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Role of the tautomerism of 2-azaadenine and 2-azahypoxanthine in substrate recognition by xanthine oxidase

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Summary

The tautomerism of 2-azaadenine and 2-hypoxanthine has been examined in the gas phase and in aqueous solution. The tautomerism in the gas phase has been studied by means of semiempirical and ab initio quantum-mechanical computations, as well as density-functional calculations. The influence of the aqueous solvent on the relative stability between tautomers has been estimated from self-consistent reaction field calculations performed with different high-level continuum models. The results provide a detailed picture of the tautomeric preference for these purine bases. The importance of tautomerism in the substrate recognition by xanthine oxidase is discussed. Finally, the rate of oxidation of 2-azaadenine and 2-hypoxanthine by xanthine oxidase is discussed in terms of the recognition model at the enzyme active site.

Introduction

The degradation metabolism of purines is mainly controlled by two enzymes: adenosine deaminase (EC 3.5.4.4) and xanthine oxidase (xanthine:O_2 oxidoreductase; EC 1.2.3.2) [1]. The former enzyme deaminates adenosine yielding inosine, which is subsequently decomposed into the ribose and the purine base, hypoxanthine (1,7-dihydro-6*H*-purin-6-one). Oxidation at position 2 of hypoxanthine by xanthine oxidase yields xanthine (1,7-dihydro-6*H*-purin-2,6-dione), which is also an intermediate product in the catabolism of guanine. Indeed, this enzyme catalyzes oxidation at position 8 of xanthine leading to uric acid, the final degradation product of purines in humans.

Xanthine oxidase is a very important pharmacological target, since a malfunctioning of this enzyme increases the content of uric acid and eventually leads to the deposition of sodium hydrogen urate monohydrate crystals in joints. This gives rise to a painful disease known as gout, which is clinically treated by the antihyperuricemic drug allopurinol [2] (pyrazolo[3,4-*d*]pyrimidin-6-one), a drug structurally related to hypoxanthine. Allopurinol is converted by xanthine oxidase into alloxanthine (pyrazolo[3,4-*d*]-pyrimidin-2,6-one). This latter compound inactivates the enzyme [3], thus inhibiting the formation of uric acid. The treatment of gout with allopurinol leads to the excretion of purines mainly as hypoxanthine and xanthine.

Owing to the biochemical and pharmacological relevance of xanthine oxidase, a great deal of research has focused on the structural and kinetic aspects of the enzymatic mechanism [4]. Xanthine oxidase and the related xanthine dehydrogenase (xanthine:NAD^+ oxidoreductase; EC 1.2.1.37) both comprise two equivalent, independent subunits [4], each containing one atom of molybdenum, one molecule of flavin adenine dinucleotide and two distinct iron-sulfur clusters. There are two spatially separated binding sites for the reducing (hypoxanthine, xanthine) and oxidizing (O_2, NAD^+) substrates. The enzyme follows a two-site ping-pong mechanism [4,5], in which all the prosthetic groups participate in catalysis forming an electron transport chain connecting the two binding sites. The basic catalytic unit consists of a MoOS moiety [4c,d].

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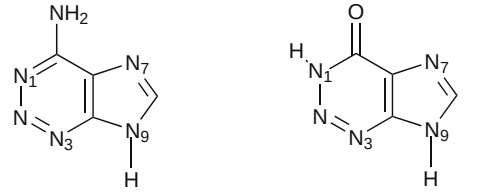

Fig. 1. Chemical structure of 2-azaadenine and 2-azahypoxanthine.

A rationale for the requirement of a sulfido group is that the sulfur atom is involved in abstraction of the acidic proton from the substrate. This step will trigger the carbanion attack on the oxygen of the Mo=O unit. Experimental studies suggest that the oxygen incorporated into the substrate is most likely that of the Mo=O unit, which is regenerated from the solvent water molecules in the course of each catalytic cycle. The molybdenum seems to be coordinated to a dithiolene side chain of a pterin cofactor, which may modulate the reactivity of the molybdenum center and/or its reduction potential [6]. Analysis of magnetic circular dichroism spectra from molybdenum complexes suggests that π-electron donation from filled sulfur orbitals of the dithiolene ligand may facilitate both the oxygen atom and electron transfer reactions that are proposed to occur in the catalytic cycle of the enzyme [7]. The spectral change associated with reduction of the molybdenum center suggests the presence of at least one ionizable group in the immediate vicinity of the molybdenum site [8].

Unfortunately, there is yet no structural information at atomic detail about the specific interactions that modulate substrate recognition and binding. Such information would be very valuable in the design of new potent, tight inhibitors [3,9] of xanthine oxidase lacking undesirable side effects [10]. Fortunately, information can be obtained from indirect approaches, i.e., from the study of the recognition patterns of powerful inhibitors or substrates of the enzyme. In this context, we have recently proposed a model for the interaction pattern that presumably modulates anchoring of the natural substrates hypoxanthine and xanthine, as well as of the inhibitors allopurinol and alloxanthine, to the enzyme active site [11]. In the present study our aim is to analyze the ability of such a model to explain the recognition of 2-azaadenine and 2-azahypoxanthine, which act as substrates of the enzyme [12].

2-Azaadenine and 2-azahypoxanthine differ from their parent compounds (adenine and hypoxanthine) in the replacement of the carbon atom at position 2 by nitrogen (Fig. 1). They are susceptible to the action of xanthine oxidase, which catalyzes oxidation at position 8 [12]. However, their susceptibility to the enzyme action is very different. Thus, the oxidation rate of 2-azaadenine is greater than that of hypoxanthine, and nearly equal to that of xanthine. Nevertheless, 2-azahypoxanthine exhibits a relative inertness to the attack of xanthine oxidase compared to 2-azaadenine.

