Molecular Orbital Study on the Reaction Mechanism of Irreversible Enzyme Inhibitors

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By means of the molecular orbital method, the reaction mechanism of the specific and irreversible enzyme inhibitors, such as cycloserine, L-2-amino-4-methoxy-*trans*-3-butenoic acid (AMB), and vinylglycine (2-amino-3-butenoic acid), was studied. Firstly, it was attempted to know which pathway is probable between the transamination process and the isomerization one. By comparing the energy increments for these two reactions, the transamination reaction was predicted to be energetically favorable, supporting the proposition of Rando. Upon complexing with the coenzyme–pyridoxal moiety of alanine racemase or aminotransferase, the reactivity of the inhibitors toward the nucleophile was found to be considerably increased due to the lowering of the lowest unoccupied molecular orbital (LUMO), and this was considered to be the reason why the inhibitors become bound with the enzyme irreversibly. The LUMO of aspartate, substrate of aspartate aminotransferase, is higher than those of the inhibitors in the free state, as well as in the pyridoxal-linked state. This difference in the energy of the molecular orbital between substrate and inhibitors was considered to be correlated with the difference in the complex-forming properties of these compounds toward the nucleophile in the enzyme.

Many compounds including antibiotics have been known to act as specific and irreversible enzyme inhibitors. Such compounds possessing latent reactive groups become reactive after being acted upon by the target enzyme and bind covalently with the enzyme, leading to its irreversible inhibition. These types of inhibitors have been termed "K_{cat}" inhibitors, since they require catalytic conversion into their active forms by the target enzyme.[1] Rando has presented very attractive reaction schemes of many types of K_{cat} inhibitors, among which pyridoxal-linked inhibitors have especially been studied.[2-4] Scheme I was postulated by him for the inhibitory action of an antibiotic, cycloserine, toward alanine racemase.[4] Thus, the compound becomes reactive by forming a Schiff base with the coenzyme part, pyridoxal, and binds covalently with the nucleophile in the apoenzyme, leading to irreversible inhibition.

The reason why the pyridoxal-linked state is far more reactive than the free cycloserine is of utmost importance in clarifying the mechanism of K_{cat} inhibitors. In the present paper, it is attempted, from the electronic viewpoint, to rationalize the mechanism proposed by Rando for the activation of pyridoxal-linked inhibitors. Furthermore, the different behavior of enzyme inhibitors from the substrate is discussed based on their electronic structures.

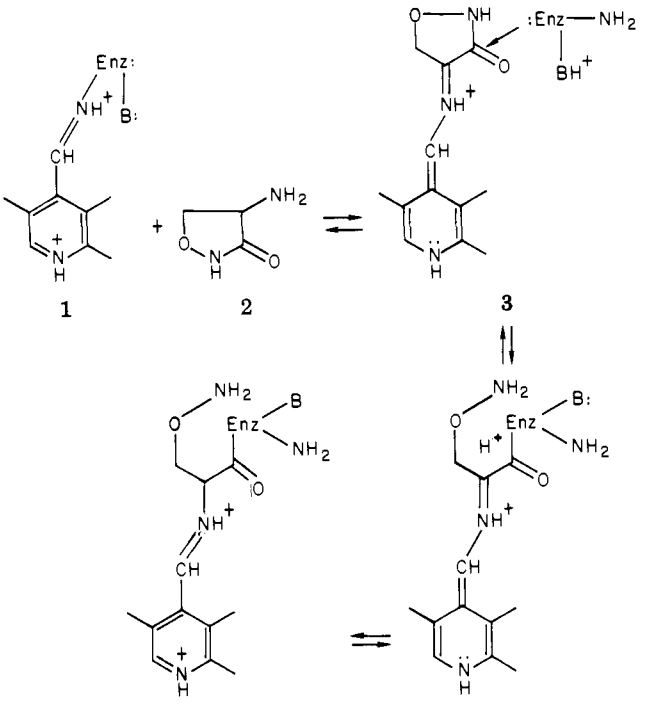
Method of Calculation. The method used in our calculation is the CNDO/2 method developed by Pople and Segal,[5] details of which are not described here. This method has been proved to be reliable in predicting the properties of chemical compounds, and it has successfully been applied to the biologically important molecules, such as the charge relay system of serine proteases,[6,7] coenzyme NADH,[8] poly(amino acids),[9,10] nucleic acids,[11-13] and pharmacological compounds.[14,15]

