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Theoretical study on the distribution of atomic charges in the Schiff bases of 3-hydroxypyridine-4-aldehyde and alanine. The effect of the protonation state of the pyridine and imine nitrogen atoms

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ABSTRACT

The protonation state of the pyridine and imine nitrogen atoms on the "electron-sink" effect was studied by DFT(B3LYP/6-31+G*) calculations in the Schiff bases formed between 3-hydroxypyridine-4-aldehyde and alanine and their Cα-carbanionic counterparts.

Results indicate that the protonation of pyridine nitrogen promotes the enolimine–ketoenamine tautomerism. The importance of pyridine nitrogen on the "electron-sink" effect in the carbanionic molecules clearly depends on the protonation state of the imine nitrogen: in the enolimine tautomers, where the imine nitrogen is deprotonated, a 70% of the electron charge is delocalized on the pyridine ring, whereas in the ketoenamine type structures, where the imine nitrogen is fully protonated, just a 20% of this charge is delocalized in this molecular moiety. The results are discussed in relation to the chemistry of some PLP-dependent enzymes and the structure of their active sites.

1. Introduction

Pyridoxal-5′-phosphate (PLP) is a form of Vitamin B6 acting as cofactor of more than one hundred enzymes which catalyze the phosphorolytic cleavage of glycogen and a wide variety of metabolic reactions of amino acids (racemizations, transaminations, α-decarboxylations, aldolic cleavages, β- and γ-eliminations and replacements) [1–4]. In all the PLP-dependent enzymes, the coenzyme binds to protein by forming a Schiff base with an ε-amine group from a lysine residue (internal aldimine).

Except in glycogen phosphorylases, where the phosphate group of PLP acts as an acid–base catalyst [4], the first step in the PLP-dependents enzymes involves the replacement of the amine group of the lysine residue by the amine group of the amino acid substrate and subsequent formation of a new Schiff base (external aldimine) [1]. Experimental [5,6] and theoretical [7,8] studies on the mechanism of the formation processes of those Schiff bases and their respective transaldiminization reactions, highlight the importance of the coenzyme ionisable groups protonation state for such reactions to occur, as well as of the involvement of external molecules acting as proton acceptor or donor.

Once the external aldimine is formed, reactions catalyzed by PLP-enzymes proceed throughout the heterolytic cleavage of any of the Cα bonds and the subsequent formation of intermediate carbanionic species [2]. According to the hypothesis put forward by Dunathan [9], the preferred orientation of the Cα bond that will be broken is the one perpendicular to the aromatic pyridine ring, so the nascent π orbital would be aligned with those of the conjugated imine/pyridine π system. In consequence, the architecture of the active site in the enzyme would control this first step by means of the orientation of the Cα bond.

It is widely accepted, that the PLP Schiff bases with protonated pyridine nitrogen are specially prepared for stabilization of carbanionic intermediates due to their high molecular electronic resonance (electron-sink effect). The participation of quinonoid type electronic structures in this stabilization by electronic resonance is crucial [10–12]. Nevertheless, several theoretical studies performed by Bach et al. [13] and Toney [14,15] on the contribution of the protonation state of pyridine nitrogen to stabilization of carbanions generated in amino acids decarboxylation processes, predict that pyridine ring does not play such a crucial role, but is the protonation state of imine nitrogen what is really important. Thus, the PLP-dependent enzymes behave as other decarboxylases [16] and aldolases [17] that stabilise carbanions by forming Schiff bases directly with the substrate and are not PLP-dependent enzymes.

Typically, in most the PLP-dependent enzymes, pyridine nitrogen is protonated and stabilised by specific interactions with enzymatic residues [1,2,11,12]. In the transaminases family, the enzymatic residues have carboxylate groups (Asp,Glu). In the Tryptophan synthase family, the residues are polar (Ser,Thr). In

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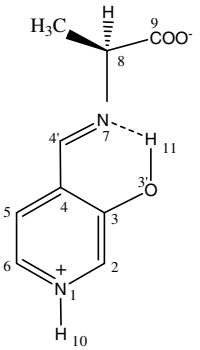
the particular case of Alanine racemase (AlaR) and according to the X-ray diffractions of its crystallographic structures [18,19], the pyridine nitrogen of coenzyme interacts with the NH moiety of the guanidinium group of an arginine residue, precluding the formation of the pyridinium cation and, in consequence, the formation of catalytic intermediates with quinonoid type structures. Nevertheless, AlaR is able to activate the heterolytic cleavage of H–Cα bonds as efficiently as any transaminase [20,21]. Recent QM/MM studies on the racemization mechanism catalysed by AlaR indicate that enhancement of carbon acidity of the alanine is due to solvation effects on the carbanion intermediate and to formation of specific interactions in the active site, rather than to intrinsic electronic withdrawing stabilization [22,23]. In this context, it seems of interest to carry out further studies on the relative importance of the ionization state of the different molecular ionisable groups on the charge delocalization processes in the carbanionic intermediate. Specially, it is a matter of concern to know the influence of two specific molecular atomic sets: the one formed by the 3′OH bound to pyridine plus the imine nitrogen, and the one formed by the pyridine nitrogen plus the hydrogen donor/acceptor group of the interacting external molecule. More precisely it is important to get further information on the effect of these external groups on the molecular distribution of atomic charges and electrostatic potentials, which are two main factors in the molecular reaction mechanisms.