The two compounds can exist as a mixture of different tautomers resulting from prototropic tautomerism at the imidazole and triazine moieties and from lactam–lactim or amino–imino equilibria. Since each tautomer has a specific pattern of hydrogen-bond donors and acceptors, inspection of the tautomeric preference allows us to discuss the recognition of these 2-azapurines by the enzyme, and to examine the reliability of the proposed model for substrate recognition at the active site.

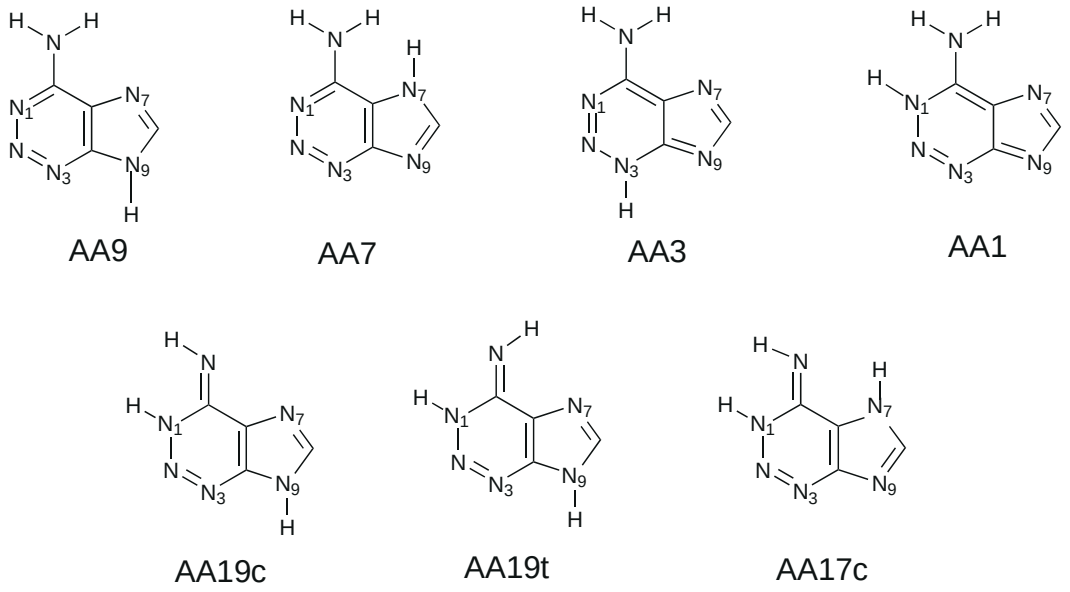

Fig. 2. Representation of the seven tautomers of the neutral 2-azaadenine included in the final study after the stepwise elimination process (see the text for details).

Methods

The tautomerism of 2-azaadenine and 2-azahypoxanthine was studied in the gas phase and in aqueous solution. Ab initio quantum-mechanical (QM) methods at the self-consistent field (SCF) and Møller–Plesset levels, and density functional (DFT) calculations were used to explore the tautomeric preference in the gas phase. The solvent effect was introduced by means of self-consistent reaction field (SCRF) continuum methods.

Owing to the large number of tautomers and the expensiveness of ab initio QM calculations, it was necessary to adopt the stepwise elimination scheme used in our previous studies [11] in order (i) to consider all the tautomeric species; and (ii) to obtain accurate estimates of the relative stability between tautomers. In this scheme the relative stability of all the tautomers in the gas phase was determined at the semiempirical level using the AM1 Hamiltonian [13], and the influence of hydration on tautomerism was estimated from AM1-SCRF calculations (see below). Those tautomers whose stability with respect to the most stable species was less than 20 kcal/mol (either in the gas phase or in aqueous solution) were considered for further analysis at the ab initio level. Initially, a gas-phase geometry optimization at the HF/6-31G(d) [14] level was performed, and the solvent effect was estimated by means of ab initio 6-31G(d) SCRF calculations (see below). Those tautomers whose free energy difference relative to the most stable species was greater than 15 kcal/mol (either in the gas phase or in aqueous solution) were excluded. In the final part of the study, single-point calculations at the SCF and second-order Møller–Plesset perturbation [15] (MP2) levels were carried out with the 6-31+G(d,p) basis [16]. In addition, DFT calculations were performed using the Becke3–Lee–Yang–Parr (B3LYP) functional [17]. The HF/6-31G(d) optimized geometries were used for all these calculations. Previous studies have shown that the refinement of structural parameters at higher computational levels has a very small effect on the tautomerism of closely related structures [18]. Thermal

TABLE 1
DIFFERENCES IN ENERGY, ENTHALPY AND FREE ENERGY IN THE GAS PHASE, RELATIVE FREE ENERGIES OF SOLVATION, AND FREE ENERGIES OF TAUTOMERIZATION IN AQUEOUS SOLUTION FOR SELECTED TAUTOMERS OF 2-AZAADENINE

Tautomer[a]	Gas phase[b]				Free energy of solvation[c] ($\Delta\Delta G^{hyd}_{A\to B}$)			Aqueous solution[d] ($\Delta G^{aq}_{A\to B}$)	
	Method	ΔE	ΔH	$\Delta G^{gas}_{A\to B}$	MST^{AM1}	$MST^{6\text{-}31G(d)}$	μ_{aq}/μ_{gas}	MST^{AM1}	$MST^{6\text{-}31G(d)}$
AA7	I	8.0	8.0	8.0					
	II	8.2	8.1	8.2					
	III	7.1	7.1	7.1	–5.3	–5.1	12.17/9.12	1.8	2.0
	IV	8.0	8.0	8.0					
AA3	I	6.6	7.0	6.0					
	II	6.4	6.7	5.8					
	III	4.2	4.6	3.6	1.3	1.4	3.40/2.21	4.9	5.0
	IV	2.9	3.3	2.3					
AA1	I	13.6	13.8	13.8					
	II	13.5	13.7	13.7					
	III	10.5	10.7	10.7	–5.9	–4.8	11.39/8.50	4.8	5.9
	IV	10.3	10.6	10.5					
AA17c	I	12.1	12.7	13.0					
	II	12.0	12.6	12.8					
	III	11.7	12.4	12.6	1.0	0.1	5.21/3.96	13.6	12.7
	IV	10.2	10.9	11.1					
AA19c	I	14.9	15.5	15.7					
	II	15.1	15.7	15.9					
	III	14.7	14.7	15.5	0.2	–1.7	4.56/3.30	15.7	13.8
	IV	12.8	13.5	13.7					
AA19t	I	9.0	9.8	10.1					
	II	9.0	9.9	10.1					
	III	9.3	10.1	10.4	4.5	1.9	5.63/4.31	14.9	12.3
	IV	7.5	8.3	8.6					

All values (in kcal/mol) are given relative to the tautomer AA9. The dipole moments (in D) in the gas phase and in aqueous solution determined from SCF and SCRF MST calculations with the 6-31G(d) basis set are also given.