Theoretical indices used in the present paper are total energy, energy increment, electronic charge, and molecular orbital energy. The total energy is the sum of the electronic energy of all valence electrons and core-repulsion energy. The latter was calculated by the following equation

$$E_{\text{core rep}} = \frac{1}{2}\sum_{A}\sum_{B(\neq A)}\frac{Z_A Z_B e^2}{R_{AB}} \qquad (1)$$

where R_{AB} is the distance between the atom A and B and where Z_A and Z_B are the effective nuclear charges on the atom A and B. As a reactive index for predicting the ease of reaction, the "energy increment", ΔE, for the reaction

Scheme I



has been used.[16,17] This index for the reaction, A + B → C + D, was defined as

$$\Delta E = \{E_C + E_D\} - \{E_A + E_B\} \qquad (2)$$

where E_A and E_B are the total energies calculated by means of the CNDO/2 method for the initial reactants; E_C and E_D, for the final products in the reaction. In general, the chemical reaction proceeds when the energy of the products is smaller than that of the reactants, i.e., $\Delta E < 0$. The chemical noncrossing rule is considered to be valid for many chemical reactions; that is, the smaller the value of ΔE is, the lower the activation energy is. Accordingly, ΔE can be used as a measure of the reaction rate when no data on the activation energy are available, and the relative rate of the two reactions is predictable by comparing the energy increments of the two reactions. Thus, when the value of ΔE for a reaction is smaller than that for another reaction, the former reaction is predicted to proceed more easily than the latter one.

Among the molecular orbitals, the highest occupied

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Table I. Total Energies for Various Conformations (A-D)[a] of AMB-Pyridoxal (I), Vinylglycine-Pyridoxal (II), and Aspartic Acid-Pyridoxal (III) Calculated by Means of the CNDO/2 Method

compd	total energy (eV) for conformation			
	A	B	C	D
I	-4519.5130	-4519.5689	-4520.0417	-4520.1872
II	-3781.4543	-3781.5035	-3781.9660	-3782.1040
III	-4769.0427	-4769.0446	-4769.4049	-4769.6218

[a] For a definition of conformations A-D, see the text.

molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) are especially remarked, because the HOMO and LUMO reflect well the electron-donating and -accepting capacities of a molecule, respectively. Recently, the importance of the HOMO and LUMO in predicting the molecular properties of amino acids was stressed.[18]

More than one conformation are considered for the pyridoxal-linked states of vinylglycine, AMB, and aspartate: thus, the planar conformation (A), the conformation with the rotated pyridinium ring by 90° around the C_4-CH_2 (pyridinium ring) axis (B), the conformation with 90° rotation around the N-CH_2 axis (C), and the conformation with 90° rotation around C_4-CH_2 (pyridinium ring) axis in addition to 90° rotation around the N-CH_2 axis (D). In comparing the stability of various conformations of a molecule, the total energy has usually been used; thus, the larger the absolute value of the total energy of a conformation, the more stable is the conformation. Calculated total energies for these conformations are indicated in Table I, from which conformation D is found to be the most stable for three compounds. Accordingly, conformation D is taken into consideration throughout the present paper. In the calculation of the pyridoxal-linked states, the SCF calculation was frequently diverged. In order to overcome the difficulty of divergence, we used the density matrix method,[19] by which the energies for all conformations were calculated without divergence.