The present work is a DFT study of the molecular distribution of atomic charges of the Schiff base formed between 3-hydroxypyridine-4-aldehyde and alanine and of its carbanionic intermediate yielded by heterolytic cleavage of the H–Cα bond. Four different protonation states for the pyridine nitrogen have been considered: fully protonated (pyridiniun cation); fully protonated and interacting with a carboxylate group from an acetate molecule; deprotonated but forming acceptor hydrogen bond with a guanidinium molecule and fully deprotonated. These four states have been considered for the enolimine species as well as for the ketoenamine species.

2. Methodology

Scheme 1 shows the structural formula, and the atomic position numbers assigned in this work, of the Schiff base formed between 3-hydroxypyridine-4-aldehyde and alanine (**PA–Ala**) in its enolimine tautomeric form with the protonated pyridine nitrogen.

DFT calculations were performed using the Gaussian03 program package [24] running in an Alphaserver GS1280. All the structures were fully optimized at the B3LYP level of the theory [25,26]. The 6-31+G(d) basis set and the water solvent in the cosmo polarizable continuum method (CPCM) [27,28] have been used in this study. The solvation spheres used have been those determined by the simple united atom topological model (UAO). Atomic charges were calculated using the CHelpG method described by Breneman and Wiberg [29].



Scheme 1. Schiff base of 3-hydroxypyridine-4-aldehyde and alanine (**PA–Ala**).

In the optimization process of molecular structures where cationic pyridinium nitrogen interacts with an acetate molecule, nitrogen and carboxylate groups were initially situated as their equivalents (coenzyme pyridine nitrogen and Asp222) in the active site of Aspartate aminotransferase (pdb code: 1ars) [30]. For the molecular structures where a neutral pyridine nitrogen forms acceptor hydrogen bond with a cationic guanidinium molecule, the interacting atoms were initially positioned as in the active site of Alanine racemase (pdb code: 2sfp) [18]. For all the Schiff base molecular structures, the H–Cα bond has been taken perpendicular to the conjugated pyridine ring.

Further, on the MP2/6-31+G(d)//B3LYP/6-31+G(d) wave function of each structure, a topological study of the density charge function was performed according to Bader's AIM theory [31] using the AIM2000 program [32] running on a PC. Once bond and ring critical points were located, molecular paths connecting bonds critical points and bond ring critical points were also computed for all structures. Additionally, we determined the electrostatic potential surfaces and electronic densities on C4′ and C8 atoms. Facio program [33] has been used to plot electrostatic potential surfaces.

3. Results and discussion

Fig. 1a and b shows the ball and sticks representation of the optimized molecular structures of **PA–Ala**. All the molecular structures named as ***1a***, ***1b***, ***1c*** and ***1d*** have a non ionised 3′OH group and non protonated imine nitrogen (enolimine tautomeric form). In ***1a*** structure the pyridine nitrogen is unprotonated, whereas in ***1d*** structure it is fully protonated giving rise to a pyridinium cation. In ***1b*** structure, a neutral pyridine nitrogen forms an acceptor hydrogen bond with a cationic guanidinium molecule, and in ***1c*** structure, H–N1 bond in pyridinium cation is modified by its interaction with the carboxylate group of an acetate molecule. Structures labelled as ***2a***, ***2b***, ***2c*** and ***2d*** are the corresponding zwitterionic tautomers, bearing a 3′-phenolate group and an iminium cation. The most representative interatomic distances are given in Table 1. N1–H10 values indicate the protonation state of pyridine nitrogen: covalently bound to a proton in ***1c***, ***1d***, ***2c*** and ***2d*** structures, and interacting to a NH of a guanidinium molecule throughout a hydrogen bond in the ***1b*** and ***2b*** structures.

As a consequence of the tautomeric equilibrium enolimine–ketoenamine [10] (Scheme 2), independently of the N1 protonation state, C3–O3′ and C4–C4′ distances are considerably shorter in zwitterionic structures (***2a***, ***2b***, ***2c***, ***2d***) than in the respective enolimine ones (***1a***, ***1b***, ***1c***, ***1d***). Calculated formation free energies of zwitterionic structures are lower than those of enolimine structures, which means that the former ones must be predominant in aqueous solutions. The free energy differences between ketoenamine and enolimine tautomers are slightly dependent on the protonation state of the pyridine nitrogen. This value is approximately 8.6 kJ/mol, which is equivalent to a tautomeric equilibrium constant ([enolimine]/[ketoenamine]) value of 0.032, slightly lower than those experimentally found to PLP and α-aminoacid Schiff bases (0.09) [34] or primary amines (0.12) [35].

