Transamination of γ-Aminobutyric Acid; a Semiempirical Molecular Orbital Study of the Transaminase Mechanism

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The mechanism of the pyridoxal-5-phosphate (PLP) dependent enzyme γ-aminobutyric acid aminotransferase (EC 2.6.1.19, GABA-T) was investigated using the AM1 semiempirical molecular orbital method. The atomic charges calculated for the isolated reaction intermediates and the differences in the HOMO and LUMO energy levels are consistent with the experimentally determined mechanism. To examine the role of the ring nitrogen, a pyridoxal analogue in which the pyridine ring nitrogen is replaced by CH is considered in the model reaction steps of the same enzyme.

Pyridoxal-5-phosphate (PLP, Fig. 1) is the cofactor (or coenzyme) for a variety of enzymes including aminotransferases,[1-6] racemases[1,2,5,6] and decarboxylases.[1,2,5,6] In the holoenzyme, the cofactor is bound to the active site by the 5'-phosphate group, the 3-hydroxy (3-OH), the 4'-aldehydic group and the ring nitrogen.[4,5,7-10] Usually the 4'-carbon plays a principal part in the catalysis. The interaction between the 4'-carbon of PLP and the amino group of the amino acid substrate results in the formation of the imine (Fig. 2) of a Schiff's base commonly known as the external aldimine.[11,12] The internal aldimine is the Schiff's base formed between PLP and the ε-amino group of an active-site lysine residue. The reactivity of the 4'-carbon is greatly affected by the presence of both the adjacent hydroxy group in the 3 position and the ring nitrogen in the 1 position. The 3-OH and ring nitrogen can undergo changes in their states of protonation depending on the pH of the system and the mechanism stage. The other groups, 2-methyl and 5'-dibasic phosphate anion, serve to anchor the cofactor in a particularly effective conformation.[13] The 2-methyl and 5'-dibasic phosphate anion can be substituted by a variety of groups while maintaining the functionality of the cofactor.[12,14]

In the case of α-amino acids, the cofactor, through the formation of this Schiff's base, labilizes all the bonds around the α-carbon of the amino acid. The catalytic effect of a particular enzyme will then be determined by which bond (a, b or c, Fig. 2) is broken by the surrounding catalytic groups of that enzyme. According to the hypothesis put forward by Dunathan,[15] the bond to be broken would be perpendicular to the imine π system. The loss of a group from the α-carbon in this manner results in a gain in resonance energy and the extension of the π system.[15] This hypothesis has been supported experimentally.[16-22] Therefore, for example, if bond a in Fig. 2 is broken transamination results, breakage of bond b would result in decarboxylation and breakage of bond c leads to retro-aldol cleavage.

It has been experimentally determined that the PLP-amino acid Schiff's base adopts a *cis* configuration in solution around the 4-4' carbon bond with the amino nitrogen pointing toward the 3-OH (see Fig. 1 for the numbering system) and that the π systems on the pyridine ring and Schiff's base are coplanar.[15,21,23,24] This *cis* configuration is also seen in the crystal structures of both the isolated Schiff's base and the enzyme aspartate aminotransferase (EC 2.6.1.1, AAT). The presence of an intramolecular hydrogen bond between the 3-OH and the imine nitrogen has been suggested by NMR

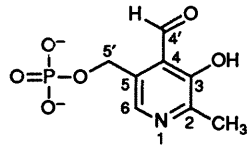

Fig. 1 Structure of pyridoxal-5-phosphate (PLP) showing the numbering scheme used in this study

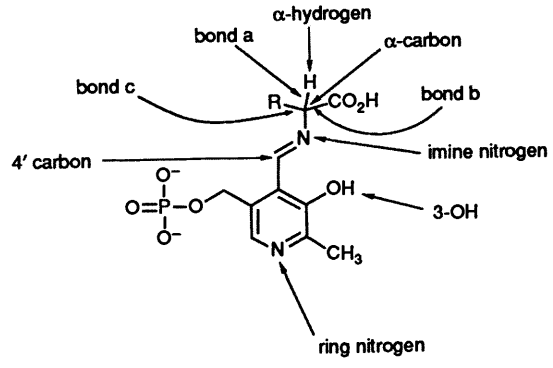

Fig. 2 Schematic diagram of an α-amino acid Schiff's base. Breakage of bond a results in transamination, bond b in decarboxylation and bond c in retro-aldol cleavage.

spectroscopy, polarized-light spectroscopy experiments and numerous theoretical calculations.[1,25-29] Such a hydrogen bond would obviously help to stabilize a planar *cis* conformation.