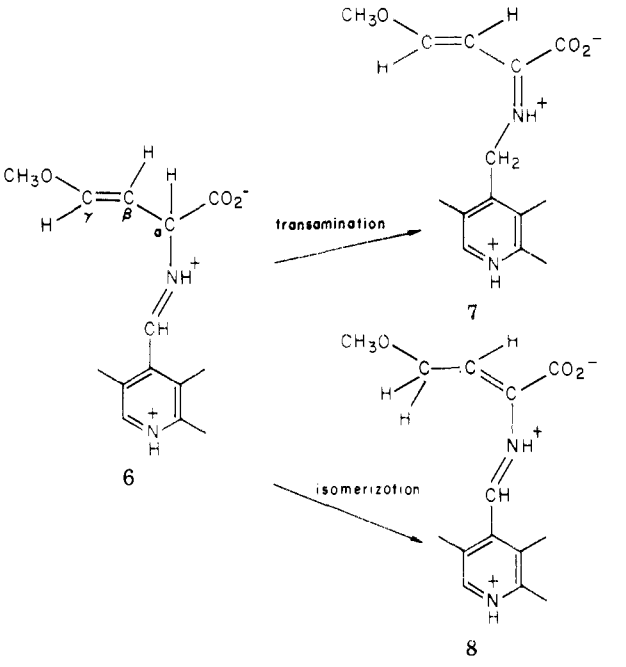
The bond lengths and bond angles for cycloserine, aspartate, vinylglycine, AMB, serine, and cysteine are determined from the X-ray diffraction data for similar compounds.[20] The same values for the free compounds were adopted for the pyridoxal-linked compounds, and values for the pyridoxal part were taken from the data for pyridinium compounds. In calculating pyridoxal, substituents in the benzene ring, such as methyl, phosphoryl, and hydroxyl groups, were not included because of the shortage of memory of our computer (HITAC 8350). However, this does not alter the conclusion, because such groups are common for both free and pyridoxal-linked states.

Results and Discussion

Pathways of the Irreversible Inhibition of Aspartate Aminotransferase by AMB. As indicated in Scheme II, two possible pathways have been considered for the irreversible inactivation of aspartate aminotransferase by the naturally occurring toxin, AMB. Thus, 7 is considered to be formed as a consequence of a normal transamination, and the 8 would result from a $\beta\gamma \rightarrow \alpha\beta$ isomerization. Based on the data of the irreversible inactivation of aspartate aminotransferase by 2-keto-4-methoxy-*trans*-3-butenoic acid and also from the model studies on the transamination or/and isomerization of AMB by pyridoxal, Rando et al. proposed that inactivation of aminotransferase by AMB would occur via a transamination process.[21]

In this article, it is attempted to predict theoretically which pathway in Scheme II is more preferable by comparing energy increments for these two reaction pathways. Since the second term on the right side of eq 2 is common for the two pathways, it is sufficient to compare the total energy for 7 and 8. The total energies for these compounds were calculated as -4520.19 and -4518.04 eV, respectively, predicting that the pathway via transamination is energetically favorable by 49 kcal/mol (1 eV = 23 kcal/mol), and this supports the mechanism proposed by Rando et al.[21] Similarly, the total energy for the transaminated product of vinylglycine is calculated as -3782.10 eV, and this is more stable than the isomerized product by 38 kcal/mol. This seems to be in contradiction with the experimental finding that vinylglycine undergoes an isomerization reaction almost exclusively.[21,22] However, this is not necessarily the case, because isomerization of vinylglycine occurs only when Al^{3+} is added in the reaction mixture, and when Al^{3+} was omitted the transamination occured almost exclusively.[21] This means that the occurrence of a transamination reaction is easier than isomerization in the absence of Al^{3+}, in agreement with the calculated total energy. The fact that AMB and vinylglycine undergo different reactions in the presence of Al^{3+} is of interest in connection with the possible function of Al^{3+}. Rando et al. suggested that the double bond in AMB was stabilized by the electron-donating methoxyl group.[21] However, the bond orders for the β-γ double bond of AMB and vinylglycine were calculated as 0.827 and 0.904, respectively, and this shows that the methoxyl group has no stabilizing effect on the double bond but rather has a destabilizing effect. Two atomic energy terms, which are also a reflection of the bond stability, were calculated as -43.590 and -44.631 eV for AMB and vinylglycine, giving the same prediction as the bond order. Thus, another