Values in Table 1 show that O3′–H11 and N7–H11 distances are dependent on N1 protonation state. In enolimine type tautomeric structures, C3–O3′ distance is progressively changing: 1.350 Å in ***1a***, 1.347 Å in ***1b***, 1.338 Å in ***1c*** and 1.334 Å in ***1d***. Similar behaviour can be observed for N7–H11 distance that goes from 1.706 Å in ***1a*** to 1.658 Å in ***1d***. In other words the protonation of pyridine nitrogen shifts the proton, in the intramolecular phenol–imine hydrogen bond, from phenolic oxygen to imine

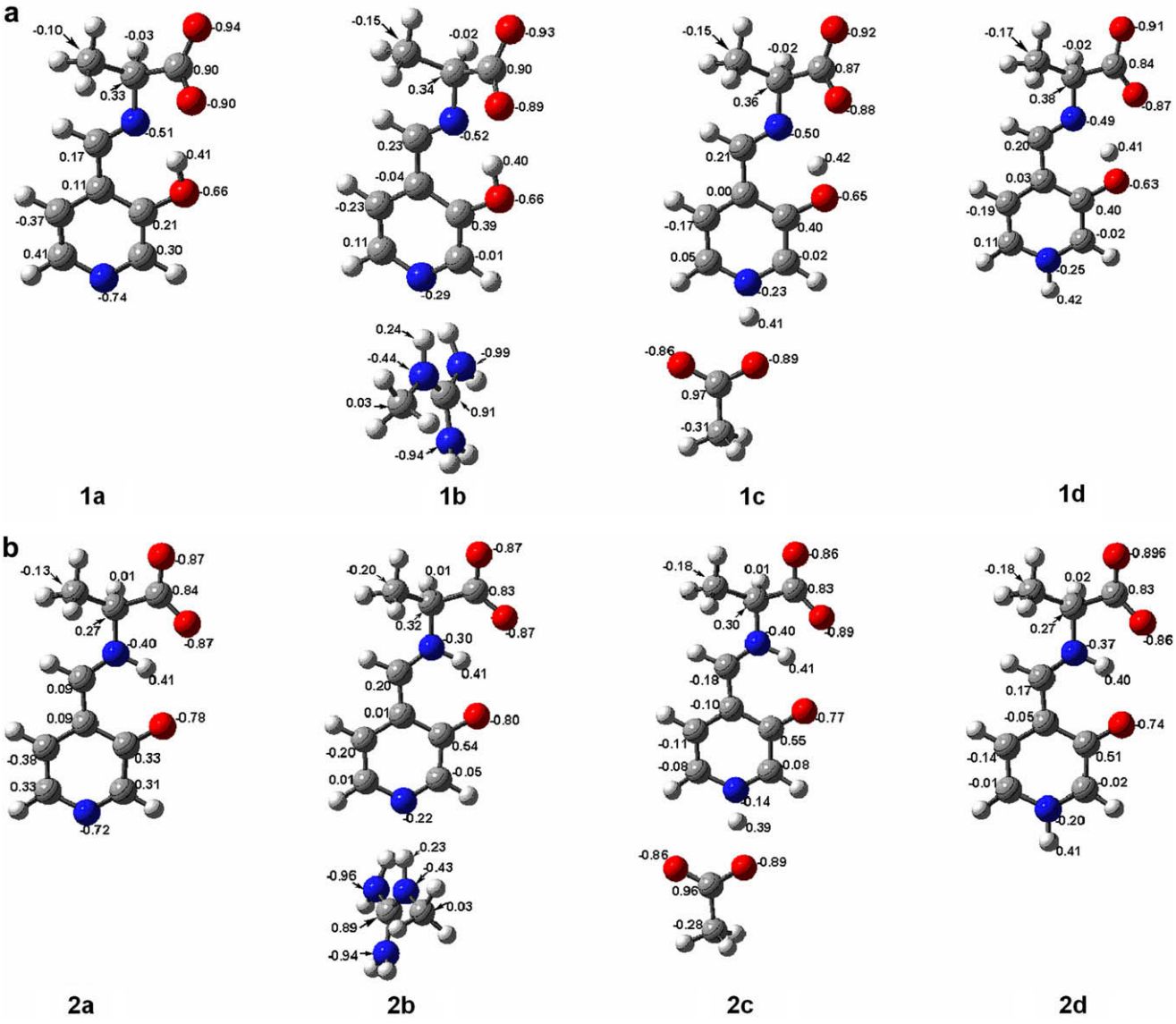
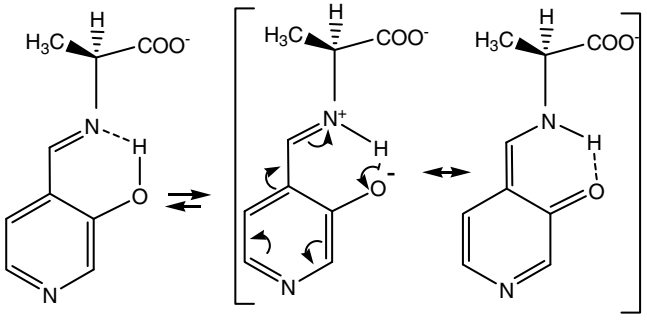


Fig. 1. Optimized molecular structures of Schiff base of 3-hydroxypyridine-4-aldehyde and alanine, enolimine tautomers (a) and ketoenamine tautomers (b). The numbers near the atoms are the CHelpG charges.

nitrogen. This is in agreement with X-ray diffraction results of crystallographic structures of pyridoxal Schiff bases [36]. In the case of zwitterionic structures, besides the clear variation of C3–O3′ distance, there are also changes in C3–C4, C4–C4′ and C4′–N7 distances with the protonation state of N1, indicating that the formation of pyridinium cation considerably increases the

Table 1
Interatomic distances (Å) of the molecular forms of Schiff base of 3-hydroxypyridine-4-aldehyde and alanine (**PLP–Ala**).