The mechanism for transamination has been extensively reviewed.[4,5,10,15] In this study the mechanism for the transamination of γ-aminobutyric acid (4-aminobutyric acid, GABA) by the enzyme GABA aminotransferase (EC 2.6.1.19, GABA-T) was modelled as depicted in Fig. 3. The amino group of GABA interacts with the 4'-carbon of PLP to form the external aldimine **1**. The protonation of the ring nitrogen and the departure of the proton from the γ-carbon lead to the formation of the quinonoid intermediate **2**. This results in the unification of the initial base into a large resonating system. Protonation of the quinonoid intermediate at the 4'-carbon gives the pyridoxamine ketimine **3** which then undergoes hydrolysis to release pyridoxamine-5-phosphate (PMP) and succinic semialdehyde. In the actual enzyme mechanism PLP is the coenzyme, not pyridoxal as is used here. The pyridoxal (PL) form can undergo identical reactions and is often used in

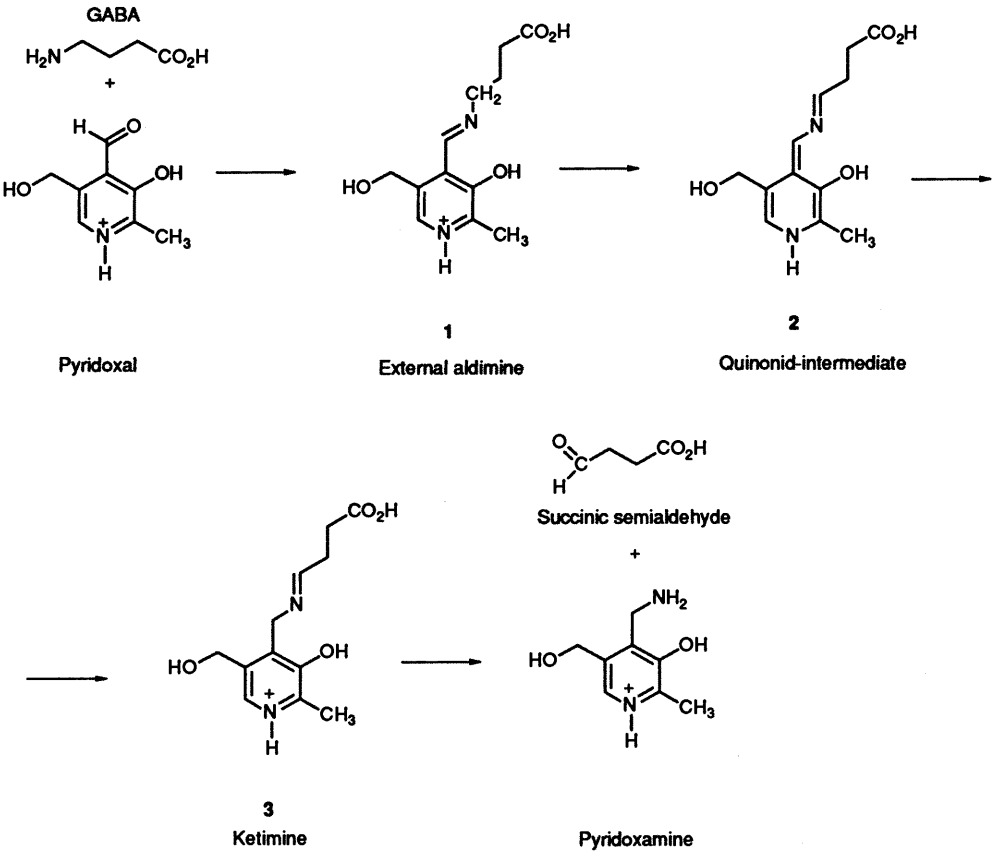

Fig. 3 Experimentally identifiable steps in the mechanism for the transamination of GABA

experimental and theoretical modelling.[6,10,25,29-31] It was decided to omit the phosphate group for simplicity in calculations since parameters for phosphorous have not given satisfactory results in the past.[32]