Scheme II



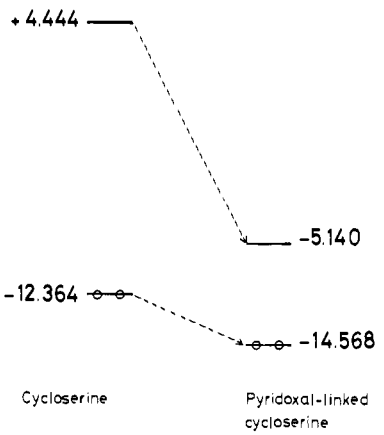


Figure 1. The HOMO and LUMO calculated by the CNDO/2 method for cycloserine (2) and its pyridoxal-linked state (3).

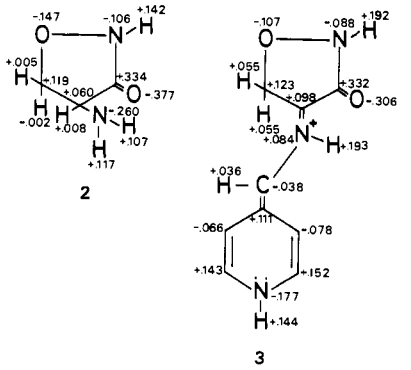


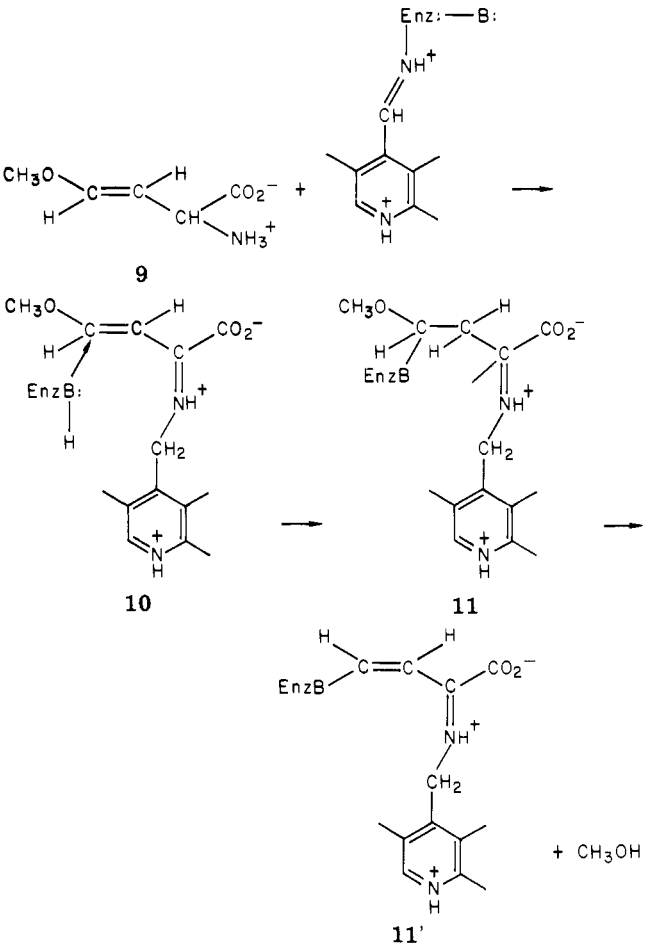
Figure 2. Electronic charges calculated by the CNDO/2 method for cycloserine (2) and its pyridoxal-linked state (3).

explanation is necessary to understand the different behaviors of AMB and vinylglycine. A plausible explanation is that chelation of aluminum ion to the γ-carbon in AMB is difficult due to the steric effect of the bulky methoxyl group.