	1a	*1b*	*1c*	*1d*	*2a*	*2b*	*2c*	*2d*
N1–C2	1.337	1.337	1.341	1.344	1.319	1.320	1.326	1.330
C2–C3	1.405	1.404	1.399	1.398	1.450	1.448	1.442	1.441
C3–O3′	1.350	1.347	1.338	1.334	1.276	1.275	1.269	1.266
O3′–H11	1.010	1.015	1.020	1.023	1.933	1.928	1.929	1.934
C3–C4	1.415	1.416	1.421	1.423	1.446	1.446	1.450	1.451
C4–C4′	1.468	1.468	1.471	1.471	1.425	1.426	1.434	1.437
C4′–N7	1.283	1.282	1.281	1.280	1.308	1.306	1.301	1.299
N7–C8	1.467	1.465	1.464	1.464	1.473	1.473	1.474	1.474
C4–C5	1.403	1.403	1.401	1.401	1.420	1.418	1.412	1.410
C5–C6	1.392	1.391	1.386	1.385	1.378	1.378	1.378	1.378
C6–N1	1.348	1.347	1.347	1.350	1.367	1.366	1.361	1.363
N7–H11	1.706	1.685	1.668	1.658	1.032	1.032	1.034	1.034
N1–H10	–	1.889	1.096	1.039	–	1.873	1.088	1.040



Scheme 2. Enolimine–ketoenamine tautomeric equilibrium and zwitterionic molecular resonant forms.

ketoenamine character of the molecule, according to previous hypothesis of Martell [37].

Atomic charge values of ***1a***, ***1b***, ***1c***, ***1d*** and ***2a***, ***2b***, ***2c***, ***2d*** are depicted in Fig. 1a and b, respectively. For a particular atom type, differences in its atomic charge can be roughly correlated to differences in its nucleophilic power. In this way, decrease of approximately 0.1 charge units on imine nitrogen in zwitterionic structures respect to the phenolic ones, is indicative of the lowest nucleophilicity of ketoenamine type structures. In PLP-dependent enzymes the increase of positive charge of imine nitrogen is a pre-requisite for reactivity of the enzyme and enhancement of Cα acidity. However, this increase of positive charge hinders the proton acceptance from the incoming cationic amino acid, the next step in the transaldiminization reaction. In fact, in native transaminases and other related enzymes the coenzyme is in its dipolar ionic form [38].

The influence of amino acid carboxylate moiety on the whole molecular charge distribution has been investigated performing the topological study of the density charge function according to Bader's AIM theory. In Fig. 2, bond and ring critical points of ***1d*** (Fig. 2a) and ***2d*** (Fig. 2b) structures representation are shown. In the ketoenamine type structure, the presence of a weak, but clear, ring critical point involving Cα, carboxylate and protonated imine, can be seen. This behaviour has been detected in all the studied zwitterionic structures, independently of the pyridine nitrogen protonation state. On the contrary, no such ring and bond critical points have been found in any of the studied enolimine type structures.

The theoretical results above described explain some proved experimental facts of PLP Schiff bases chemistry. Nevertheless, PLP Schiff bases reactivity is conditioned by the molecular parameters of their α-carbanionic structures, since they are compulsory reaction intermediates. Fig. 3a and b shows the structures and atomic charge values of studied enolimine and ketoenamine carbanion molecules. The most significant bonds and interatomic distances of these structures are given in Table 2. Positional numbers are in these molecules the same to those used for the respective Schiff bases.

In enolimine type carbanionic molecules, ***3a***, ***3b***, ***3c*** and ***3d***, the N7–H11 distance depends on protonation state of pyridine nitrogen. As in **PA–Ala** structures, formation of pyridinium cation decreases electronic charge on the ring atoms, enhances the 3′OH acidity and imine nitrogen basicity and promotes proton transfer from phenol to imine group. In zwitterionic type carbanionic molecules (***4a***, ***4b***, ***4c***, ***4d***) C3–O3′ distances are shorter than those of enolimine type, showing the presence of ketoenamine type resonance forms.

Formation of quinonoid type resonance forms (Scheme 3) is the paradigm to justify the high stability of pyridinium carbanionic intermediates [10–12].

Presence of quinonoid forms must be detected in the molecular geometry; C2–C3, C4–C4′, N7–C8 and C5–C6 double bond character must increase and N1–C2, C3–C4, C4′–N7, C4–C5 and C6–N1 double bond character must decrease. Results in Tables 1 and 2 show irregular variations of above mentioned distances as a consequence of the carbanion electronic resonance in Scheme 3; meanwhile N7–C8, C4′–N7 and C4–C4′ distances change strongly from neutral to carbanionic structures, C2–C3, N1–C2, C3–C4, C4–C5 and C3–O3′ are little affected and N1–C6 and C5–C6 remain almost invariable. It is interesting to point out that changes in N7–C8, C4'–N7 and C4–C4' distances are present not only in ketoenamine carbanionic structures but also in the enolimine ones. For both enolimine and ketoenamine structures, data in Table 2 show a clear dependence of most of the significant bond distances with the protonation state of pyridine nitrogen. If N7–C8 bond distance is taken as a reference, its value goes from 1.323 Å in ***3a*** structure to 1.306 Å in ***3d***, which means a decrease, in relation to their respective neutral structure values, of 0.144 Å for the first structure and of 0.158 Å for the second. This increment is 0.145 Å for the ***3b*** molecule and 0.155 Å for ***3c***. Similar variations are found for ***4a***, ***4b***, ***4c*** and ***4d*** structures. On the other hand, meanwhile N7–H11 distance values remain almost invariant, there is an observed decrease in O3′–H11 distance values when comparing those of ***4a*** with ***4b***, ***4c*** and ***4d***. This behaviour is due to small changes in 3′O–C3–C4, C3–C4–C4′ and C4–C4′–N7 bond angle values caused by the

