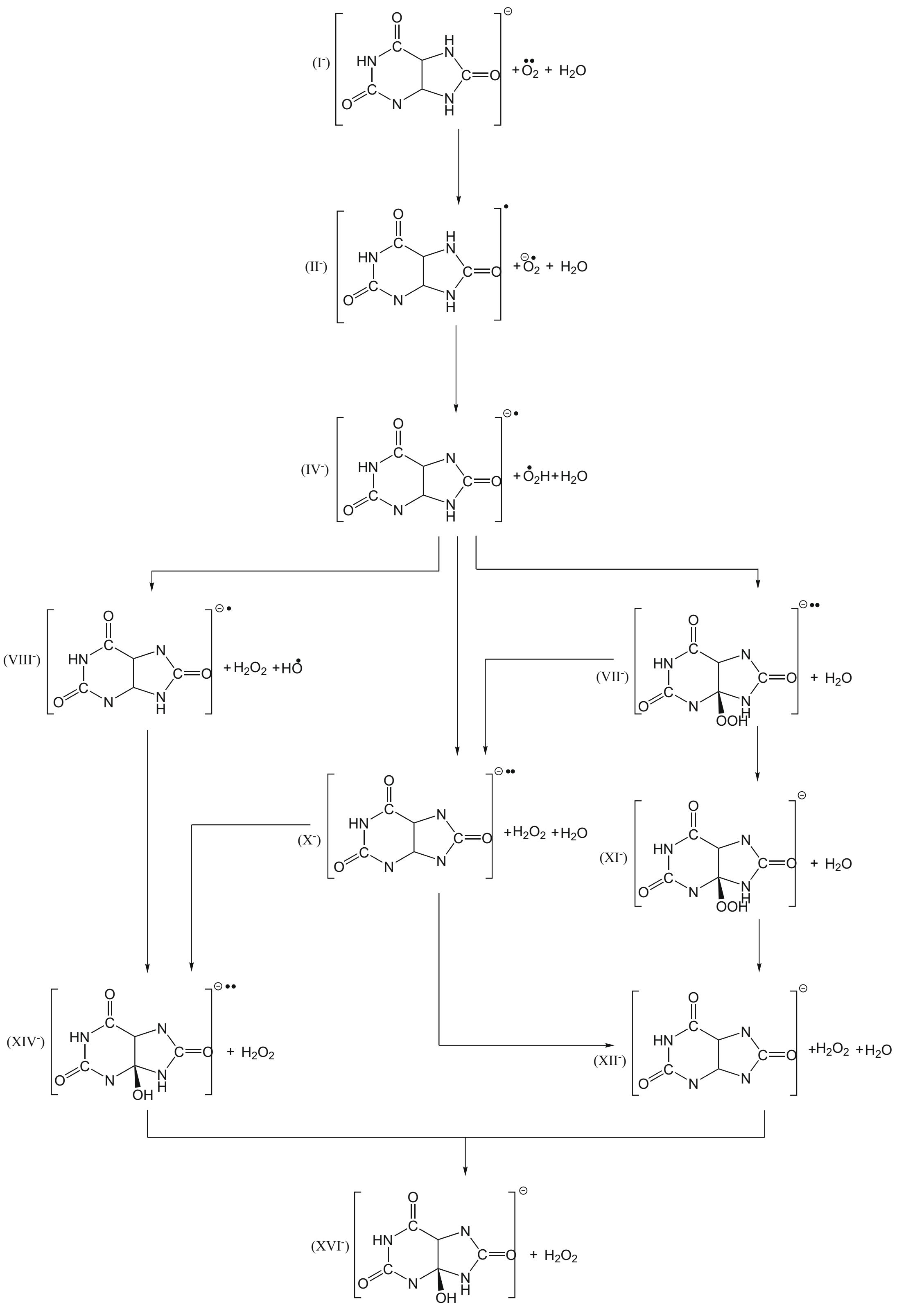

Fig. 7. Reactivity of the 3-monoanion derived from uric acid with dioxygen according to Hypothesis [H-7 → 4-OH].

gives two main pathways: one formed by intermediates $I^{2-}, II^{2-}, III^{2-}, V^{2-}$, and XIV^{2-}, and one formed by intermediates $I^{2-}, II^{2-}, III^{2-}, VII^{2-}, X^{2-}, XI^{2-}$, and XIV^{2-}. In the latter case, it is possible that intermediate VII^{2-} can be skipped. This will correspond to the third mechanism envisaged before.