Cycloserine, an Inhibitor of D-Alanine Racemase. Calculated HOMO and LUMO for the pyridoxal-linked cycloserine are given in Figure 1 in comparison with those of isolated cycloserine. As was expected, the LUMO of cycloserine is considerably lowered by linking with the pyridoxal, due to the positive charge in the molecule. However, the degree of alteration in the energy was largely different between the HOMO and LUMO; that is, lowering of the LUMO was especially large in comparison with the HOMO. In the quantum chemistry of organic reactions, a well-known theorem states that the lower the LUMO of a molecule, the more electron accepting and, accordingly, the more reactive is the molecule toward the nucleophile. On the other hand, the higher the HOMO of a molecule, the more electron donating and more reactive is the molecule toward the electrophile.[23,24] Accordingly, we can anticipate that the pyridoxal-linked cycloserine becomes extremely reactive toward the nucleophile. Thus, the lowering of the LUMO of cycloserine by forming the Schiff base with pyridoxal seems to be an essential factor for the conversion of the unreactive cycloserine into a reactive inhibitor of racemase.

The positive charge at the carbonyl carbon to which the nucleophile attaches covalently is considered to be an important factor in determining the reactivity of cycloserine, as was pointed out by Rando.[4] However, the positive charge at the carbonyl carbon of the pyridoxal-linked cycloserine is rather small compared to that of the free cycloserine (Figure 2). Thus, it is not the positive charge but rather the molecular orbital energy which seems

Scheme III



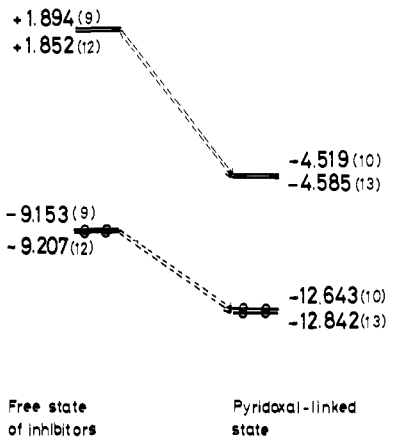
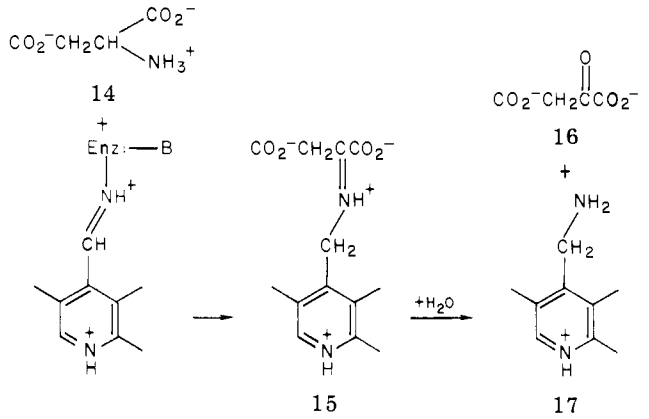


Figure 3. The HOMO and LUMO calculated by the CNDO/2 method for AMB (9), vinylglycine (12), and their pyridoxal-linked state (10 and 13).

to be a primary factor in altering the reactivity of cycloserine toward the nucleophile. Of course, as will be described later, the positive charge at the reaction site may be of importance in determining the reactivity toward the nucleophile, even if it is not a decisive factor.