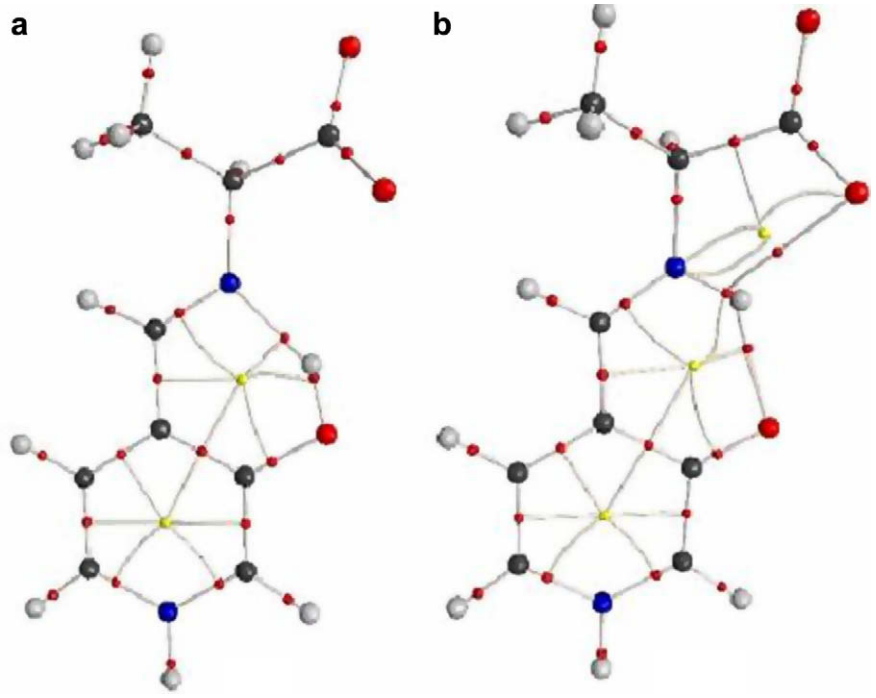


Fig. 2. Molecular graph of ***1d*** (a) and ***2d*** (b) structures, showing the bond and ring critical points and the paths connecting them.

