A Model for Enzyme−Substrate Interaction in Alanine Racemase

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Abstract: We report on a theoretical model for the complex of the enzyme alanine racemase with its natural substrate (L-alanine) and cofactor (pyridoxal 5′-phosphate). Electrostatic potentials were calculated and ionization states were predicted for all of the ionizable groups in alanine racemase. Some rather unusual charge states were predicted for certain residues. Tyr265′ has an unusually low predicted pK_a of 7.9 and at pH 7.0 has a predicted average charge of −0.37, meaning that 37% of the Tyr265′ residues in an ensemble of enzyme molecules are in the phenolate form. At pH 8−9, the majority of Tyr265′ side groups will be in the phenolate form. This lends support to the experimental evidence that Tyr265′ is the catalytic base involved in the conversion of L-alanine to D-alanine. Residues Lys39 and Lys129 have predicted average charges of +0.91 and +0.14, respectively, at pH 7.0. Lys39 is believed to be the catalytic base for the conversion of D-alanine to L-alanine, and the present results show that, at least some of the time, it is in the unprotonated amine form and thus able to act as a base. Cys311′, which is located very close to the active site, has an unusually low predicted pK_a of 5.8 and at pH 7.0 has a predicted average charge of −0.72. The very low predicted charge for Lys129 is consistent with experimental evidence that it is carbamylated, since an unprotonated amine group is available to act as a Lewis base and form the carbamate with CO_2. Repeating the pK_a calculations on the enzyme with Lys129 in carbamylated form predicts trends similar to those of the uncarbamylated enzyme. It appears that the enzyme has the ability to stabilize negative charge in the region of the active site. Implications for selective inhibitor design are discussed.

Introduction

The bacterial enzyme alanine racemase catalyzes the interconversion of L-alanine and D-alanine. The production of D-alanine in this manner is a required step in bacterial cell wall construction. In addition, there is no known human alanine racemase, and thus alanine racemase represents an ideal target for new antibiotic design. Inhibitors of alanine racemase, such as D-cycloserine, are of particular interest as agents against *Mycobacterium tuberculosis*.[1] However, D-cycloserine and most other known alanine racemase inhibitors are suicide inhibitors that form a covalent adduct with the pyridoxal 5′-phosphate (PLP) cofactor or the protein. In addition, clinical application of these inhibitors results in an array of serious side effects, especially neurological, presumably because these inhibitors are not specific for alanine racemase but are specific for PLP. In the present paper, we report on a theoretical model for alanine racemase complexed with its natural substrate and cofactor, and we discuss the unique features of the interaction between the substrate and the alanine racemase active-site region.

Catalysis by alanine racemase depends on the cofactor pyridoxal 5′-phosphate, a phosphorylated and oxidized form of vitamin B6. Pyridoxal phosphate (**I**) combines with an α-amino acid (**II**) to form a Schiff base (**III**) as in Figure 1. When this Schiff base remains bound in the protein but is not covalently attached to the protein, it is called an *external aldimine*. When PLP is covalently attached to a protein, generally by formation of a Schiff base with the ϵ-amino group of the side chain of a lysine residue, this is called an *internal aldimine*.

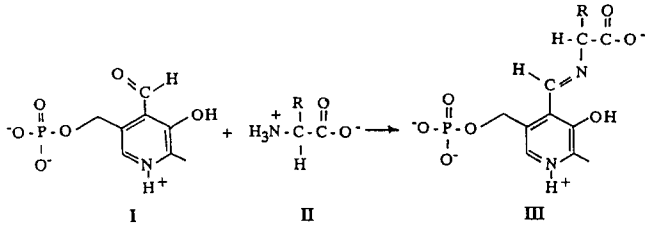

Figure 1. The reaction of pyridoxal phosphate (**I**) with an α-amino acid (**II**) to form a Schiff base (**III**).

The class of enzymes which utilize a vitamin B6 derivative as a cofactor is of particular interest because the Schiff base **III** may proceed along many different possible reaction paths toward completely different products, depending on which enzyme serves as the catalyst. Enzymes in this class catalyze a diverse set of reactions that, in addition to racemization, include transamination, decarboxylation, and reactions of the amino acid side chain. It is of particular interest to understand the structural and electronic features of the enzymes in this class that enable them to promote selectively one particular reaction.