Inhibitors of Aspartate Aminotransferase. Although the microbial toxin AMB and vinylglycine have no reactive groups, they are transformed into a reactive inhibitor by the target enzyme, aspartate aminotransferase.[2] Rando has assumed that these compounds, just as the case of cycloserine, become sufficiently reactive to engage in a chemical reaction with the target enzyme by forming a Schiff base with the pyridoxal moiety[2] (Scheme III). As indicated in Figure 3, the LUMO of AMB and vinylglycine

Scheme IV



14 15 16 17

are greatly lowered by forming a Schiff base with pyridoxal. As described in the case of cycloserine, these changes in the molecular orbital levels are considered to be intimately correlated with the enzyme-catalytic inhibition by these compounds. As was previously described, the fact that vinylglycine undergoes an isomerization reaction in the presence of Al^{3+} is considered to be due to its easier chelate formation with Al^{3+}. As in the case of the transamination reaction, the energy of the LUMO of vinylglycine is progressively lowered by forming pyridoxal-linked complex in the isomerization reaction too, i.e., from +1.852 to -6.796 eV. Accordingly, in this case too, an increase of the reactivity toward the nucleophile is expected as in the case of the transamination reaction.

Electronic Aspect of Different Behavior between Substrate and Inhibitors. It is of interest to compare the molecular orbital energy of the inhibitors with that of the substrate aspartate, because in the latter case the reaction is not inhibited but proceeds by the addition of water. This means that the complex between the pyridoxal and aspartate (Scheme IV) is not covalently bound but loosely bound. Pyridoxal-linked aspartate has α-carbon alone as a susceptible site to be attacked by nucleophile, whereas pyridoxal-linked inhibitors have an additional two sites to be susceptible to the attack of reagents (compare compounds 10 and 15). This difference is considered to be a crucial factor to discriminate between the substrate and inhibitors. However, it should also be remarked that relative values of the LUMO for the substrate and inhibitors are largely different. For the reason given below, this difference is also considered to be correlated with the different behavior between the substrate and inhibitors. Thus, Nagakura and Tanaka[25] have found an important relation between the orbital energy and chemical reaction. That is, in the case of the chemical reaction of benzene with electrophilic reagents, such as NO^+, I^+, Br^+, and Cl^+, the LUMO of the reagents lie lower than that of the HOMO of benzene, whereas in the case of molecular complex formation the HOMO of benzene lies lower than the LUMO of the partner, such as a silver ion or an iodine molelcule. As indicated in Figures 3 and 4, the LUMO of aspartate, a substrate of aspartate aminotransferase, is higher than those of inhibitors of the enzyme in the free state, as well as in the pyridoxal-linked state. This means that the electron-accepting capacity and reactivity toward the nucleophile of two inhibitors are larger than that of the substrate. Although the nucleophile involved in the covalent binding is not identified, it is sure that one of the electronegative atoms, such as O, S, and N, is involved.[21] The HOMO of the nucleophilic groups $-O^-$ of serine and $-S^-$ of cysteine are calculated as -3.18 and -3.40 eV, respectively. It is worth noting that these values are higher

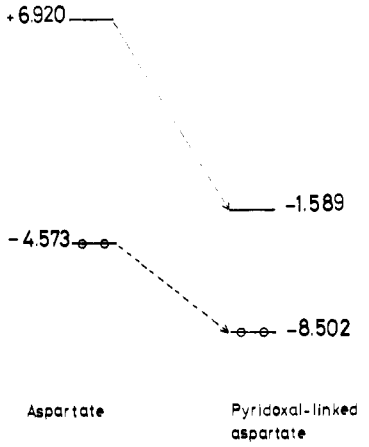
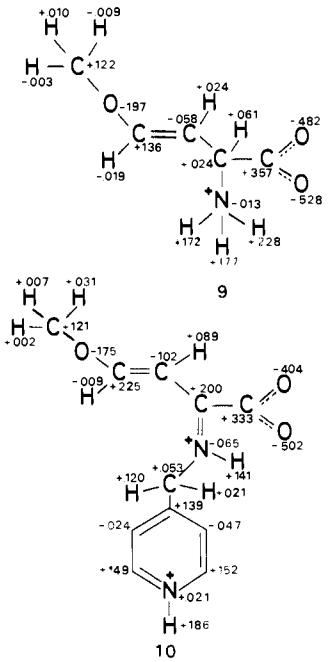


Figure 4. The HOMO and LUMO calculated by the CNDO/2 method for aspartate (14) and its pyridoxal-linked state (15).