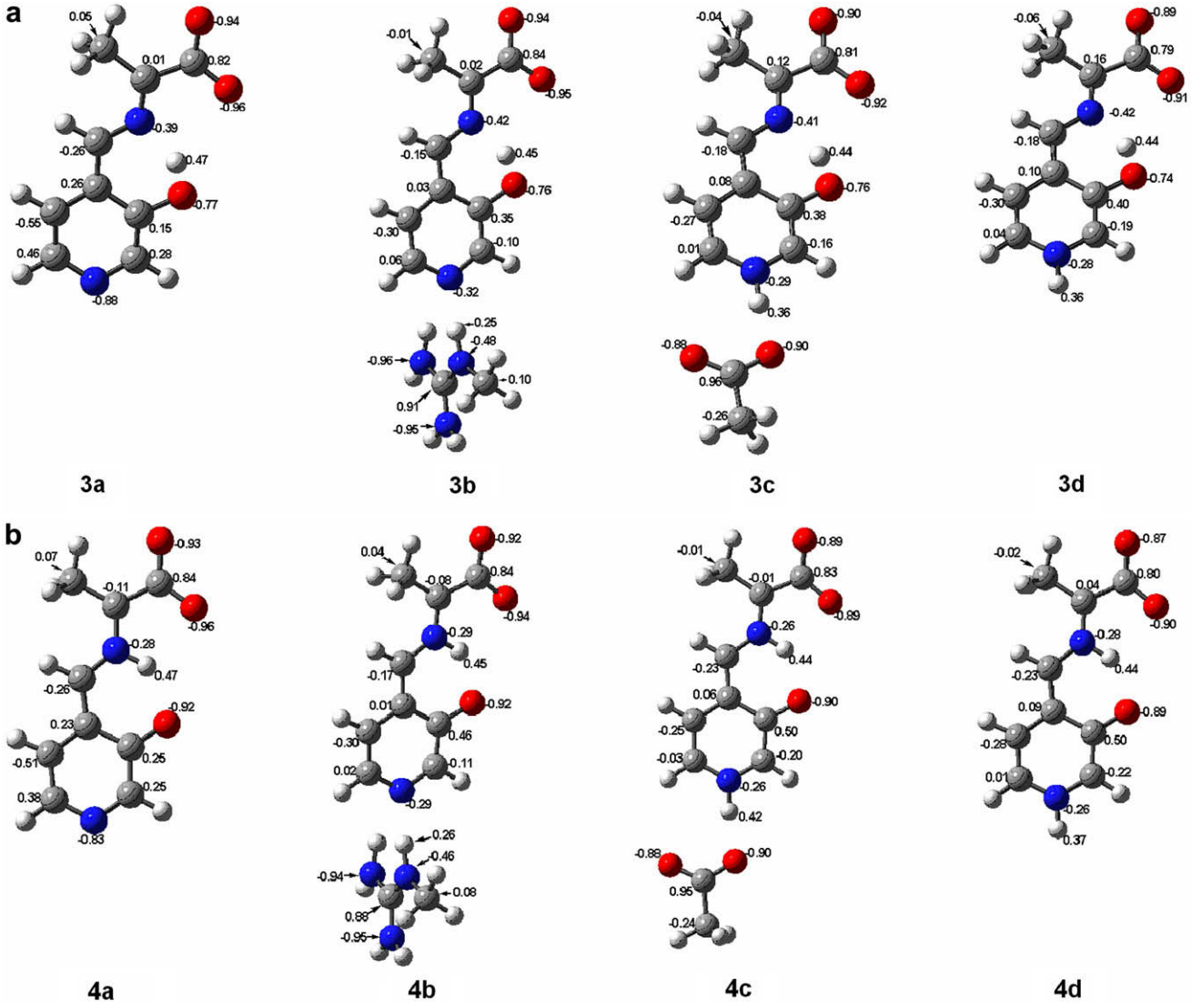


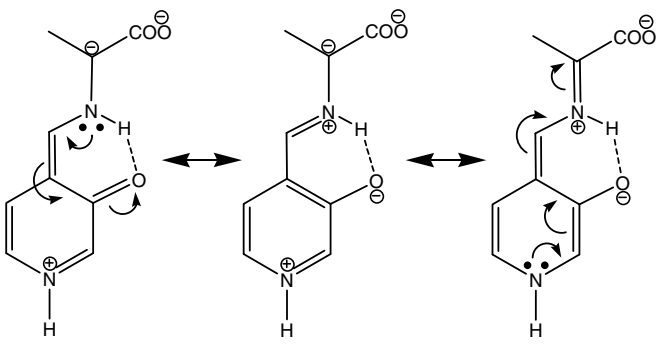
Fig. 3. Optimized molecular structures of the carbanions, enolimine tautomers (a) and ketoenamine tautomers (b). The numbers near the atoms are the CHelpG charges.

dependence of the ratio of ketoenamine to quinonoid type resonance forms with the protonation state of pyridine nitrogen.

Previous DFT [13] and semiempiric [14] studies on PLP catalysed decarboxylation reactions of amino acids indicate that protonation of pyridine nitrogen has a minor influence on the delocalisation of carbanion electronic charge. According to these studies, the presence of a protonated imine nitrogen would be the important factor for such delocalisation and, therefore, the important factor for α-carbanion stabilisation. Atomic charge values given in Fig. 3a and b indicate which molecular atom positions are mainly affected by carbanion generation on Cα and can be used

Table 2
Interatomic distances (Å) of the carbanions of Schiff base of 3-hydroxypyridine-4-aldehyde and alanine.

	3a	*3b*	*3c*	*3d*	*4a*	*4b*	*4c*	*4d*
N1–C2	1.356	1.358	1.369	1.374	1.346	1.348	1.361	1.366
C2–C3	1.389	1.386	1.374	1.371	1.422	1.419	1.406	1.404
C3–O3′	1.369	1.367	1.361	1.335	1.306	1.305	1.299	1.267
O3′–H11	1.019	1.021	1.020	1.021	1.851	1.832	1.820	1.812
C3–C4	1.436	1.439	1.453	1.456	1.449	1.452	1.464	1.467
C4–C4′	1.427	1.423	1.403	1.400	1.434	1.431	1.417	1.412
C4′–N7	1.349	1.352	1.364	1.367	1.345	1.347	1.357	1.360
N7–C8	1.323	1.320	1.309	1.306	1.348	1.345	1.332	1.329
C4–C5	1.422	1.424	1.435	1.438	1.414	1.414	1.420	1.432
C5–C6	1.390	1.387	1.372	1.369	1.392	1.390	1.380	1.378
C6–N1	1.350	1.350	1.358	1.361	1.350	1.349	1.351	1.354
N7–H11	1.668	1.656	1.659	1.649	1.039	1.040	1.041	1.042
N1–H10	–	1.762	1.045	1.027	–	1.767	1.051	1.029



Scheme 3. Cα carbanion electronic resonance. Ketoenamine type resonance form is on the left and quinonoid type resonance form is on the right.