A large number of derivatives of PLP and PMP have been synthesized and many have been investigated as possible inhibitors of enzymes or as substitutes for the cofactor.[6,33-38] However, analogues with the ring nitrogen of PLP replaced by CH or other atoms and groups have yet to be synthesized and tested. The ring nitrogen of PLP has an important role in catalysis and plays a significant part in both resonance energy and electronic distribution for the transformation of the external aldimine into the transitional forms.[25,30] Replacement of the ring nitrogen would alter the cofactors' ability to act as an electron sink and such a compound may affect the catalytic process by simply blocking the cavity normally occupied by PLP. To provide an insight into the catalytic effect of the ring nitrogen and indicate if such analogues could be useful as inhibitors or as substitutes for PLP, the ring nitrogen will be replaced by CH in each of the model mechanism steps.

Computational Methods

The structures representing model GABA-T transamination reaction steps were built up from the crystal structure of PLP[39] by using standard bond lengths and angles. The GABA backbone was maintained in an extended conformation since constrained GABA analogues indicate this to be the active conformation in GABA-T inhibition.[40] Viewing and manipulation of these structures was carried out using CRYSX, a program within the MORPHEUS[41] molecular modelling package. All structures were optimized using the AM1 option within the AMPAC[42] and MOPAC version 5.0[43] packages. PRECISE was not specified unless the gradient normal was above 4. Comparison of the atom charges for structures optimized without PRECISE and gradient normals less than four, with those then run with PRECISE showed differences only in the third decimal place. There were no significant differences in the structures provided the gradient was below 4. The geometries of the AM1 optimized structures were consistent with those containing pyridoxal nuclei found in the Cambridge Crystallographic Database.[44] Charges obtained from the AM1 calculations were used in ELCPOT, also part of the MORPHEUS package, to generate molecular electrostatic potential maps.

The programs CRYSX, AMPAC and MOPAC were run on either a Vax 11/750 or a Vax 3400 at The Victorian College of Pharmacy, Monash University, while ELCPOT and ENMAP were implemented on a Cyber 760 at the Royal Melbourne Institute of Technology.

Results and Discussion

The charges for selected atoms, heats of formation, energy of the highest occupied molecular orbital (HOMO) and energy of the lowest unoccupied molecular orbital (LUMO) for structures **1–3** are given in Table 1.

The transamination of GABA involves the deprotonation of the external aldimine **1** at the γ-carbon by an active-site basic residue (Fig. 3). Deprotonation is favoured at this position due to the negatively charged carbon (allylic carbon) and the resulting resonance stabilization upon formation of the quinonoid intermediate **2**. This implies that the γ-carbon should be more negative in **1** than the 4'-carbon and the results listed in Table 1 show this to be the case. Whereas in **2** and **3** the 4'-carbon carries a greater negative charge than the γ-carbon. This is consistent with the protonation of the 4'-carbon (by an active-site basic residue) in the mechanism stage going from **2** to **3**. The hydrolysis of **3** to give PMP and succinic semialdehyde is reflected in the charge distribution of **3**; the γ-carbon carries a

Table 1 Physical data for **1** to **3** and **1'** to **3'** obtained using the AM1 method