To further discriminate between these mechanisms and to identify which is used by the enzyme, energetics for the different envisaged reaction paths in Table 8 must be more deeply analyzed. In the first mechanism, the electronic charge is conserved for each entities from III^{2-} to XIV^{2-}, but intermediate V^{2-} seems too high

Table 4
Relative internal energies (ΔE), relative internal energies with ZPE corrections at 0K (ΔE_{ZPE}), relative enthalpies (ΔH), and relative free energies (ΔG) for the oxidation mechanism of urate 3-monoanion using Hypothesis [H-9 → 5-OH] and B3LYP/6-31G** (in kcal/mol).

UAN_3^-	ΔE			ΔE_{ZPE}			ΔH			ΔG		
I^-	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0
II^-	73.2	73.2	73.2	72.8	72.8	72.8	72.8	72.8	72.8	72.9	72.9	72.9
III^-	31.6	31.6	31.6	29.9	29.9	29.9	29.9	29.9	29.9	28.3	28.3	28.3
V^-	–	–	58.5	–	–	57.8	–	–	57.2	–	–	67.5
VI^-	64.2	–	–	62.1	–	–	62.1	–	–	60.9	–	–
IX^-	–	–	−7.0	–	–	−5.4	–	–	−5.9	–	–	6.0
X^-	–	59.9	–	–	56.6	–	–	56.6	–	–	54.9	–
XII^-	–	16.3	16.3	–	15.3	15.3	–	15.3	15.3	–	13.9	13.9
XIII^-	65.7	–	–	65.9	–	–	65.3	–	–	74.0	–	–
XV^-	−0.9	−0.9	−0.9	1.6	1.6	1.6	1.0	1.0	1.0	11.4	11.4	11.4

Table 5
Relative internal energies (ΔE), relative internal energies with ZPE corrections at 0K (ΔE_{ZPE}), relative enthalpies (ΔH), and relative free energies (ΔG) for the oxidation mechanism of urate 3-monoanion using Hypothesis [H-7 → 4-OH] and B3LYP/6-31G** (in kcal/mol).

UAN_3^-	ΔE			ΔE_{ZPE}			ΔH			ΔG		
I^-	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0
II^-	73.2	73.2	73.2	72.8	72.8	72.8	72.8	72.8	72.8	72.9	72.9	72.9
IV^-	24.3	24.3	24.3	23.0	23.0	23.0	23.0	23.0	23.0	21.6	21.6	21.6
VII^-	–	–	48.4	–	–	48.0	–	–	48.1	–	–	56.2
VIII^-	57.0	–	–	55.2	–	–	55.2	–	–	54.2	–	–
X^-	–	59.9	–	–	56.6	–	–	56.6	–	–	54.9	–
XI^-	–	–	9.5	–	–	−0.4	–	–	−1.0	–	–	10.9
XII^-	–	16.3	16.3	–	15.3	15.3	–	15.3	15.3	–	13.9	13.9
XIV^-	54.1	–	–	54.0	–	–	54.3	–	–	64.3	–	–
XVI^-	6.0	6.0	6.0	8.1	8.1	8.1	7.5	7.5	7.5	17.5	17.5	17.5

Table 6
Relative internal energies (ΔE), relative internal energies with ZPE corrections at 0K (ΔE_{ZPE}), relative enthalpies (ΔH), and relative free energies (ΔG) for the oxidation mechanism of urate 3-monoanion using Hypothesis [H-9 → 5-OH] and B3LYP/6-31+G** (in kcal/mol).