[a] See Fig. 2 for nomenclature.

[b] The calculational levels used are I: HF/6-31G(d)//HF/6-31G(d); II: HF/6-31+G(d,p)//HF/6-31G(d); III: MP2/6-31+G(d,p)//HF/6-31G(d); IV: B3LYP/6-31+G(d,p)//HF/6-31G(d).

[c] Values estimated from SCRF MST-AM1 and MST-6-31G(d) calculations.

[d] Values estimated from the addition of the free energy of tautomerization in the gas phase at the MP2/6-31+G(d,p)//HF/6-31G(d) level to the relative free energy of hydration determined from MST-AM1 and MST-6-31G(d) calculations (see Eq. 1).

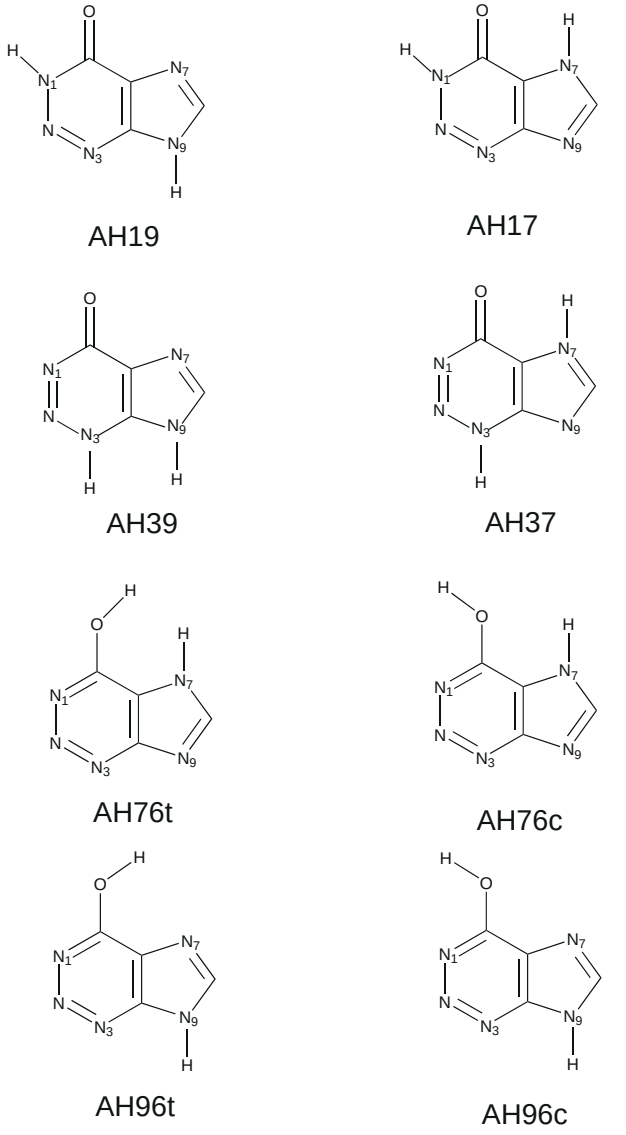

Fig. 3. Representation of the eight tautomers of the neutral 2-azahypoxanthine included in the final study after the stepwise elimination process (see the text for details).

and entropic corrections were computed from the HF/6-31G(d) geometries using the standard formula [19] for a harmonic oscillator–rigid rotor as implemented in GAUSSIAN94 [20].

The free energy of tautomerization in aqueous solution was determined according to Eq. 1:

$$\begin{aligned}\Delta G^{aq}_{A\rightarrow B} &= \Delta G^{gas}_{A\rightarrow B} + \Delta G^{hyd}_{B} - \Delta G^{hyd}_{A} \\ &= \Delta G^{gas}_{A\rightarrow B} + \Delta\Delta G^{hyd}_{A\rightarrow B}\end{aligned} \quad (1)$$

The relative free energies of hydration ($\Delta\Delta G^{hyd}_{A\rightarrow B}$) were computed from the absolute free energies of hydration (ΔG^{hyd}_{A},ΔG^{hyd}_{B}) as determined from SCRF calculations using the AM1 [21] and ab initio 6-31G(d) [22] optimized versions of the continuum model developed by Miertus, Scrocco and Tomasi (MST; also called polarizable continuum model) [23]. The corresponding gas-phase optimized geometries were used in the calculations, since small geometrical effects are expected for rigid molecules like those considered here [11,24]. Nevertheless, in order to assess the relevance of solvent-induced geometrical changes on the stability between tautomers, AM1-SM2 calculations [25] were performed using gas-phase and solvent-optimized geometries. An inspection of these results allowed us to verify the goodness of the use of gas-phase geometries (see below).

Calculations in the gas phase were carried out using the MOPAC93-Rel A [26] and GAUSSIAN94 [20] computer programs. MST calculations were performed with locally modified versions of MOPAC93-Rel A and MONSTERGAUSS [27]. AM1-SM2 calculations were performed using the AMSOL program [28] developed by Cramer and Truhlar. All the simulations were performed on the IBM-SP2 of the Centre de Supercomputació de Catalunya, and on HP and SGI workstations in our laboratory.