Structure	Charges e				$\Delta H_f°$/ kcal mol^{-1}	*E*/eV		
	Ring N	4'-Carbon	Imine N	γ-Carbon		HOMO	LUMO	LUMO–HOMO
Protonation conditions as depicted in Fig. 3								
1	−0.05	−0.04	−0.18	−0.11	−2.3	−13.48	−5.25	8.23
2	−0.23	−0.16	−0.12	−0.09	−129.6	−7.29	−0.10	7.19
3	−0.06	−0.08	−0.26	0.01	2.7	−13.42	−5.43	7.99
Physiological conditions; imine nitrogen protonated, 4'- and γ-carbons deprotonated								
1'	0.02	0.20	−0.13	−0.07	−103.7	−8.07	−1.73	6.34
2'	−0.06	−0.38	0.05	−0.10	−147.0	−4.43	1.62	6.06
3'	0.01	−0.04	−0.10	−0.12	−100.3	−8.53	−1.49	7.04

Table 2 Physical data for **1c** to **3c** and **1c'** to **3c'** obtained using the AM1 method

Structure	Charges e				$\Delta H_f°$/ kcal mol^{-1}	*E*/eV		
	Ring carbon[a]	4'-Carbon	Imine N	γ-Carbon		HOMO	LUMO	LUMO–HOMO
Protonation conditions as depicted in Fig. 5								
1c	−0.09	−0.00	−0.24	−0.09	−157.3	−9.09	−0.40	8.69
2c	−0.08	−0.03	−0.18	−0.01	−142.2	−8.13	−0.28	7.85
3c	−0.10	−0.07	−0.22	−0.04	−160.0	−9.09	−0.09	9.00
Physiological conditions: imine nitrogen protonated, 4'- and γ-carbons deprotonated								
1c'	−0.08	0.14	−0.24	−0.05	−181.5	−4.54	1.53	6.07
2c'	−0.06	−0.24	−0.02	0.02	−135.0	−4.27	1.05	5.32
3c'	−0.06	−0.03	−0.08	−0.05	−165.2	−4.21	1.52	5.73

[a] Carbon corresponding to the ring nitrogen in **1**–**3**.

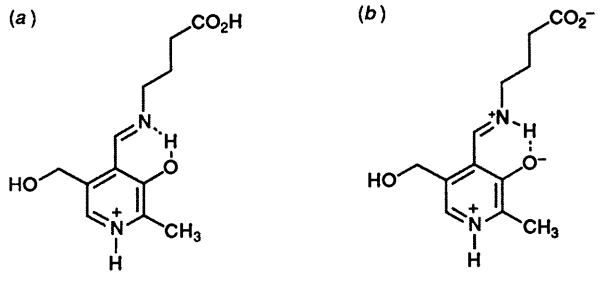

Fig. 4 (*a*) The initial protonation states of the imine nitrogen, 3-OH, carboxyl group and the pyridine ring nitrogen used in modelling the transamination mechanism; (*b*) the protonation states of these atoms expected at physiological pH. The dashed line indicates a possible hydrogen bond.

more positive charge (+0.01 e) than either the imine nitrogen (−0.26 e) or 4'-carbon (−0.08 e) and would be the site for the fixation of water. Therefore, it appears that atomic charges calculated using the AM1 method for the isolated intermediates **1**–**3** are consistent with the known transamination mechanism for GABA.

In proceeding from **1** to **2** there is a large change in the ring nitrogen charge (becoming more negative), while going from **2** to **3** the ring nitrogen charge returns to approximately the same value as that of **1**. This illustrates the ability of the ring nitrogen to act as an electron sink (going from **1** to **2**) and then to act as an electron source (going from **2** to **3**). For structures **1** and **2** the 4'-carbon, imine nitrogen and γ-carbon all carry negative charges giving a chain of three atoms all bearing a negative charge. A similar charge distribution has been previously reported by Perault *et al.*[30] in their study on PLP dependent enzymes using a classical LCAO approximation molecular orbital method, the method is fully described in ref. 45.

The external aldimine **1** has a heat of formation of −2.3 kcal mol^{-1} * however the next step in the transamination mechanism *i.e.* the quinonoid intermediate **2** is far more stable ($\Delta H_f°$ = −129.6 kcal mol^{-1}). Once the ketimine **3** is formed the heat of formation rises to +2.7 kcal mol^{-1}.