Experimental Background

In 1997 Shaw, Petsko and Ringe[2] reported the structure of alanine racemase from *Bacillus stearothermophilus* to 1.9 Å

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resolution. The structure is a dimer where the two chains have the same amino acid sequence and are structurally very similar but not identical. Following ref 2, we shall designate the two subunits as the unprimed and primed chains. Note that the homodimer has two active sites which are both completed upon dimer formation. Each active site is made up primarily of residues from one chain (unprimed) but is completed by several residues from the other chain (primed). In the reported structure, PLP forms an internal aldimine with the side chain of Lys39. Upon introduction of free alanine into the active site, the covalent attachment of the PLP to the side chain of Lys39 is broken, and the PLP forms an external aldimine with the alanine. Alanine undergoes enzyme-catalyzed racemization while covalently attached to the PLP. In other words, the reactive species is the external aldimine, the Schiff base formed from PLP and alanine. It is believed that the first step in the racemization reaction is abstraction of the α-hydrogen atom. Shaw et al. suggest that the enzyme has two bases involved in this step, one for each enantiomer.[2] The first of these bases is believed to be Lys39 itself, the residue that forms the Schiff base linkage with the PLP cofactor before the substrate binds. The authors further suggest that the OH group of Tyr265′ (from the other monomer) is the second base. Tyr265′ was found to be part of a hydrogen-bonded network consisting of Tyr265′-His166-Arg219-His200-His127-Glu161.

Later, Stamper, Morollo, and Ringe[3] reported the structure of alanine racemase from *Bacillus stearothermophilus* complexed with an inhibitor, an external aldimine of PLP and alanine phosphonate. In this inhibitor, the carboxylate group of alanine is replaced by a phosphonate. These authors report that the Tyr265′ is in position to abstract the α-hydrogen atom from the L-alanine moiety of the external aldimine, while Lys39 is in position to abstract the α-hydrogen atom from the corresponding D-enantiomer. Thus, a two-base mechanism is supported by this structure.

Very recently, Watanabe et al.[4] reported on site-directed mutagenesis studies in which the Tyr265′ residue was replaced, in that case by an amino acid which is completely unable (or significantly less able) to function as a base (phenylalanine, alanine, or serine). The mutant enzymes (Y265F, Y265A, and Y265S) are poor catalysts for racemization. Rates for racemization of alanine are reported to decline by a factor of about 10^4.[4] These authors conclude that Tyr265′ is the catalytic base for the conversion of L-alanine to D-alanine. They further conclude that Tyr265′ is the counterpart residue to Lys39, which catalyzes the conversion of D-alanine to L-alanine.

Alanine racemase has been shown to be bifunctional, in that at lower pH, transamination is also catalyzed. The optimum pH for racemization was reported to be around 9-10, whereas the rate of transamination is enhanced around pH 6.[5]

In the present work, we construct a theoretical model for interaction between alanine racemase, its substrate and cofactor. We examine how the Schiff base influences the enzyme and how the enzyme influences the Schiff base.

Theoretical Background

A number of theories concerning the mechanism of enzyme action have been proposed. These include the effects of solvation and desolvation in the region of the active site[6−8] and the suggestion that electrostatic effects can play a major role.[7−12]

Warshel[7,11,13] has argued that enzymes function by solvent substitution in the active site in the immediate region around the substrate and therefore lower the electrostatic energy difference between ground and transition states.

Others suggest that low-barrier hydrogen bonds between the substrate and multiple adjacent side groups in the active site serve to activate the ground state or stabilize the transition state and thus reduce the activation energy barrier.[14−17]

To date there have been a few calculations reported on the electronic structure of PLP Schiff bases. The electronic structure at the AM1 level of some pyridoxal phosphate derivatives has been reported by Nero et al.,[18] with a focus on the structures believed to be the intermediates in transamination. In that paper, the Schiff base was formed using γ-aminobutyric acid (GABA) instead of an α-amino acid. Also, an OH group was used in place of the phosphate. Calculations were performed on structure **III** and on the presumed transamination intermediates, namely a quinonoid structure and a ketimine structure. On the basis of the atomic charges and HOMO and LUMO energies for the structures at physiological pH, the authors conclude that the AM1 results predict labilization at the 4′-carbon atom of the pyridoxal moiety, rather than at the γ-carbon atom of the amino acid moiety. This prediction is contrary to observation for the transamination reaction. However, this calculation was performed for the free Schiff base and not for the enzyme-bound Schiff base.