Results

Gas phase

Seven tautomers of the neutral 2-azaadenine were considered after the screening in the stepwise elimination protocol (detailed results are available upon request from the authors; see also the Methods section): four amino (AA9, AA1, AA3 and AA7) and three imino (AA19t, AA19c and AA17c) species, which are shown in Fig. 2. The relative stabilities estimated at the SCF, MP2 and DFT levels with the 6-31 + G(d,p) basis for the selected tautomers are given in Table 1. All levels of computation clearly indicate that the amino tautomer AA9 is the most stable in the gas phase. A large difference in stability is observed for the prototropic tautomerism between nitrogens N7 and N9, since AA7 is around 7 kcal/mol less stable, which can be explained by the increased repulsion between lone pairs of nitrogens N3 and N9, and between the hydrogen atoms of the amino group and that attached to N7. Tautomerism between imidazo and triazine moieties favors those species bearing the proton at the imidazole ring. Thus, tautomer AA3 is disfavored by 3.6 kcal/mol at the MP2/6-31 + G(d,p) level with regard to the preferred species AA9. Such a destabilization amounts to nearly 11 kcal/mol for AA1, which again reflects the enlarged repulsion between vicinal lone pairs and acidic protons. All the imino tautomers are greatly destabilized (by more than 10 kcal/mol) with respect to AA9, which indicates a clear preference for amino tautomers.

Eight tautomers of the neutral 2-azahypoxanthine (see Fig. 3) were selected according to the stepwise selection protocol: four keto (AH19, AH17, AH39 and AH37) and four enol (AH96c, AH96t, AH76c and AH76t) forms. The keto tautomer AH17 is the most stable species in the gas phase at all the levels of theory (see Table 2). Tauto-

merism at the imidazole ring of AH17 leads to a small destabilization, the AH19 form being disfavored by around 1 kcal/mol. Nevertheless, prototropic tautomerism between nitrogens N1 and N3 of AH17 promotes a great destabilization, since AH37 is less stable by around 10 kcal/mol. This effect is even larger (around 20 kcal/mol) for the tautomer AH39. The enol forms are less stable than AH17 by around 7 kcal/mol (AH96c) or even more, thus indicating a clear preference for the keto species in the gas phase.

Aqueous solution

The solvent effect on the tautomerism of these 2-azapurines was examined by means of SCRF calculations, which were performed using our AM1 and 6-31G(d) optimized versions [21,22] of the polarizable continuum model (MST) developed by Tomasi's group [23]. However, in order to examine the influence of solvent-induced geometrical changes on the relative stability between tautomers, AM1-SM2 [25] calculations were also performed using the geometry optimized in the gas phase or with geometry optimization in aqueous solution. The results showed that changes in structural parameters induced upon solvation do not significantly modify the relative free energy of hydration, which differs on average by 0.3 kcal/mol (root-mean-square deviation of 0.8 kcal/mol). This finding agrees with our previous results on the small magnitude of this effect for hypoxanthine, xanthine, allopurinol and alloxanthine [11], as well as for other heterocyclic compounds [24a,29]. Accordingly, gas-phase optimized geometries can be used confidently in MST calculations, which allows one to gain a remarkable saving in computer time.

The differences in the free energy of hydration ($\Delta\Delta G^{hyd}_{A\rightarrow B}$) for selected tautomers of 2-azaadenine and 2-azahypoxanthine are given in Tables 1 and 2, respectively. In the case of 2-azaadenine the amino tautomers AA7 and AA1 are greatly stabilized upon solvation (by around 5 kcal/mol) with respect to the reference species AA9, while AA3 is disfavored by 1.3 kcal/mol. The preferential hydration of AA7 and AA1 can be understood from the

TABLE 2
DIFFERENCES IN ENERGY, ENTHALPY AND FREE ENERGY IN THE GAS PHASE, RELATIVE FREE ENERGIES OF SOLVATION, AND FREE ENERGIES OF TAUTOMERIZATION IN AQUEOUS SOLUTION FOR SELECTED TAUTOMERS OF 2-AZAHYPOXANTHINE

Tautomer[a]	Gas phase				Free energy of solvation ($\Delta\Delta G^{hyd}_{A\rightarrow B}$)			Aqueous solution ($\Delta G^{aq}_{A\rightarrow B}$)	
	Method	ΔE	ΔH	$\Delta G^{gas}_{A\rightarrow B}$	MST^{AM1}	$MST^{6\text{-}31G(d)}$	μ_{aq}/μ_{gas}	MST^{AM1}	$MST^{6\text{-}31G(d)}$
AH17	I	–1.0	–0.9	–0.9					
	II	–1.2	–1.2	–1.2					
	III	–1.1	–1.1	–1.1	–0.3	0.8	4.69/3.73	–1.4	–0.3
	IV	–1.0	–0.9	–1.0					
AH37	I	10.6	10.3	10.0					
	II	9.9	9.7	9.4					
	III	10.1	9.9	9.6	–0.5	–1.9	7.97/6.07	9.1	7.7
	IV	8.6	8.3	8.0					
AH39	I	21.7	21.0	20.2					
	II	21.3	20.6	19.9					
	III	22.3	21.6	20.9	–5.8	–8.0	14.33/10.48	15.1	12.9
	IV	20.3	19.5	18.8					
AH76c	I	11.9	11.2	11.2					
	II	10.0	9.3	9.3					
	III	9.1	8.4	8.4	–3.1	–5.0	10.18/7.50	5.3	3.4
	IV	11.1	10.3	10.3					
AH76t	I	22.4	21.2	20.5					
	II	20.3	19.1	18.4					
	III	19.2	18.0	17.2	–7.6	–7.3	12.85/9.77	9.6	9.9
	IV	20.8	19.6	18.8					
AH96c	I	9.0	8.3	8.4					
	II	7.2	6.5	6.6					
	III	6.9	6.2	6.3	–1.1	1.2	5.44/4.12	5.2	7.5
	IV	8.5	7.8	7.9					
AH96t	I	12.0	11.2	11.3					
	II	10.0	9.3	9.3					
	III	9.5	8.8	8.8	–1.5	–0.9	9.30/7.18	7.3	7.9
	IV	11.2	10.5	10.5					

All values (in kcal/mol) are given relative to the tautomer AH19. The dipole moments (in D) in the gas phase and in aqueous solution determined from SCF and SCRF MST calculations with the 6-31G(d) basis set are also given. See the footnotes in Table 1 for the computational methods used.