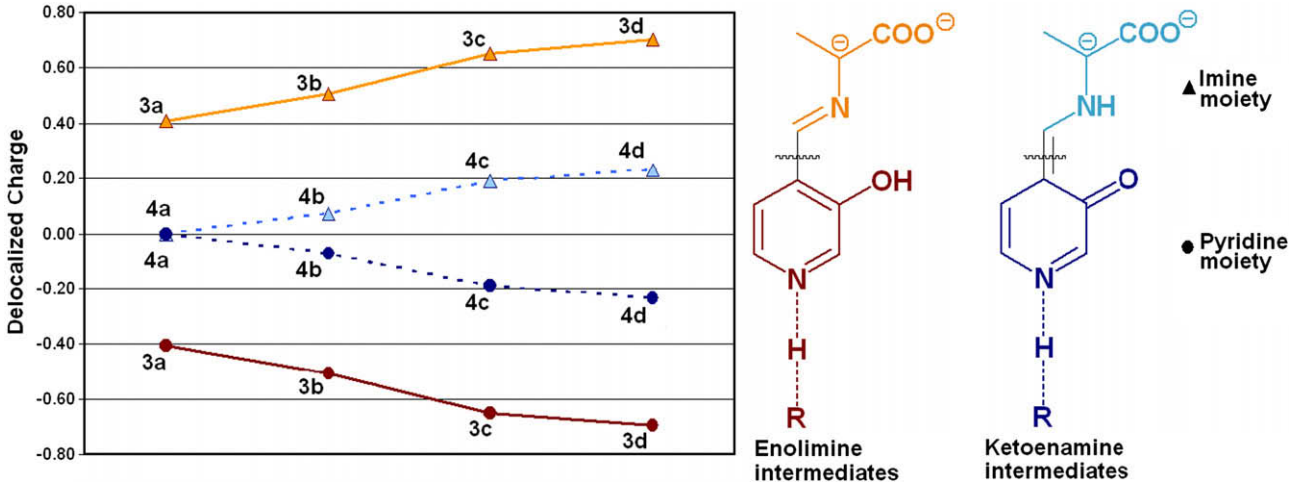


Fig. 4. Variation of the delocalization of C8 carbanion charge in the *imine moiety* (triangles) and *pyridine moiety* (circles) depending on the protonation state of pyridine nitrogen. Charge variation of the enolimine intermediate tautomers, ***3a***, ***3b***, ***3c*** and ***3d*** is in solid line, and charge variation of the ketoenamine intermediate tautomers, ***4a***, ***4b***, ***4c*** and ***4d*** in dotted line. The delocalized charge of *imine* and *pyridine-moieties* are obtained by adding the charge of all the atoms of each moiety and subtracting the sum of hypothetical localized charges of the respective moiety.

to evaluate which part of the molecule is mainly involved in delocalisation effect of such electron charge, the 3-hydroxypyridine part of the Schiff base molecule (as it was traditionally accepted in PLP chemistry) or the imine double bond (as Bach et al. [13] and Toney [14,15] suggested). For further charge consideration, all the molecular structures have been divided in two parts (Fig. 4). The *imine moiety* is formed by the carboxylate and methyl groups and the C8 (Cα) from the initial alanine, as well as the imine double bond formed by the azomethine nitrogen from amino acid and the C4′ from the initial aldehyde. The *pyridine moiety* is formed by the rest of the molecule as well as guanidinium and acetate when is the case.

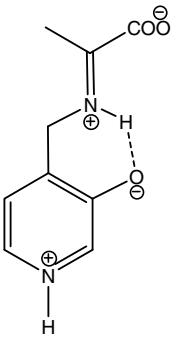
Solid lines in Fig. 4 show variations with protonation state of pyridine nitrogen of electron charge in *imine moiety* (triangles) and *pyridine moiety* (circles) in enolimine type structures. Values have been evaluated taking into account the CHelpG charge calculated on each moiety of the molecule and the virtual charge if the carbanion generated on H–Cα cleavage were totally localised on Cα atom. Obviously the total electronic charge gained by *pyridine moiety* is equal to that lost by *imine moiety*. As it can be seen, there is an important electron charge shift from *imine* to *pyridine moiety*, which can rise 0.70 e in the full protonated pyridine nitrogen structure, ***3d*** which means that for that kind of structures the pyridine electron sink effect is the predominant factor. Even in structure with unprotonated pyridine nitrogen, ***3a***, electron charge transferred is about 0.40 e. In other words, protonation of pyridine nitrogen increases the electron sink effect approximately in 0.30 e.

Dotted lines in Fig. 4 present variation of atomic charges in *imine moiety* (triangles) and *pyridine moiety* (circles) in ketoenamine type structures. Clearly no sink effect of the *pyridine moiety* is detected in ***4a*** structure, furthermore, protonation of pyridine nitrogen (***4d***) involves just a 20% of the Cα negative charge generated by the heterolytic cleavage of Cα–H bond. Therefore, even for carbanions with fully protonated pyridine nitrogen, the *imine moiety* of molecules with protonated imine nitrogen retains almost 80% of electron charge, in agreement with previously cited DFT and semiempiric studies [13,14].

Atomic charge values in Fig. 3a and b also give useful information to explain some well known facts in PLP-dependent enzyme catalysed processes. On one hand, it can be observed that C4′ atomic charge is always negative, no matter what protonation state of imine and pyridine nitrogens are. Therefore C4′ is a nucleophilic atom, suitable to accept a proton from any acid residue and to form new ketimine type Schiff bases (Scheme 4), as it happens in transamination processes [1]. In order to check these results, electrostatic potential surfaces and atomic electronic densities have been determined by MP2/6-31+G(d)//B3LYP/6-31+G(d)// calculations. In Fig. 5, electrostatic potential surfaces for ***4c*** (Fig. 5a) and ***3c*** (Fig. 5b) are plotted. In both structures there is a negative electrostatic potential zone (in blue[1] colour) centred on carboxylate group, phenol oxygen, imine nitrogen and C4′ and does not include Cα atom.