More recently, higher-level ab initio calculations were reported at electron-correlated levels for the Schiff base formed

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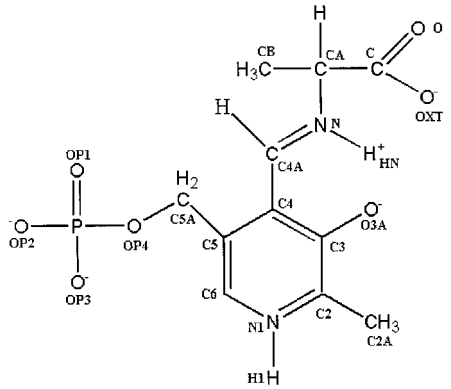

Figure 2. The atom naming scheme for the Schiff base (**III**) or aldimine, formed from the addition of alanine to pyridoxal phosphate (**I**).

from pyridoxal phosphate and alanine.[19] These authors address how the pyridoxal functionality may facilitate enzyme-catalyzed decarboxylation. It was also found that the Hartree−Fock level of theory overestimates the activation energy barrier, indicating that electron correlation is important, particularly in the characterization of the transition state.

Methods

First an ab initio calculation was performed at the Hartree−Fock level for the Schiff base (**III**) formed from PLP and L-alanine, using the GAMESS package.[20] A double-ζ basis set[21] was used for the O, N, C, and H atoms. The correlation consistent polarization valence triple-ζ (cc-pVTZ) basis set of Woon and Dunning[22] was used for the P atom. Full geometry optimization was performed. These calculations were performed using parallel processors on a CRAY T3E supercomputer.

The atom numbering scheme for the Schiff base is shown in Figure 2. Partial atomic charges needed for molecular modeling force field parameters for the Schiff base (**III**) were obtained based on the Löwdin atomic charges from the ab initio calculation.

A model structure for the protein-bound Schiff base (external aldimine) was made using the bond distances and the bond angles from the geometry optimization and the dihedral angles of the inhibitor structure reported by Stamper et al.[3]. We refer to this structure as the model protein-bound external aldimine (MPBEA).

Potentials were calculated and protonation states were predicted for all of the titratible groups in alanine racemase, using the pK_a procedure[23,24] implemented in the UHBD program.[25] This calculation was performed on the protein without the Schiff base and again on the protein with the MPBEA present in the active site. The calculations were also performed on the protein with Lys129 in carbamylated form.[26] Parameters for carbamylated lysine (KCX) were based on an ab initio calculation on *N*-methyl carbamate [$CH_3NHCO_2^-$].[27]

Table 1. Löwdin Atomic Charges for the Schiff Base

atom	charge	atom	charge	atom	charge	atom	charge
N1	−0.14	P	−1.99	C4	−0.09	HA	+0.14
H1	+0.28	OP1	−0.06	C4A	+0.20	CB	−0.29
C2	+0.03	OP2	−0.05	H4A	+0.20	HB1	+0.14
C2A	−0.26	OP3	−0.02	C5	−0.00	HB2	+0.12
H2A1	+0.15	C6	−0.05	C5A	+0.09	HB3	+0.13
H2A2	+0.14	H6	+0.16	H5A1	+0.13	C	+0.22
H2A3	+0.09	N	−0.16	H5A2	+0.10	O	−0.55
C3	+0.14	HN	+0.34	OP4	+0.05	OXT	−0.54
O3A	−0.57	CA	−0.07				

Table 2. Predicted Average Net Charge for Selected Residues of Alanine Racemase

		calculated charge at pH 7.0	
residue	AA	MPBEA not bound	MPBEA bound*
39	LYS	0.91	0.92
39′	LYS	0.94	0.99
129	LYS	0.14	0.14
129′	LYS	0.17	0.08
265	TYR	−0.37	0.00
265′	TYR	−0.37	−0.38
311	CYS	−0.73	−0.07
311′	CYS	−0.72	−0.70

[a] MPBEA bound to unprimed (B) chain.