UAN_3^-	ΔE			ΔE_{ZPE}			ΔH			ΔG		
I^-	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0
II^-	60.9	60.9	60.9	60.4	60.4	60.4	60.4	60.4	60.4	60.4	60.4	60.4
III^-	31.0	31.0	31.0	29.3	29.3	29.3	29.4	29.4	29.4	27.6	27.6	27.6
V^-	–	–	56.0	–	–	55.8	–	–	55.5	–	–	65.1
VI^-	63.7	–	–	61.6	–	–	61.6	–	–	60.3	–	–
IX^-	–	–	−4.9	–	–	−3.2	–	–	−3.8	–	–	8.0
X^-	–	48.4	–	–	46.2	–	–	46.2	–	–	44.1	–
XII^-	–	15.7	15.7	–	14.7	14.7	–	14.7	14.7	–	13.3	13.3
XIII^-	66.9	–	–	67.1	–	–	66.5	–	–	75.1	–	–
XV^-	1.3	1.3	1.3	3.8	3.8	3.8	3.2	3.2	3.2	13.4	13.4	13.4

Table 7
Relative internal energies (ΔE), relative internal energies with ZPE corrections at 0K (ΔE_{ZPE}), relative enthalpies (ΔH), and relative free energies (ΔG) for the oxidation mechanism of urate 3-monoanion using Hypothesis [H-7 → 4-OH] and B3LYP/6-31+G** (in kcal/mol).

UAN_3^-	ΔE			ΔE_{ZPE}			ΔH			ΔG		
I^-	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0
II^-	60.9	60.9	60.9	60.4	60.4	60.4	60.4	60.4	60.4	60.4	60.4	60.4
IV^-	24.4	24.4	24.4	23.2	23.2	23.2	23.2	23.2	23.2	21.6	21.6	21.6
VII^-	–	–	50.5	–	–	50.3	–	–	49.7	–	–	60.5
VIII^-	57.1	–	–	55.4	–	–	55.4	–	–	54.3	–	–
X^-	–	48.4	–	–	46.2	–	–	46.2	–	–	44.1	–
XI^-	–	–	9.3	–	–	9.2	–	–	9.2	–	–	9.0
XII^-	–	15.7	15.7	–	14.7	14.7	–	14.7	14.7	–	13.3	13.3
XIV^-	56.0	–	–	56.7	–	–	56.1	–	–	65.4	–	–
XVI^-	7.3	7.3	7.3	9.5	9.5	9.5	9.3	9.3	9.3	19.0	19.0	19.0

in energy to be considered as a viable intermediate in the reaction. A possible mechanism could only use intermediates $\mathrm{I}^{2-}, \mathrm{II}^{2-}, \mathrm{III}^{2-}$, and XIV^{2-} in Fig. 8 by suggesting a direct attack mechanism of the water molecule on the dehydrourate radical with a concerted hydrogen transfer to the perhydroxyl radical. This could be the role of the W1 water molecule identified in some UOx X-ray structures [16,22], but the role of this water molecule has been recently challenged [18]. In the second mechanism, intermediates VII^{2-} and X^{2-} are much lower in energy than the final product XIV^{2-}. The thermodynamics of this reaction, previously suggested by Kahn [21], would imply the true product of UOx should be a dehydrourate instead of a 5-hydroxyisourate, unless one of the roles of UOx could