It has been proposed[30] that the driving force responsible for transformation of the external aldimine **1** into the quinonoid intermediate **2** is the gain in resonance energy of approximately 10 kcal mol^{-1}. Such a gain in resonance energy should be reflected in the energy difference between the LUMO and HOMO for **1** and **2**. The energy of (LUMO–HOMO) for **1** is 8.23 eV, which is about 1 eV and 0.24 eV more than for **2** and **3** respectively. An increase in resonance energy would correspond to a decrease in the energy difference between LUMO and HOMO levels and this was consistent in proceeding from **1** to **2**. Similarly, from **2** to **3** a decrease in resonance energy, indicated by an increase in the energy difference between LUMO and HOMO levels, was obtained.

There have been many calculations reported with the 3-OH, amino acid carboxylate and the amine groups all in various states of protonation/deprotonation.[24,29,30,46,47] The protonation states of these groups in the initial calculations are shown in Fig. 3. At physiological pH the imine nitrogen is expected to be protonated, whilst both the 3-OH and the amino acid carboxyl group would be deprotonated (Fig. 4).[48] The effect of altering the protonation states of these atoms was examined and the results are also given in Table 1.

When simulating physiological conditions the 4'-carbon in the external aldimine **1'** becomes electron deficient (−0.04 e in **1** becoming +0.20 e in **1'**). The γ-carbon is negatively charged

* 1 cal = 4.184 J.

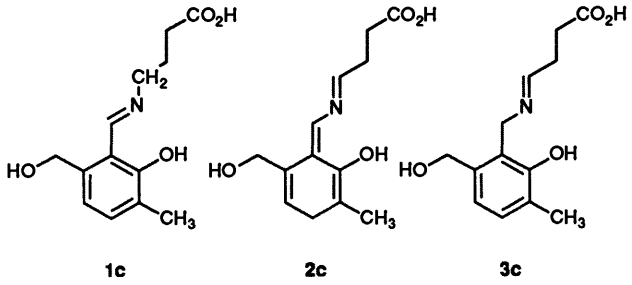

Fig. 5 Structures resulting from the replacement of the pyridine ring nitrogen by CH in the transamination mechanism

and would be the site for deprotonation. Although the imine nitrogen is also negatively charged, abstraction of the proton from the imine nitrogen is unlikely since this is involved in a hydrogen bond with the ionized 3-OH and is not situated on the *si* face of the molecule which is required for deprotonation by an active-site basic residue. Studies on model reactions and various transaminases have established that the deprotonation and subsequent protonation of the 4′-carbon occurs in a *cis* manner, that is, removal and addition of the proton occur on the *si* face of the molecule.[2] The quinonoid intermediate **2′** would be protonated at the 4′-carbon, this atom carries a greater negative charge (−0.38 e) when compared to the γ-carbon (−0.10 e). Under these conditions the imine nitrogen is now slightly electron deficient (+0.05 e). The charge distribution of the 4′-carbon (−0.04 e), imine nitrogen (−0.10 e) and γ-carbon (−0.12 e) of the ketimine **3′** would indicate the fixation of water to be favoured at the 4′-carbon. This is in contrast to the initial protonation conditions where the γ-carbon **3** carrying a charge of +0.01 e is the site for water fixation. Although the atom charges obtained for **1′** and **2′** when simulating physiological conditions are consistent with the transamination mechanism, there appears to be an inconsistency for the hydrolysis step, involving the ketimine **3′**. The heats of formation for structures **1′–3′** under these protonation conditions indicate an increase in the stability of these isolated intermediates. Trends in the variation of the HOMO and LUMO energies are similar to those under the initial protonation conditions, although the energy values have decreased.