Table 3. Predicted Net Charge as a Function of pH for Selected Residues of Alanine Racemase

	calculated charge at pH					
residue	5.0	6.0	7.0	8.0	9.0	10.0
TYR 265′	−0.19	−0.28	−0.38	−0.51	−0.66	−0.77
CYS 311′	−0.38	−0.54	−0.70	−0.83	−0.92	−0.96
LYS 39	0.98	0.96	0.92	0.81	0.68	0.56
LYS 129	0.58	0.35	0.14	0.06	0.03	0.02

Results and Discussion

Table 1 gives the Löwdin charges obtained by the ab initio calculation for the atoms of the Schiff base. These charges were used for the molecular modeling force field parameters for the Schiff base.

Upon calculation of the electrostatic potentials and prediction of the charge states for the ionizable groups of alanine racemase, some rather unusual charge states were found for certain residues. Table 2 shows the calculated net average charge for selected residues of the apoenzyme (the protein with no Schiff base bound) and for the protein with the MPBEA present in the active site of the unprimed chain. (For purposes of the present calculation, the B chain is the unprimed chain, and the A chain is the primed chain.) Table 3 shows the predicted net average charge for some selected residues as a function of pH. Results were obtained in the same fashion as for Table 2. The pK_a's for the individual residues were calculated by interpolation. Table 4 shows the predicted pK_a's for selected residues.

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(27) The following effective charge parameters were used for carbamylated lysine (KCX): NZ −0.400; HZ1 +0.250; CO +0.300; OC1−0.575; OC2−0.575. For the remaining atoms, the parameters used were the same as for lysine.

Table 4. Predicted pK_a for Selected Residues of Alanine Racemase

residue	AA	predicted pK_a
129	LYS+	5.3
180	TYR	10.1
182	TYR	10.7
201	CYS	8.4
265′	TYR	7.9
311	CYS	8.6
311′	CYS	5.8

[a] Note: LYS+ denotes the conjugate acid of lysine.

All of the LYS residues, with the exception of Lys39 and Lys129, are predicted to be fully protonated, or almost fully protonated, at neutral pH and all the way up to the pH 9−10 range. Lys39 has a predicted average net charge of 0.92 at pH 7.0 and of 0.68 at pH 9.0. Thus, some of the Lys39 residues in an ensemble of alanine racemase protein molecules are unprotonated in the 7−9 pH range and thus are able to act as a base. Another way to think of this is that the Lys39 has a shifted pK_a such that it is not protonated 100% of the time. This is consistent with the earlier assertions[3,4] that Lys39 serves as one of the bases in the catalytic racemization.

Even more unusual are the predicted charges for Lys129, which are far less than the typical value of +1.00 at pH 7. There is structural evidence that this residue is carbamylated in nature.[26] The results of Table 2 are consistent with this observation; a Lys129 residue is predicted to be most likely neutral at pH 7. Thus, it is able to act as a Lewis base and react with CO_2 to form the carbamate. Indeed, the conjugate acid of Lys129 has a very low pK_a, predicted to be 5.3.

Of particular interest is Tyr265′, which is predicted to have significant negative charge at pH 7 and therefore is able to function as a Brønsted base. This is consistent with the structural data of refs 2 and 3, and with the kinetics and site-directed mutagenesis results of ref 4. In nature, this enzyme is most likely active at around pH 8. From Table 3, we see that for a pH greater than 8.0, the Tyr265′ residues are mostly negatively charged and therefore available to serve as a Brønsted base. Tyr180 and Tyr182 are listed in Table 4 as representative TYR residues. They have predicted pK_a's of 10.1 and 10.7, respectively, which are typical values for a TYR side chain. Tyr265′ has an unusually low predicted pK_a of 7.9.