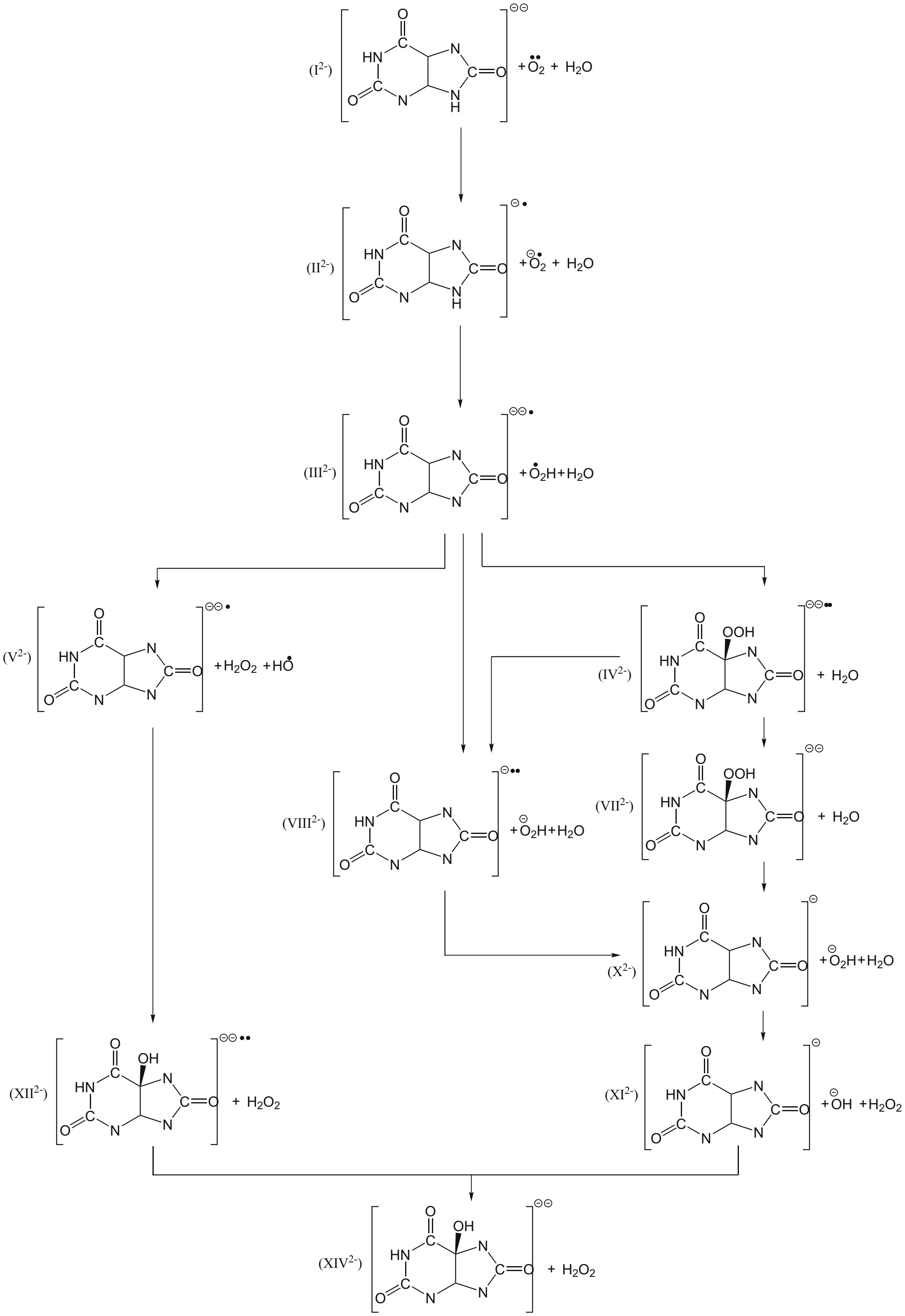

Fig. 8. Reactivity of the urate 3,7-dianion derived from uric acid with dioxygen according to Hypothesis [H-9 → 5-OH].

be to destabilize these intermediates to make them higher in energy than the final product through enzyme/substrate interactions.

In addition, from Table 8, one can note that intermediate III^{2-} is rather high in energy as compared to intermediates II^{2-}, VII^{2-}, or X^{2-}. The transformation of intermediate II^{2-} in intermediate X^{2-} is globally a hydrogen transfer. It is reasonable to assume this hydrogen transfer could happen in only one step instead of being decomposed in a proton transfer followed by an electron transfer. Fig. 10 summarized the reaction path we suggest for urate oxidase which skips this high energy intermediate III^{2-}. Energy values