[a] See Fig. 3 for nomenclature.

enhanced charge separation of these tautomers. Thus, their dipole moments in the gas phase are 3.90 and 3.28 D larger than the dipole of AA9, which is estimated to be 5.22 and 6.82 D in the gas phase and in aqueous solution from ab initio 6-31G(d) SCF and MST calculations. The relative free energy of hydration for the imino tautomers AA17c and AA19c is small, but AA19t is clearly destabilized upon solvation in aqueous solution. In the case of 2-azahypoxanthine hydration stabilizes preferentially the keto tautomer AH39 by 6–8 kcal/mol with regard to AH19, which is the reference tautomer. The enol forms AH76c and AH76t are also better hydrated by around 4 and 7 kcal/mol. Again this reflects the larger polarity of these tautomers compared to the dipole moment of AH19 (see Table 2), which amounts to 4.97 and 6.65 D in the gas phase and in aqueous solution. The relative free energy of hydration lies in the range of 1 kcal/mol for the rest of the tautomers.

The results derived from SCRF calculations indicate that the tautomeric preference in the gas phase is in general not drastically altered upon transfer to aqueous solution, since the tautomers that are better solvated are notably unstable in the gas phase (see Tables 1 and 2). This is stated from an inspection of the relative free energies of tautomerization in aqueous solution ($\Delta G^{aq}_{A\rightarrow B}$) for tautomers of 2-azaadenine and 2-azahypoxanthine. The values of $\Delta G^{aq}_{A\rightarrow B}$ were estimated (see Eq. 1) from addition of the MST relative free energy of hydration ($\Delta\Delta G^{hyd}_{A\rightarrow B}$) to the free energy of tautomerization in the gas phase ($\Delta G^{gas}_{A\rightarrow B}$) determined at the MP2/6-31 + G(d,p) level.

The results in Table 1 indicate that the tautomer AA9 of 2-azaadenine is the predominant species in aqueous solution. Despite the greater solvent-induced stabilization of tautomer AA7, this form is less stable by 2 kcal/mol in aqueous solution. The rest of the tautomers are destabilized by more than 5 kcal/mol. Therefore, around 97% of 2-azaadenine is expected to exist as tautomer AA9. In the case of 2-azahypoxanthine (see Table 2) the difference in stability between tautomers AH17 and AH19 is not greatly affected upon solvation, the former being still the most stable species, while the rest of the tautomers are sensibly disfavored. Accordingly, AH17 is the main tautomer of 2-azahypoxanthine, but a significant population of AH19, which ranges from 10% to 40% depending on the AM1 and 6-31G(d) MST estimate of the free energy of hydration, is also expected.

Discussion

A determination of the tautomeric preferences of 2-azaadenine and 2-azahypoxanthine would be valuable to understand the different susceptibilities of these compounds to the action of xanthine oxidase. Since each tautomer can be assigned a specific scheme of hydrogen-bond donors and acceptors that characterizes the potential interactions with groups at the substrate binding site of the enzyme, an inspection of the predominant tautomeric forms can be useful to understand their recognition by xanthine oxidase. Unfortunately, to our knowledge there is no experimental evidence available on the tautomerism of these 2-azapurines. In this context, a combination of QM ab initio calculations and SCRF continuum methods allows us to identify with confidence the most stable tautomers in the gas phase and in aqueous solution, and to discuss the biochemical implications of the preferred tautomers.

With regard to the results determined in the gas phase, the size of the molecules prohibits the use of very high levels of theory in QM calculations. Nevertheless, calculations at the MP2/6-31 + G(d,p) level are accurate enough to determine the main trends of the tautomeric equilibria for these 2-azapurines. This is supported by the results derived at this calculational level for tautomerism of the closely related compounds hypoxanthine, xanthine, allopurinol and alloxanthine, which were found to be in very good agreement with the experimental data [11]. Additional support for the reliability of the MP2/6-31 + G(d,p) results is provided by the convergence achieved as the computational level is increased. Thus, similar estimates of the relative stability between tautomers are obtained at the HF and MP2 levels of theory when the 6-31 + G(d,p) basis set is used. Moreover, this agreement also extends reasonably well to the values computed at the HF/6-31G(d) level, which gives confidence in the stepwise elimination protocol followed to select the most stable tautomers. Finally, further support also stems from the similarity with the hybrid nonlocal DFT results.

The tautomeric preferences of the 2-azapurines in the gas phase are clear from the results in Tables 1 and 2. In the case of 2-azaadenine, AA9 is expected to be the only tautomeric form, since it is stabilized by more than 3.6 kcal/mol at the MP2/6-31 + G(d,p) level over the rest of the species. Our MP2/6-31 + G(d,p) estimates of the relative stability for tautomers of 2-azahypoxanthine (see Table 2) suggest that AH17 is predominant in the gas phase. However, a nonnegligible population of AH19 is also expected, since the free energy difference between AH17 and AH19 amounts to around 1 kcal/mol.

Regarding the magnitude of the solvent effect, there is general agreement between the relative free energies of hydration determined from AM1 and ab initio 6-31G(d) MST calculations. The results indicate that the solvent-induced stabilization does not alter the gas-phase tautomeric preference of either 2-azaadenine or 2-azahypoxanthine. Thus, AA9 is still the predominant form of 2-azaadenine in aqueous solution. In the case of 2-azahypoxanthine, AH17 and AH19 are the only species in aqueous solution, the population of the former tautomer being expected to be somewhat larger.