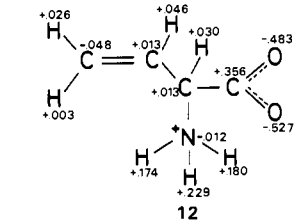


9 10

Figure 5. Electronic charges calculated by the CNDO/2 method for AMB (9) and its pyridoxal-linked state (10).

than the LUMO of inhibitors but lower than that of substrate. Applying the Nagakura and Tanaka's theorem, the $-O^-$ and $-S^-$ are considered to engage in a chemical reaction with the inhibitors resulting in covalent binding, whereas formation of a molecular complex is expected with the substrate. Of course, the values of the HOMO of the nucleophile in the enzyme may not necessarily be the same as those of $-O^-$ of serine or $-S^-$ of cysteine, but even when this is not the case the relative reactivity of inhibitors and substrate to the nucleophile may be retained. That is, the capacity of the complex formation of inhibitors is far larger than that of the substrate, and this difference in complex-forming force is considered to be one of the factors that lead to different behaviors between the substrate and inhibitors.

Electronic Charges and Positions of Attack. The β-γ double bond in AMB and vinylglycine is susceptible to the simultaneous addition reaction. As shown in Figures 5 and 6, γ carbons are positively charged and β carbons are negatively charged in the pyridoxal-linked states of AMB and vinylglycine. This corresponds well with the scheme of Rando in which the nucleophilic EnzB: attacks γ carbon and the electrophilic H^+ attacks β carbon simultaneously (Scheme III). Thus, the positive charge at



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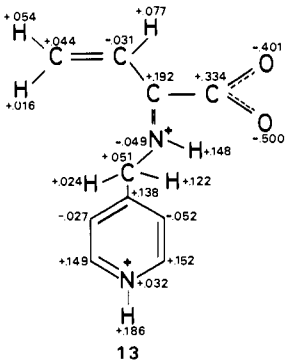

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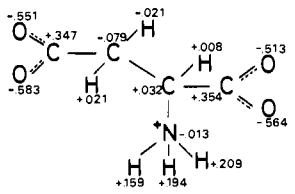
Figure 6. Electronic charges calculated by the CNDO/2 method for vinylglycine (**12**) and its pyridoxal-linked state (**13**).



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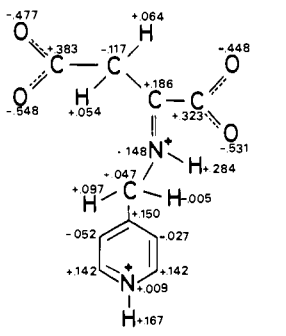

15

Figure 7. Electronic charges calculated by the CNDO/2 method for aspartate (**14**) and its pyridoxal-linked state (**15**).

the γ carbon of AMB increased from +0.136 to +0.225 and the negative charge at the β carbon increased from −0.058 to −0.102 by linking with pyridoxal, making these positions more favorable to the nucleophilic and electrophilic attacks, respectively (Figure 5). It is also worth noting that negative charge at the γ carbon and positive charge at the β carbon in vinylglycine are altered to become positive and negative, respectively, by linking with pyridoxal, making these sites susceptible to the nucleophilic and electrophilic attacks, respectively (Figure 6). Accordingly, in addition to the lowering of the values of LUMO, the electronic charges at the β and γ carbons are altered to make the β–γ bond favorable to the simultaneous addition reaction. The α carbon of pyridoxal-linked aspartate is considered to be susceptible to the attack of the nucleophile via an addition reaction (Figure 7). However, as described earlier, the value of the LUMO of aspartate is far higher than those of the inhibitors. Accordingly, even if the nucleophile attacks the α carbon of aspartate, it is not considered to be covalent but rather a weakly bound molecular complex.

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