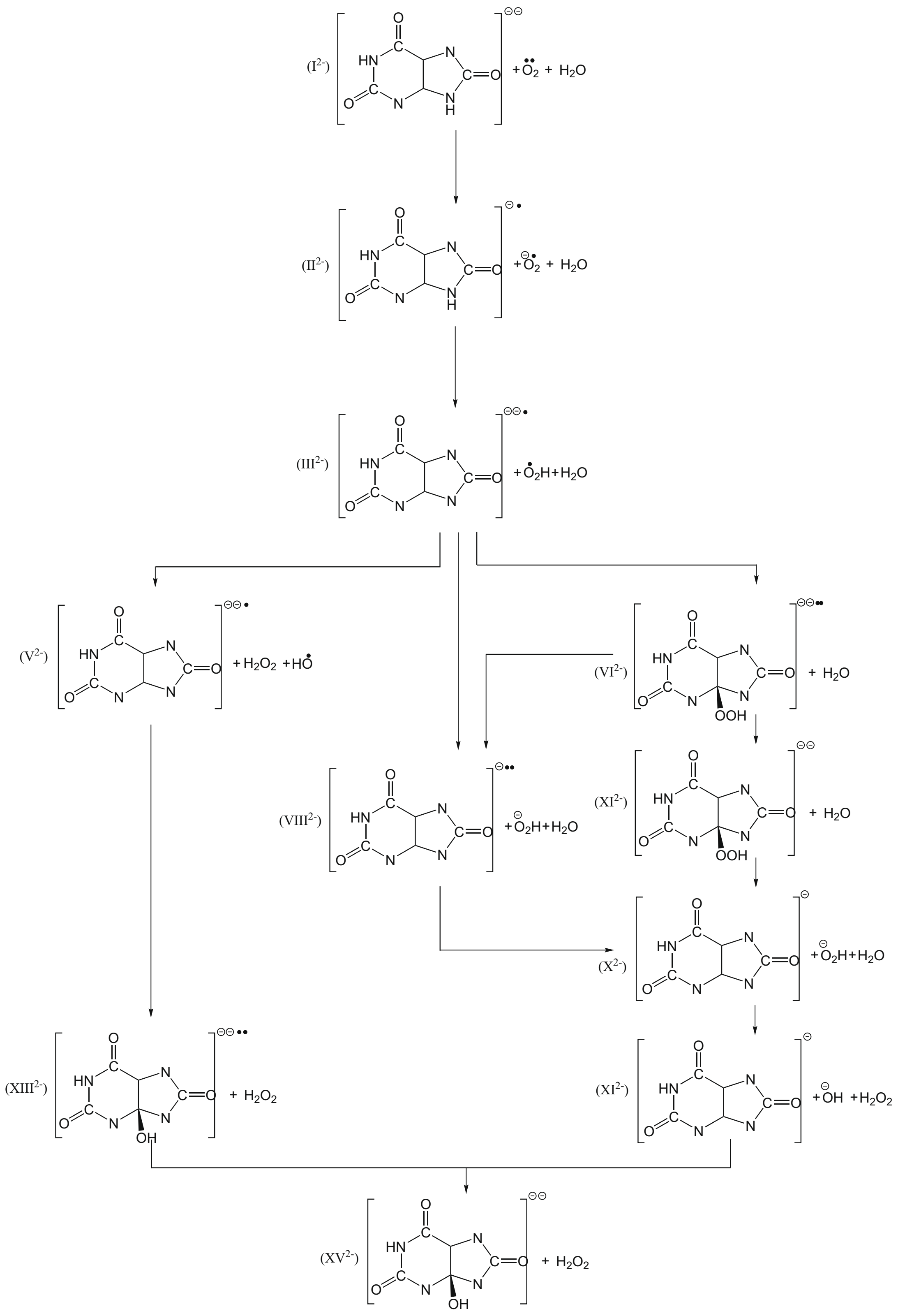

Fig. 9. Reactivity of the urate 3,7-dianion derived from uric acid with dioxygen according to Hypothesis [H-9 → 4-OH].

reported in this figure have been computed both at the MP2/6-31++G** level and at the B3LYP/6-31++G** in the same manner as the B3LYP/6-31G** results from Table 8 (i.e., all structures were fully optimized, Δ*E* is indicated using the infinitely separated reactants as the energy reference). These additional computations are expected to give better energetical results, because they employ a larger basis set including diffuse and polarization functions in addition to reliable electronic correlation evaluation. DFT and MP2 results from Fig. 10 are very similar except for the first step of the reaction ($I^{2-} \rightarrow II^{2-}$). There is a 14.8 kcal/mol difference

Table 8
Relative reaction energies for the oxidation mechanism of urate 3,7-dianion using Hypothesis [H-9 → 5-OH] according to different quantum methods (Δ*E* in kcal/mol).