The replacement of the pyridine ring nitrogen with CH in each of the steps **1–3** was carried out to investigate what the effect would be on the charges of the 4′- and γ-carbons, as well as on the imine nitrogen. The authors point out that there is no experimental evidence to date suggesting that such an analogue of PL would undergo a similar mechanism or even interact with the apoenzyme. Fig. 5 depicts the structures that would result from the replacement of the ring nitrogen with CH (they will be referred to as the carbon analogues). All of the structures **1c–3c** (Fig. 5) were optimized using AM1 and Table 2 shows the comparative results of those given in Table 1. There are differences, as expected, in the charges on the 4′- and γ-carbons and the imine nitrogen when the ring nitrogen is replaced by CH. The carbon analogue **1c** has a neutral 4′-carbon and the imine nitrogen has a greater negative charge than in structure **1**. The carbon analogue of the quinonoid intermediate **2c**, in contrast to **2**, has only slight negative charges on the 4′- and γ-carbons. The negative charge on the γ-carbon in **3c** of −0.04 e contrasts with structure **3** where the γ-carbon is slightly electron deficient (+0.01 e). The charge on the ring carbon (corresponding to the ring nitrogen of **1–3**) can be regarded as constant for **1c–3c**. This is in contrast to the pyridoxal ring nitrogen where the nitrogen carries a greater negative charge in the quinonoid intermediate **2** than in either **1** or **3**. The heats of formation for the carbon analogues **1c–3c** indicate that they are more stable than the original PL structures, with **2c** being the least stable of the three. Similar trends are seen for the difference

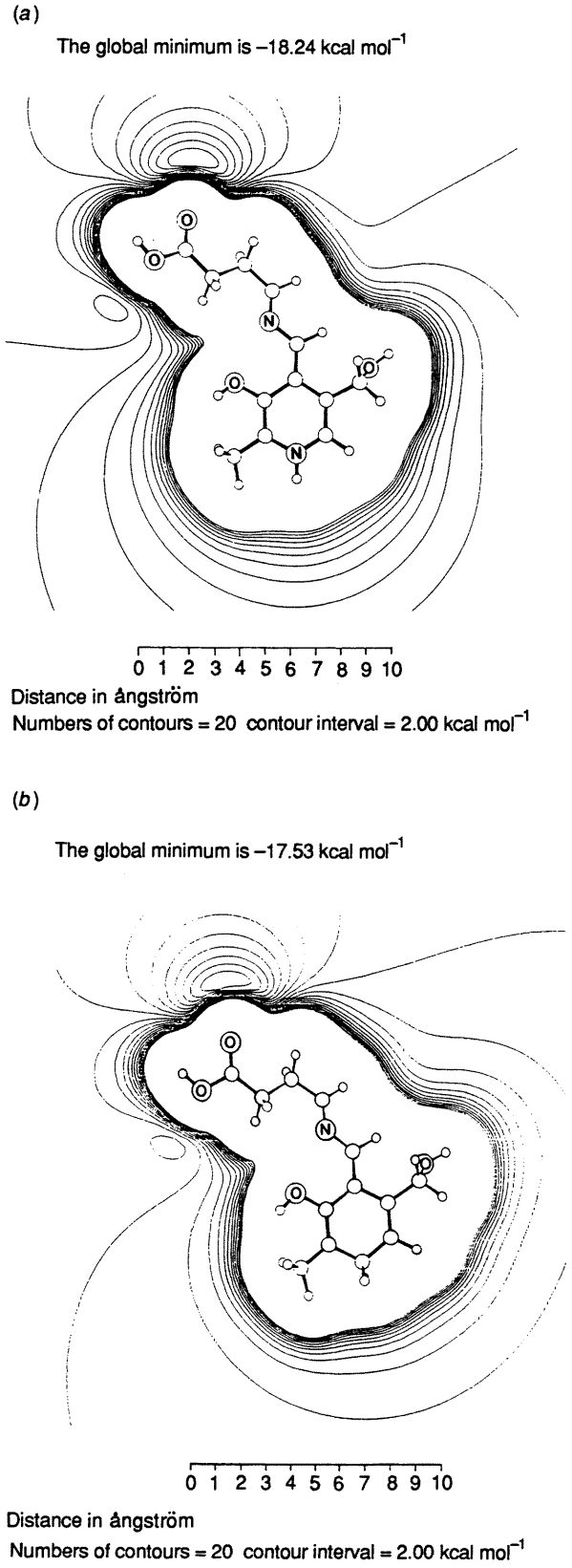

Fig. 6 (*a*) Electrostatic potential map of the quinonoid intermediate **2** in the plane of the ring. (*b*) Electrostatic potential map of the carbon analogue **2c** in the plane of the ring. The oxygen and nitrogen atoms are labelled, the carbon atoms are the larger of the unlabelled circles and the hydrogens are the smaller ones. Multiple bonds are not shown in the diagram.