Since the species AA9 of 2-azaadenine as well as tauto-

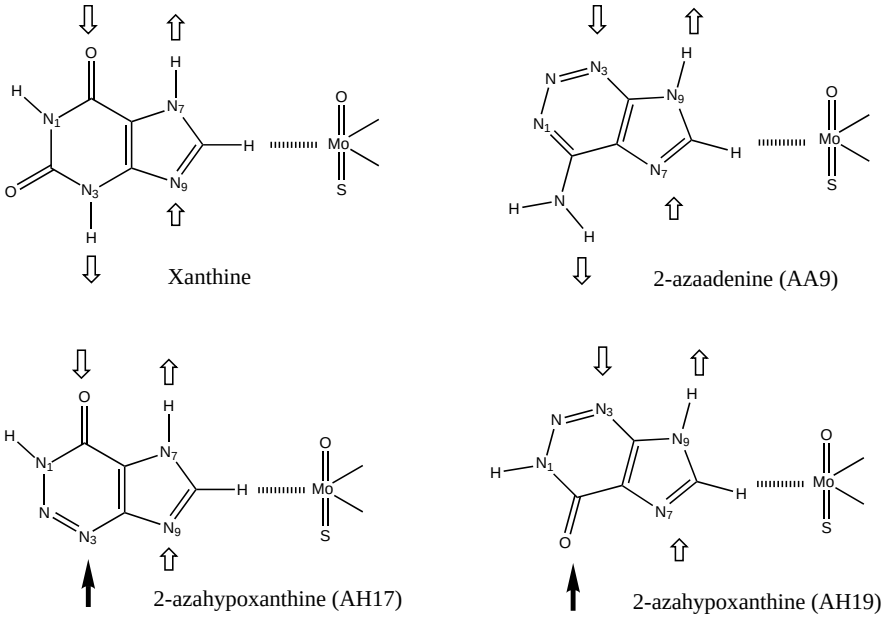

Fig. 4. Scheme of the potential hydrogen-bond interactions for the bioactive tautomers of xanthine, 2-azaadenine (AA9) and 2-azahypoxanthine (AH17 and AH19). White arrows denote the proposed interaction pattern that modulates substrate recognition. A black arrow at a specific site means an incorrect interaction that does not fit the recognition pattern.

mers AH17 and AH19 of 2-azahypoxanthine are the predominant species both in the gas phase and in aqueous solution, desolvation of the tautomers, which is required prior to substrate recognition by the enzyme, cannot be an interfering step in the recognition process. On the contrary, the main tautomers in the gas phase, AA9 and AH17 (AH19 cannot be completely excluded), can be assumed to be the 'bioactive' species at the enzyme. In order to verify the suitability of all these tautomers as the putative 'bioactive' species of 2-azaadenine and 2-azahypoxanthine, it is reasonable to conceive that they must share a common pattern of hydrogen-bond donors and acceptors, which will be resposible for substrate recognition and binding. Moreover, this pattern of hydrogen-bond groups must fit the scheme of interactions in the natural substrates hypoxanthine and xanthine, as well as in the inhibitors allopurinol and alloxanthine, which have been discussed in detail elsewhere [11], since all these structures are expected to interact with the same catalytic center of the enzyme.

The hydrogen-bond interaction patterns of the natural substrate xanthine and the predominant tautomers of the 2-azapurines are schematically depicted in Fig. 4. In order to analyze these patterns, it is worth noting that the proposed bioactive form of xanthine has been considered [11b]. In addition, since the oxygen incorporated in the substrate during the reduction process of the enzyme is presumably that of the Mo=O unit [4] and the three substrates are oxidized by the enzyme at position 8 [4,12,30], the carbon C8 has been placed near the molybdenum center in a fixed position. Under these restrictions, the relative orientation of the molecules was modified in order to maximize the recognition pattern defined for xanthine, which was proposed to consist of four basic hydrogen-bond interactions that modulate binding at the enzyme active site (see Fig. 4).

In the case of 2-azaadenine, such a recognition pattern is fully recovered upon rotation of the molecule by 180° around the line C8 – Mo, so that the interactions due to the >C6=O and N3-H groups in xanthine are now provided by the N3 and >C6-NH_2 groups in this compound, while the hydrogen-bond donor and acceptor contacts of N7 and N9 in xanthine are played by the atoms N9 and N7 respectively in tautomer AA9 of 2-azaadenine. However, a comparison of the interaction patterns of the two tautomers of 2-azahypoxanthine (AH17 and AH19) with that of xanthine reveals a clear difference (note the dark arrow in Fig. 4), which concerns the proposed interaction established by the hydrogen-bond donor group N3-H of xanthine with a presumed nucleophilic site in the purine binding site. Inspection of Fig. 4 reveals that such a group is replaced by the hydrogen-bond acceptors N3 in the tautomer AH17 and >C6=O in the form AH19, which would lead to unfavorable contacts upon binding to the enzyme. At this point, it is worth noting that other tautomers of 2-azahypoxanthine fulfill the proposed scheme of hydrogen-bond interactions, but their large difference in relative stability (by more than 7 kcal/mol regarding the species AH17) excludes a significant role of these tautomers in binding.