$UAN_3^-N_7^-$	PM3			HF/6-31G**			B3LYP/6-31G**			MP2//DFT/6-31G**		
I^{2-}	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0
II^{2-}	−45.0	−45.0	−45.0	−43.5	−43.5	−43.−47.7	−47.7	−47.7	−36.9	−36.9	−36.9	
III^{2-}	12.5	12.5	12.5	17.3	17.3	17.3	19.0	19.0	19.0	36.7	36.7	36.7
IV^{2-}	–	–	24.6	–	–	58.4	–	–	51.4	–	–	72.8
V^{2-}	30.7	–	–	39.2	–	–	51.6	–	–	64.4	–	–
VII^{2-}	–	–	−16.8	–	–	−1.1	–	–	−17.7	–	–	−21.4
$VIII^{2-}$	–	−20.3	–	–	25.7	–	–	17.2	–	–	43.7	–
X^{2-}	–	−33.2	−33.2	–	−17.2	−17.2	–	−26.5	−26.5	–	−24.7	−24.7
XI^{2-}	–	−14.0	−14.0	–	11.3	11.3	–	0.2	0.2	–	1.5	1.5
XII^{2-}	17.1	–	–	66.1	–	–	70.7	–	–	87.3	–	–
XIV^{2-}	−17.4	−17.4	−17.4	10.7	10.7	10.7	−1.1	−1.1	−1.1	−2.4	−2.4	−2.4

Table 9
Relative reaction energies for the oxidation mechanism of urate 3,7-dianion using Hypothesis [H-9 → 4-OH] according to different quantum methods (Δ*E* in kcal/mol).

$UAN_3^-N_7^-$	PM3			HF/6-31G**			B3LYP/6-31G**			MP2//DFT/6-31G**		
I^{2-}	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0
II^{2-}	−45.0	−45.0	−45.0	−43.5	−43.5	−43.5	−47.7	−47.7	−47.7	−36.9	−36.9	−36.9
III^{2-}	12.5	12.5	12.5	17.3	17.3	17.3	19.0	19.0	19.0	36.7	36.7	36.7
V^{2-}	30.7	–	–	39.2	–	–	51.6	–	–	64.4	–	–
VI^{2-}	–	–	19.2	–	–	43.1	–	–	35.7	–	–	48.8
$VIII^{2-}$	–	−20.3	–	–	25.7	–	–	17.2	–	–	43.7	–
IX^{2-}	–	–	−7.4	–	–	14.2	–	–	−5.2	–	–	−11.4
X^{2-}	–	−33.2	−33.2	–	−17.2	−17.2	–	−26.5	−26.5	–	−24.7	−24.7
XI^{2-}	–	−14.0	−14.0	–	11.3	11.3	–	0.2	0.2	–	1.5	1.5
$XIII^{2-}$	19.2	–	–	60.9	–	–	44.9	–	–	56.9	–	–
XV^{2-}	−8.9	−8.9	−8.9	27.6	27.6	27.6	12.5	12.5	12.5	8.4	8.4	8.4