in E(LUMO–HOMO) for both sets of structures. If such a PLP analogue was able to participate in a similar transamination mechanism then the γ-carbon of **1c** would be the site of deprotonation, as is the case for PLP. The charges on the 4′- and γ-carbons of **2c** are very similar and thus protonation could occur at either carbon. For PLP the protonation of the 4′-carbon could be driven by the increased negative charge of the ring nitrogen of the quinonoid intermediate **2**. Therefore, the absence of the ring nitrogen, an electron sink, in the carbon analogue may lead to the termination of the transamination mechanism at this step. On the basis of charge alone, hydrolysis of **3c** could occur at either of the 4′- or γ-carbons since the charges are similar (−0.07 e and −0.04 e respectively).

The calculations for **1c–3c** were repeated simulating physiological conditions, *i.e.* with both the carboxyl and 3-OH groups deprotonated and the imine nitrogen protonated. The results are also given in Table 2 (**1c′** to **3c′**). Protonation of the imine nitrogen in **1c′** leads to the 4′-carbon becoming electron deficient (+0.14 e) and again the γ-carbon would be the site of deprotonation. The 4′-carbon of **2c′** now carries a larger negative charge than previously (−0.24 e for **2c′** compared to −0.03 e for **2c**) and protonation would occur at this atom. The site of water fixation in structure **3c′** is unclear as the atom charges of the 4′-carbon, imine nitrogen and γ-carbon are within 0.05 e of each other.

Molecular Electrostatic Potentials.—The electrostatic field of the cofactor must be complementary to the electrostatic field of the GABA-T active site. Electrostatic potential energy maps were constructed for **1–3** and **1c–3c**. The characteristics of the electrostatic maps for the PL based structures were compared with the maps obtained for the corresponding carbon analogues. Due to the striking similarity, discussion will be confined to the electrostatic potential energy maps of the quinonoid intermediates (**2** and **2c**) in the plane of the ring. This view was chosen as they are easily compared. The electrostatic potential energy maps for the quinonoid intermediate **2** and the carbon analogue **2c** are given in Figs. 6(*a*) and 6(*b*). The amino acid side-chains are in similar conformations, however it is the region surrounding the ring that is specifically of interest. For **2** the electrostatic minimum is −18.24 kcal mol^{-1} compared to −17.53 kcal mol^{-1} for **2c** and in both cases this minimum is situated near the carboxyl group of the amino acid side-chain. The electrostatic potentials surrounding the ring are very similar in shape and energy. These results suggest that the carbon analogues may satisfy the electrostatic requirements of the cofactor for the GABA-T enzyme.

Conclusions

The mechanism of GABA-T was modelled using the AM1 semiempirical molecular orbital method. With the pyridine ring nitrogen protonated and all other atoms neutral the enzyme mechanism can be rationalized by the charges of the relevant atoms. However, when physiological pH conditions are simulated, hydrolysis is expected at the 4′-carbon rather than at the γ-carbon. Hydrolysis at this site would not lead to the generation of PMP and succinic semialdehyde.

Substitution of the carbon analogue into the transamination mechanism enabled the effect of the ring nitrogen to be clearly seen. Provided the external aldimine **1c** was formed, the calculations suggest that the deprotonation of **1c** would take place leading to the formation of **2c**. However, it is unclear whether the remaining mechanism steps could occur. To resolve this uncertainty, the synthesis and biological evaluation of the carbon analogue are underway.

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