Cys311′ is also likely to be ionized and could play a role in a proton-transfer system in the region of the active site. Table 2 shows a predicted net charge of about −0.7 for Cys311′ at pH 7. According to ref 5, the optimum pH for racemization is 9−10. The results of Table 3 show that both Tyr265′ and Cys311′ are well ionized in this pH range. Cys201 and Cys311 are listed in Table 4 as representative CYS residues. They have predicted pK_a's of 8.4 and 8.6, respectively, which are typical values for a CYS side chain. CYS 311′ has an unusually low predicted pK_a of 5.8.

It is important to note that Tyr265′ and Cys311′ are both located close to the amino acid moiety of the Schiff base, when the Schiff base is in its presumed reactive orientation. Figure 3 shows a stereoview of the PLP and selected residues in the active site of alanine racemase. The phenolic oxygen atom of Tyr265′ is about 3 Å away from the α-carbon atom of alanine. The sulfur atom of Cys311′ (labeled as SG in Figure 3) is about 5 Å away from the phenolic oxygen atom of Tyr265′. SG is also about 6 Å away from the α-carbon atom of alanine. Both Tyr265′ and Cys311′ have uncommonly negative charges. This suggests that the field of the protein stabilizes negative charge in this region of space. This could serve to stabilize an anionic reaction intermediate during racemization.

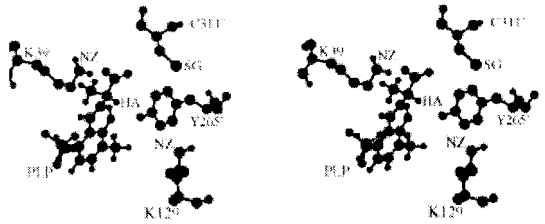

Figure 3. A stereoview of the PLP and selected residues in the active site of alanine racemase. The ϵ-nitrogen atoms of Lys39 and Lys129 are labeled NZ. The α-hydrogen atom on alanine is labeled HA. Notice its close proximity to the phenolic oxygen atom of Tyr265′. The sulfur atom of Cys311′ is labeled SG. Note its proximity to Tyr 265′ and to the amino acid moiety of the PLP Schiff base.

Table 5. Predicted Net Charges for Selected Residues for the Uncarbamylated (Lys129) and Carbamylated (Kcx129) Enzyme at pH 7.0 and pH 9.0

	pH 7.0		pH 9.0	
residue	with Lys129	with Kcx 129	with Lys129	with Kcx129
Lys39	0.94	0.98	0.78	0.88
Lys39′	0.91	0.97	0.66	0.83
Lys76	1.00	1.00	1.00	1.00
Lys76′	1.00	1.00	1.00	1.00
Lys140	1.00	1.00	0.99	0.99
Lys140′	1.00	1.00	0.99	0.99
Tyr265	−0.37	−0.18	−0.65	−0.37
Tyr265′	−0.37	−0.17	−0.68	−0.35
Tyr269	0.00	0.00	0.00	0.00
Tyr269′	0.00	0.00	0.00	0.00
Tyr284	0.00	0.00	−0.01	−0.02
Tyr284′	−0.01	0.00	−0.02	−0.02
Tyr354	−0.01	−0.01	−0.05	−0.08
Tyr354′	−0.02	−0.01	−0.09	−0.09
Cys311	−0.72	−0.54	−0.93	−0.86
Cys311′	−0.73	−0.54	−0.92	−0.86
Cys315	0.00	0.00	−0.02	−0.02
Cys315′	0.00	0.00	−0.03	−0.03
Cys358	0.00	0.00	−0.03	−0.03
Cys358′	−0.01	0.00	−0.04	−0.05

It is also important to remember that Tyr265′ and Cys311′ are actually part of the active site of the unprimed chain in the dimer. These members of the primed chain are located near the dimer interface and serve as part of the active site of the other chain. The external aldimine must be bound in the active site of the unprimed chain in order to have the unusual negative charge states for adjacent residues Tyr265′ and Cys311′. When the external aldimine is in the primed chain, the predicted values for the average net charge for Tyr265′ and Cys311′ are more typical (close to zero). In the example shown in Table 2 the active site for the primed chain is unoccupied, and the active site for the unprimed chain contains the external aldimine. The predicted net charges for Tyr265 and Cys311 are not unusual.