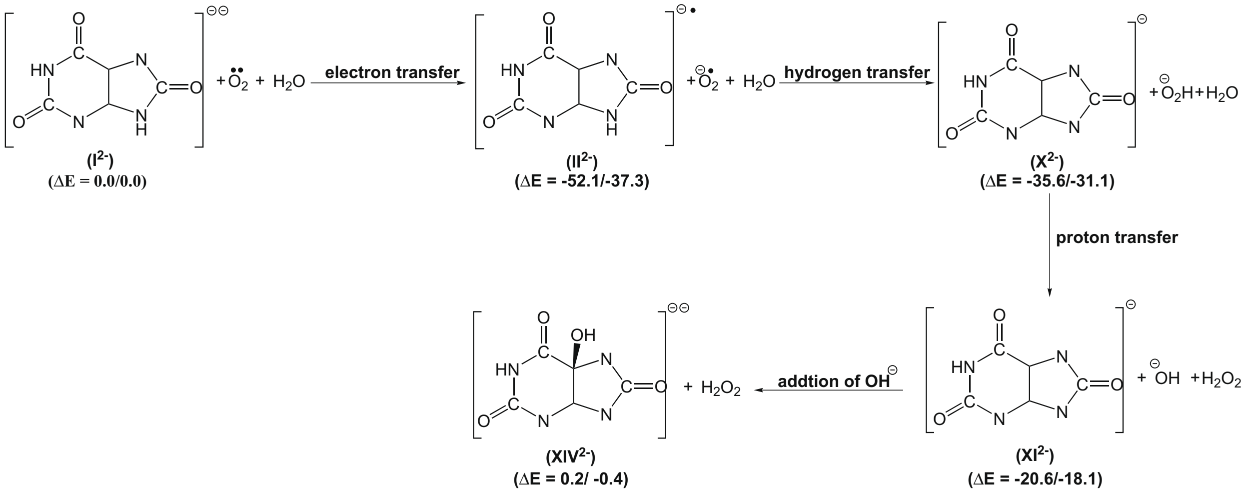

Fig. 10. Our proposal for the reactivity of urate in urate oxidase starting from urate 3,7-dianion ($UAN_3^-N_7^-$). Reported energies (Δ*E* in kcal/mol) are relative energies compared to the reactants computed from fully optimized B3LYP/6-31++G** and MP2/6-31++G** structures, respectively.

which is due to a difference between MP2 and DFT methods in calculating the ionization potential of $UAN_3^-N_7^-$ (~3.5 kcal/mol) and in calculating the electronic affinity of O_2 (~11.3 kcal/mol) [32]. By skipping intermediate III^{2-}, it is then not possible to form intermediate VII^{2-}. Moreover, our DFT calculations indicate the relative energy of the latter is −13.9 kcal/mol below the reactants, while intermediates X^{2-} and XI^{2-} are −35.6 and −20.6 kcal/mol below the reactants, respectively. The major outcome of the dehydro intermediate X^{2-} should, therefore, be intermediate XI^{2-} subsequently transformed into the final product XIV^{2-} rather than the hydroperoxy intermediate VII^{2-}. This energetic trend should be reinforced by the active site of urate oxidase: the dehydrourate is structurally very close to uric acid while the tetrahydric geometry of C5 in hydroxyisourate or hydroperoxyisourate should be sterically destabilized and favor the ejection of the final product.

Our proposed mechanism, summarized in Fig. 10, suggests a reaction in four steps: (i) a very fast electron transfer from urate to O_2 to form a superoxide with a monoanionic radicalar urate species in which singlet and triplet spin states are degenerated in energy; (ii) a hydrogen transfer from urate to the superoxide to form a dehydrourate and a hydroperoxyl anion; (iii) a proton transfer between a water molecule and the hydroperoxyl anion to form hydrogen peroxide and a hydroxyl anion; (iv) the addition of the hydroxyl anion to the dehydrourate molecule to form the final 5-hydroxyisourate product. It is worth noting here that the formation of the dehydrourate intermediate is due to the fact that the starting reactants include the $UAN_3^-N_7^-$ dianion. In this case, it is the dehydrourate intermediate that will form preferentially rather than the 5-hydroperoxyisourate. This is in opposition to the reaction starting with the UAN_3^- monoanion, where the addition of the superoxide to the urate radical would preferentially form the 5-hydroperoxyisourate intermediate.