The preceding discussion allows us to explain qualitatively the differences in susceptibility to the action of xanthine oxidase exhibited by 2-azaadenine and 2-azahypoxanthine. As mentioned before, 2-azaadenine is oxidized

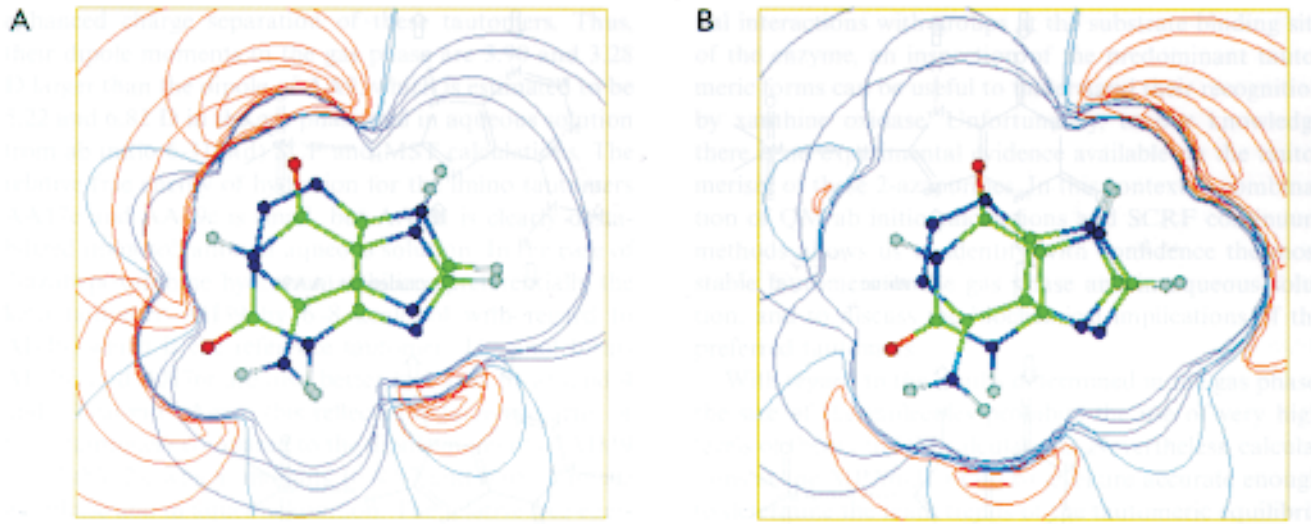

Fig. 5. Superimposition of the maps of molecular interaction potential for xanthine and 2-azaadenine (AA9). The maps correspond to the interaction with hydrogen (A) and oxygen (B) atoms. Charges of +0.5 and –0.5 were defined for hydrogen and oxygen, respectively. Negative, zero and positive isocontour lines are shown in red, blue and dark blue.

at a rate similar to that of xanthine, and even greater than that of hypoxanthine. However, 2-azahypoxanthine shows a relative inertness, since the rate of oxidation is remarkably lower [12]. This can be understood from the fact that 2-azaadenine exists in a tautomeric species which fulfills all the requirements for enzyme recognition, but none of the predominant tautomers of 2-azahypoxanthine can establish correctly all the interactions that modulate binding at the enzyme active site.

A comparison of the interaction patterns depicted in Fig. 4 provides a qualitative basis to understand the biological activity of the 2-azapurines. However, to achieve a more reliable description of the suitability of these compounds to be recognized by the enzyme, the patterns of interaction with potential electrophilic and nucleophilic groups were determined using the molecular interaction potential (MIP) [31]. Particularly, we determined the MIP maps corresponding to the interaction energy of xanthine, 2-azaadenine (AA9) and 2-azahypoxanthine (AH17 and AH19) with charged hydrogen and oxygen atoms as test probes. Standard optimized van der Waals parameters were used to describe the steric interactions of the quantum particle [31b]. In all cases the wave functions determined from calculations in the gas phase at the SCF level with the 6-31G(d) basis were used in the evaluation of the MIP.

Inspection of the maps reveals the same reactivity patterns for xanthine and tautomer AA9 of 2-azaadenine in front of an electrophilic (Fig. 5A) and nucleophilic (Fig. 5B) attack. The fitting of the MIP minima in the space surrounding the two molecules only requires a small rotation (around 15°) of the local reference system of 2-azaadenine relative to that used for xanthine. The dis-

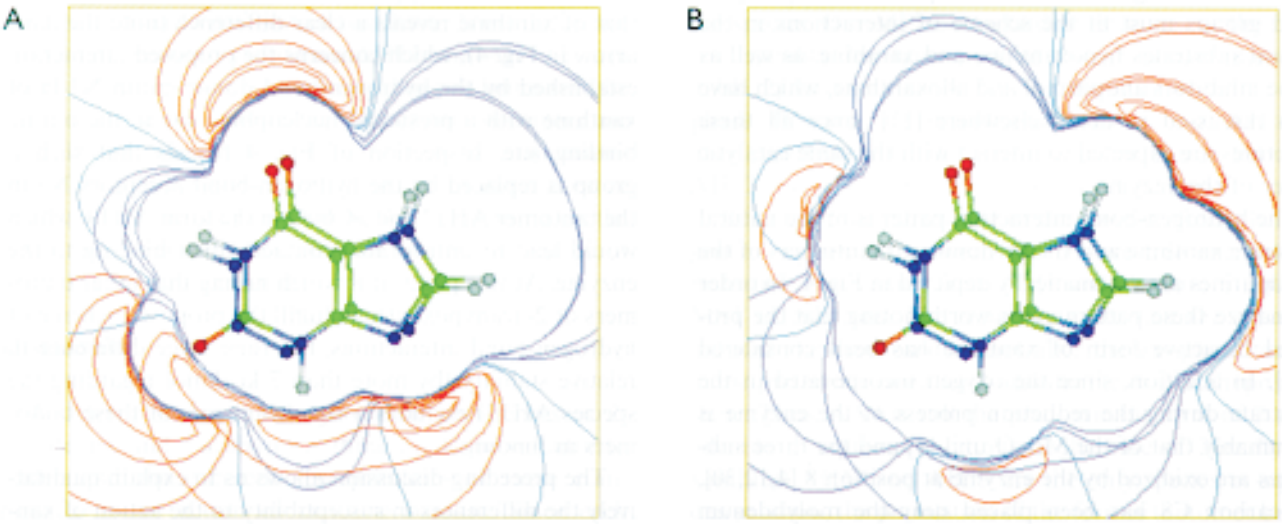

Fig. 6. Superimposition of the maps of molecular interaction potential for xanthine and tautomer AH17 of 2-azahypoxanthine. Interactions with (A) hydrogen and (B) oxygen atoms are shown. For an explanation, see the caption to Fig. 5.