Since the experimental structural data[26] suggest that Lys129 is carbamylated and since the above results are consistent with this observation, the calculations of the potentials and the titration curves were repeated for the enzyme with Lys129 in carbamylated form. Table 5 shows the predicted net charges for selected residues of alanine racemase in both uncarbamylated (Lys129) and carbamylated (Kcx129) form at pH 7.0 and pH 9.0.[28] As one might expect, the presence of a negatively charged carbamate in the active site has the effect of increasing (or making less negative) the charge on adjacent residues. However,

(28) For the example shown in Table 5, no PLP is bound. The unprimed and primed chains show slight differences because of their slight difference in structure.

the unusual charge states reported above, although somewhat less pronounced, are still predicted even in the presence of the carbamate.

Table 5 shows that a small fraction of the Lys39 and Lys39′ residues are predicted to be unprotonated at pH 7.0, although this fraction is smaller in the presence of carbamate. Lys76 and Lys140 are shown in Table 5 as more typical Lys residues. At pH 9.0, the difference between Lys39 and the more typical Lys residues is more pronounced, even in the presence of the carbamate.

Table 5 also shows Tyr265 and Tyr265′ to be different from the more typical Tyr residues, 269, 284 and 354, although the predicted charges for 265 are less negative in the presence of carbamate. Likewise, Cys311 and Cys311′ show considerable difference from the more typical Cys residues, 315 and 358.

Conclusions

The present results supply further evidence that Tyr265′ does in fact function as the catalytic base in the conversion of L-alanine to D-alanine. The predicted negative charge and the low pK_a indicate that, for a substantial fraction of an ensemble of protein molecules, this side group is in the phenolate form in the pH range where the enzyme is most active for racemization (pH 7−9).

We also note that a small fraction of the Lys39 residues are not protonated in the pH range where the enzyme is most active for racemization. Thus, we predict that Lys39 is available, for part of the time, as an unprotonated amine and therefore is able to serve as the catalytic base for the conversion of D-alanine to L-alanine.

Even more striking is the predicted charge for Lys129, which appears to be heavily unprotonated in the pH range where the enzyme is most active for racemization. Our results lend support to the interpretation of the crystal structure that this residue is carbamylated.[26] The same general trends are predicted for the carbamylated enzyme as for the uncarbamylated enzyme. The unusually low charge states and shifted pK_a's are predicted even in the presence of a carbamate in the active site.

Cys311′ is predicted to be mostly in the thiolate form at neutral pH. This residue is located just above the amino acid moiety of the Schiff base, and may play a participatory role in the catalyzed abstraction of the α hydrogen atom, as part of a proton-transfer chain.

One of the most interesting features of the present results is that, while most of the ionizable residues have predicted charges about where one would expect them to be, many residues in the region of the active site have predicted charges much lower (or more negative) than expected. It appears that the enzyme has the ability to stabilize low charge in the region of the active site, particularly in the area near the amino acid moiety of the Schiff base. It appears that the electric field of the protein matrix is able to stabilize negative charge in the region of the active site. This may help to stabilize a carbanion intermediate and to lower the activation energy for catalyzed racemization.

The experimental evidence and the present results support a two-base mechanism for the catalytic function of alanine racemase. We note that Lys39 and Tyr265′ are both only partially ionized in the pH range where the enzyme is most active for racemization. This means that they may be amphoteric; they can function as bases in the abstraction of the α-hydrogen atom and may also function as acids by donation of a proton to the intermediate, to form the other enantiomer.

The experimental structure, kinetics, and site-directed mutagenesis studies together with the present computational results all paint a consistent picture about the mechanism of catalytic function of alanine racemase. The phenolate side chain of Tyr265′ adjacent to the alpha position of the substrate appears to be a unique feature of alanine racemase. Thus, if one wishes to design a selective inhibitor for alanine racemase, strong coupling to the phenolate ion of Tyr265′ is likely to be a useful feature for selective binding.

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