Additionally, we have calculated the values of the electronic densities on the C4′ and Cα atoms at 1 Å of the molecule plane. In all the structures the electronic density on the C4′ is ~0.20 and ~0.17 on the Cα. This fact clearly shows that the re-protonation of carbanions will be preferentially at the C4′ atom.

The absolute value of the charge is modulated by the pyridine nitrogen protonation state. The ketoenamine forms protonated on the pyridine nitrogen (***4c*** and ***4d***) show the highest charge difference between the C4′ and Cα atoms of the zwitterionic species. This corroborates the well known fact that in transaminases and PLP-dependent enzymes that catalyse ketimine depending reactions the PLP pyridine nitrogen is protonated and interacts with



Scheme 4. Transamination ketimine intermediate generated by protonation on C4′.

[1] For interpretation of colour in figures, the reader is referred to the Web version of this article.

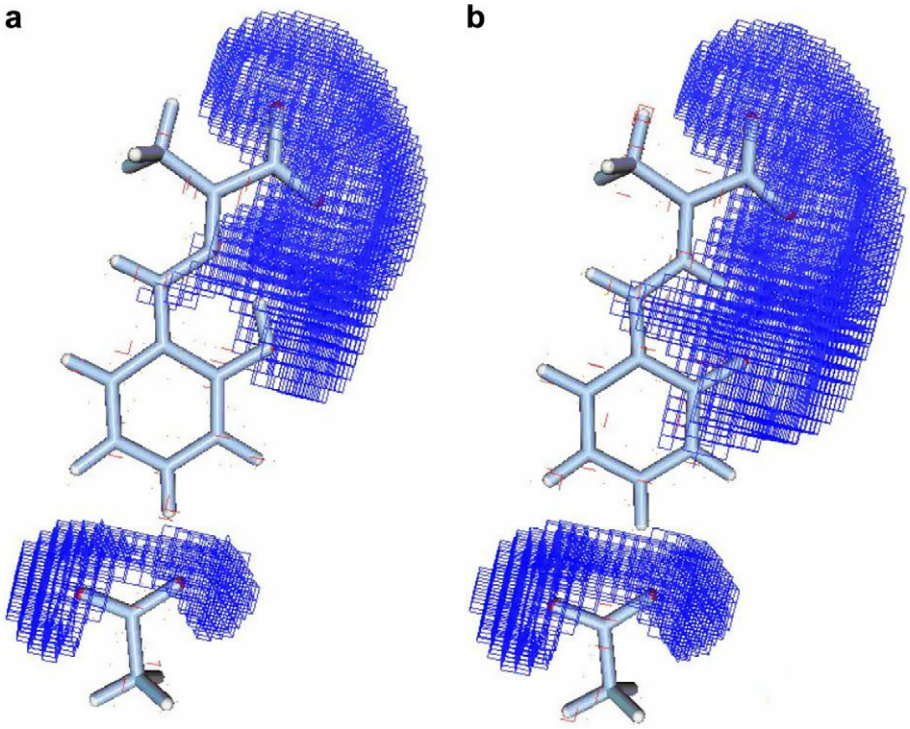


Fig. 5. Electrostatic potential surface of ***4c*** (a) and ***3c*** (b).

carboxylate groups of Asp or Glu residues. On the other hand, the ***3c*** and ***3d*** forms show a moderate negative charge on the C4′ and positive on Cα. This means that these enolimine tautomers favour the ketimine species formation.

It is clear that ketoenamine forms increase the H–Cα acidity, through the carbanion stabilization. Additionally the pyridine nitrogen protonation enlarges the C4′ nucleophilicity in the reactions with ketimine intermediates. It is also clear that proton transference from the imine nitrogen to the phenolate (O3′–H···N7) and subsequent electronic density decrease on the Cα, is a suitable mechanism to prevent the proton return on this atom.

We have already pointed out that in some PLP-dependent enzymes the pyridine nitrogen does not interact with anionic groups. However, the nitrogen interacts with polar groups, such as hydroxyl groups, in the tryptophan synthase [39], cystathionine β-synthase [40] and O-acetylserine sulfhydrylase [41], or even with NH groups of a guanidine residue, as in the Alanine racemase [18,19]. Nevertheless none of those enzymes catalyse reactions with ketimine intermediates formation. Specifically, in the D-alanine racemization catalysed by this last enzyme, once the H–Cα is transferred to the Lys39 (in the *re*-face of the internal aldimine) the Tyr265 protonates the C atom (in the *si*-face) [18].

The protonated nitrogen enolimine forms (***3c*** and ***3d***) show a positive charge on the Cα, which hinders its re-protonation, as it can be seen in Fig. 3a. In the ketoenamine structures with the deprotonated pyridine nitrogen (***4a*** and ***4b***), the Cα shows a slight negative charge (Fig. 3b). This increase of the electronic density on the Cα and the decrease of the electronic density on the C4′ explain the pyridinium cation absence in the aldimine of racemases that prevent the occurrence of non desired transamination reactions. It is well known that some racemases and other PLP-dependent enzymes catalyse collateral reactions of transamination [42–45].

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