Overall, our computational results seem to rule out the possibility of having a 5-hydroperoxyisourate intermediate in the urate oxidase catalytic mechanism. In their paper, Kahn et al. [20] characterized, by UV–visible spectroscopy combined with stopped flow experiments, an intermediate, named INT II, which they assigned to 5-hydroperoxyisourate based on comparisons with similar known stable chemical entities. However, because this experimental intermediate is too unstable, there is no definitive evidence that it is a hydroperoxy intermediate, or a dehydrourate species. In a subsequent paper, Kahn studied the energetics of the urate oxidase reaction using quantum chemistry calculations [21]. He found the 5-hydroperoxyisourate intermediate was lower in energy than the dehydrourate and concluded the former was the observed INT II. However, these calculations were performed using the urate 3-monoanion as the starting reactant. These results are similar to our data reported in Table 2 where intermediate IX^{-} is always lower in energy than intermediate XII^{-}. However, a few years after the publication of this article by Kahn, it has been found by Retailleau et al. using X-ray crystallography that the true substrate of UOx docked inside its active site is the 3,7-dianion form [16]. To the opposite of urate 3-monoanion, in the case of the urate 3,7-dianion substrate, the 5-hydroperoxyisourate intermediate is always higher in energy than the dehydrourate derivative (see intermediates VII^{2-} and X^{2-} energies from Table 8). Therefore, given our computational results presented here, we raise concerns on the identity of INT II as characterized by Kahn et al. Our opinion is that it is not a 5-hydroperoxyisourate but rather a dehydrourate. The latter is a more stable intermediate, is structurally closer from the urate reactant, and can be simply formed from the reactants without requiring high energy intermediates (i.e., through a direct hydrogen transfer $II^{2-} \rightarrow X^{2-}$ instead of $II^{2-} \rightarrow III^{2-} \rightarrow VII^{2-}$, where intermediate III^{2-} is high in energy).

5. Conclusions

Quantum chemistry enabled us to evaluate energies from all possible intermediates that could arise along a reaction mechanism involving the transformation of uric acid into 5-hydroxyisourate. Focusing on the reaction of 3,7-dianion, the true substrate of urate oxidase, we found that (i) 5-hydroxyisourate is thermodynamically the most favored product compared to 4-hydroxyisourate; (ii) the first step of the reaction can only be a spontaneous transfer of one electron from urate dianion to dioxygen, therefore, producing a superoxide inside the active site of the protein; and (iii) while dioxygen is originally in a triplet state, the spontaneous electron transfer implies an energy degenerancy between the triplet state and an overall singlet state being the sum of two double state molecules, superoxide and urate 3,7-radical monoanion: no triplet state intermediate has, therefore, to be considered in the mechanism even though this reaction can be seen as radicalar.

Overall, three possible mechanisms have been identified to form 5-hydroxyisourate starting from urate 3,7-dianion. Energetic comparisons of each intrinsic reaction pathways seem to favor a mechanism where a dehydro intermediate would be the central molecule allowing the transformation of uric acid in 5-hydroxyisourate. In this mechanism, no 5-hydroperoxyisourate intermediate would be formed. To help clarifying whether INT II as characterized by Kahn et al. [20] belongs to a hydroperoxy or a dehydro family, further calculations should be carried out. First, because the only experimental evidence of the existence of INT II is UV–visible absorbance spectra, such spectra should be computed using adequate quantum chemistry techniques. Second, the true energetics of the catalytic reaction pathway should be explored through the use of either full QM or QM/MM methodology inside the enzyme active site. Both approaches will give us insights into the true reaction mechanism of urate oxidase and clearly identify the different possible roles of 5-hydroperoxyisourate and dehydrourate.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bioorg.2009.05.004.

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[32] Comparison between the experimental gas phase electronic affinity of dioxygen and our computations shows MP2/6-31++G^{**} has trouble reproducing the experimental value while B3LYP/6-31++G^{**} gives a reasonable estimation: experimental electronic affinity for O_2 is 10.4 kcal/mol; MP2/6-31++G^{**} and B3LYP/6-31++G^{**} give 2.4 kcal/mol and 13.7 kcal/mol, respectively. Using a larger basis set (aug-cc-pVTZ) slightly corrects the MP2 value to 6.8 kcal/mol and the DFT value to 12.3 kcal/mol.