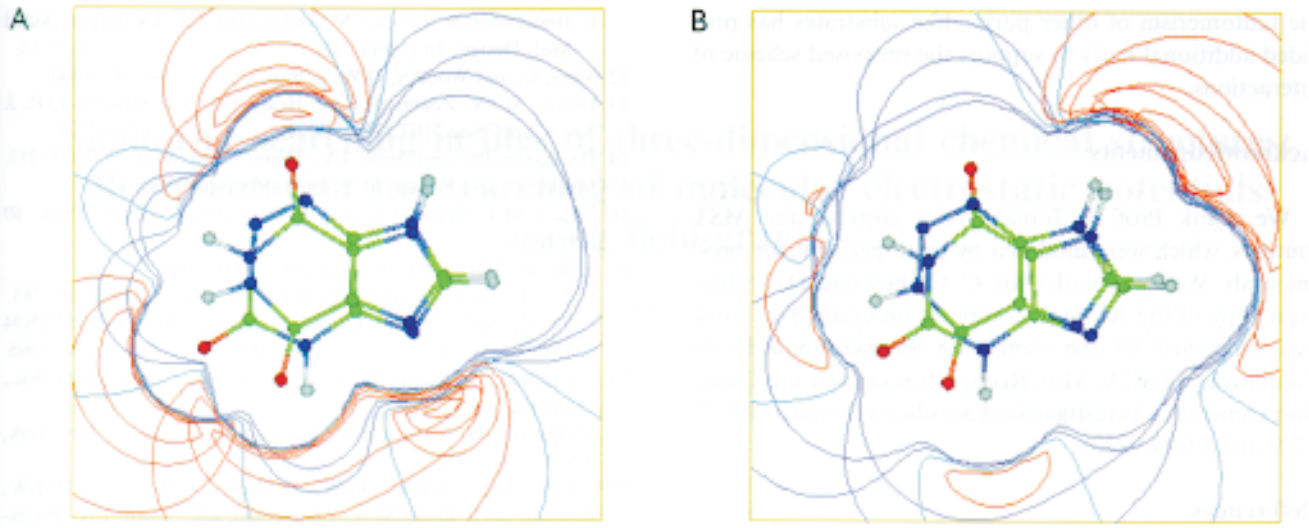

Fig. 7. Superimposition of the maps of molecular interaction potential for xanthine and tautomer AH19 of 2-azahypoxanthine. Interactions with (A) hydrogen and (B) oxygen atoms are shown. For an explanation, see the caption to Fig. 5.

tribution of the isocontour lines is very similar in the two molecules in those regions of space corresponding to the hydrogen-bond interaction pattern proposed to modulate substrate recognition (see Fig. 4). The differences are limited to the region around positions N1 and C2 of xanthine, which according to the proposed interaction pattern does not modulate recognition at the enzyme binding site. However, superimposition of the MIP maps for xanthine with those of the two tautomers of 2-azahypoxanthine (AH17: Fig. 6; AH19: Fig. 7) shows general agreement between the reactivity maps but in the region around position N3 of xanthine, which is proposed to be one of the substrate anchoring sites at the binding site. Thus, the MIP map for xanthine shows that such a position is able to interact with a nucleophilic site, whereas the corresponding center in either AH17 or AH19 is susceptible to recognize an electrophilic site. Since the two predominant tautomers of 2-azahypoxanthine do not fit completely the proposed scheme of interactions, their recognition at the enzyme binding site is expected to be less effective than that of tautomer AA9 of 2-azaadenine, which would explain the relative inertness of 2-azahypoxanthine in spite of its greater structural similarity to the natural substrate xanthine.

The preceding discussion offers a rationale for understanding the different rate of oxidation of 2-azaadenine and 2-azahypoxanthine by the enzyme xanthine oxidase. The results suggest that the different susceptibility to the enzyme likely reflects the different efficiency to fit the recognition pattern at the binding site. On this point, the role of the tautomerism equilibria in determining the proposed bioactive species involved in substrate recognition is worth noting. However, in the absence of detailed atomic information on the structure of the enzyme, caution must still be taken, since the electrostatic field generated from the residues surrounding the active site can play a decisive role in modulating the equilibrium between tautomers. Even though additional research effort is required to validate the interaction pattern proposed from our previous studies [11], the results presented here can be valuable for the design of new tight specific inhibitors lacking undesirable side effects.

Conclusions

A combination of ab initio QM methods with SCRF continuum models has been used to determine the main trends of the tautomeric equilibria for 2-azaadenine and 2-azahypoxanthine both in the gas phase and in aqueous solution. A comparison of the relative stability between tautomers in vacuo and in the condensed medium indicates that the tautomeric preference is not significantly changed upon solvation. This allows us to identify the potential bioactive species susceptible to being recognized at the purine binding site. Inspection of the reactivity patterns for the bioactive tautomers of xanthine, 2-azaadenine and 2-azahypoxanthine permits us to explain the differences in susceptibility to the enzymatic action experimentally observed for the 2-azapurines. Thus, whereas 2-azaadenine fulfills the proposed pattern of hydrogen-bond interactions that presumably modulate substrate recognition, the two predominant tautomers of 2-azahypoxanthine do not fit completely the proposed scheme of interactions.

The results in this study show the potential applications of theoretical methods to gain an insight into the relationships between molecular properties and biological activity, especially when there is no experimental evidence available about the relevant bioactive species or the nature of the interactions involved in substrate recognition and binding. Extension of our previous studies [11] about the recognition of the natural substrates (hypoxanthine and xanthine) and inhibitors (allopurinol and alloxanthine) to

the tautomerism of other purine-like substrates has provided additional basis to support the proposed scheme of interactions.

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