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Transition State Stabilization and α-Amino Carbon Acidity in Alanine Racemase

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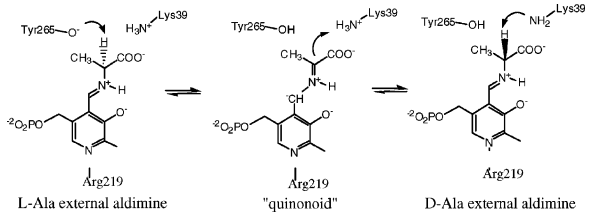
We report computational results of efficient catalysis of deprotonation of L-alanine, by pyridoxal 5′-phosphate (PLP) and by alanine racemase (AlaR), which is the result of large solvation effects of the external aldimine cofactor to lower the pK_a of the α-amino acid by 13 units and transition state stabilization (14.1 kcal/mol) by the enzyme.

AlaR catalyzes the interconversion of L- and D-alanine, an essential process required in the formation of the peptidoglycan layer of bacteria cell wall.[1] Biochemical and structural studies have established that Tyr265′ is the base that abstracts the α-proton of L-Ala, producing a carbanion intermediate, which is subsequently protonated by Lys39 on the opposite side (Scheme 1).[1−3] It is well-known that the formation of iminium ion adducts between amino acids and the cofactor PLP results in a large increase in acidity of the amino α-proton.[4] Typically, the pyridine nitrogen of PLP is protonated, PLP(H$^+$), and the effect is attributed to enhanced charge delocalization to form a quinonoid intermediate that interacts with an acidic residue (e.g., aminotransferases) or a polar residue (Ser/Thr; e.g., tryptophan synthase family).[5,6] In contrast, AlaR is unique in that the positively charged residue, Arg219, donates a hydrogen bond to the pyridine nitrogen,[3] making it necessarily unprotonated, which in turn hinders quinonoid formation (Scheme 1).[2,3,5c] Indeed, the quinonoid intermediate was not observed in the wild-type AlaR nor in Arg219Gln and Arg219Ala mutants, whereas it is formed in the Arg219Glu mutant.[2a] We now present computational results demonstrating that the unprotonated PLP can also significantly increase the acidity of α-amino acid in water, while both solvation effects and electrostatic interactions in the enzyme dramatically reduce the activation barrier of the deprotonation reaction.

Scheme 1



We employ a combined quantum mechanical and molecular mechanical (QM/MM)[7] potential to carry out explicit molecular dynamics (MD) simulations to obtain the potentials of mean force (pmf) for the deprotonation of L-Ala that is uncatalyzed, PLP-catalyzed, or AlaR catalyzed in water. In the uncatalyzed and PLP-catalyzed reactions, the phenolate ion is adopted as the base, whereas Tyr265′ anion is used in AlaR. For the QM/MM potential, we employ the semiempirical Austin model 1 (AM1) formalism,[8a] but we have reparametrized it to fit the experimental and density functional theoretical (DFT) energies for the corresponding reactions in the gas phase. Thus, this specific reaction parametrization of a semiempirical model can yield results comparable to the DFT-mPW1PW91/6-311++G(3df,2p) calculations (see Supporting Information).[8b]

Table 1. Calculated and Experimental Free Energies of Reaction and Activation (kcal/mol) for the Uncatalyzed, PLP-Catalyzed, and AlaR-Catalyzed Deprotonation of Alanine by Phenolate Ion in Water

		ΔG_{aq}			$\Delta G_{aq}^{\ddagger}$		$\Delta\Delta G_{aq}^{\ddagger}$	
	ΔG_g[a]	PCM	pmf	exp[b]	pmf	exp[b]	pmf	exp[b]
uncatalyzed	31.0	27.5	30.9	25.8	32.7	30.4	0	0
PLP(H$^+$)	−33.6	−10.9		8.2		25.5		5
PLP	67.0	5.8	13.4	>8.2	26.3	>25	6.4	5
AlaR			5.3	>4.2	18.6	12.8	14.1	17.6

[a] Computed using mPW1PW91/6-311++G(3df,2p). [b] The pK_a values are taken from refs 4, 6, 11, 12, and 13.

The pmfs are obtained from a series of umbrella sampling simulations, spanning 31 windows for the two reactions in water and 26 windows for the reaction in AlaR. In all calculations, for the MM region, we use the CHARMM22 force field for the protein and the TIP3P model for water.[9] For the uncatalyzed and PLP-catalyzed reactions, we neutralized the system by placing one and two sodium ions, respectively, in a cubic box of about 46 × 46 × 46 Å^3, containing 3375 water molecules. Periodic boundary conditions and the isothermal−isobaric ensemble at 298 K and 1 atm along with particle-mesh Ewald method are used.[10] In the AlaR enzymatic reaction, we imposed stochastic boundary conditions with a 30 Å sphere, and nonbonded interactions were switched to zero at distances beyond 12 Å. In all simulations, the integration step is 1 fs for a total of 8.7 ns.

Listed in Table 1 are the computed and experimental free energies of reaction and activation barriers for the proton abstraction of the alanine zwitterion, in the gas phase, in water, catalyzed by PLP(H$^+$) and PLP, and by the enzyme AlaR. We choose to use the zwitterion in the gas phase for comparison, although the neutral form is more stable.[5d] Table 1 shows that there is only small solvent effect on the α-proton abstraction of Ala by the phenolate ion in water both from the polarizable continuum model (PCM) and from QM/MM-MD (pmf) calculations. The overall reaction free energy is overestimated by 2−5 kcal/mol in comparison with experiments, making use of the pK_a values for glycine[6,11b] and phenol.[11a] In the gas phase, formation of an iminium ion with PLP(H$^+$) strongly increases the carbon acidity by 65 kcal/mol from DFT calculations, due to charge delocalization to form a quinonoid intermediate. On the other hand, without the pyridinium ion electron-withdrawing effect, the gas phase acidity of the PLP−Ala adduct is greatly *reduced* by 36 kcal/mol. Nevertheless, this is fully compensated by strong solvation stabilization of the carbanion species from the α-proton abstraction. On the basis of the free energies of reaction of 13.4 (5.8) kcal/mol from pmf and (PCM) calculations and a pK_a of 10 for phenol, we obtain a pK_a of 19.8 for the PLP−Ala adduct from simulations or 14.3 from a continuum solvation model. Although PLP is not as effective as PLP(H$^+$) in enhancing the

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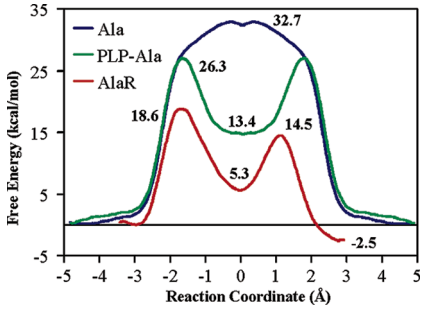

Figure 1. Computed potentials of mean force (pmf) for the uncatalyzed (blue), PLP-catalyzed (green), and AlaR-catalyzed (red) racemization of alanine in water. The reaction coordinate specifies the difference between the distances of the migrating proton with the donor and acceptor atoms, which has been shifted by ±2 Å to anchor the intermediate (transition state) in the center.

acidity of α-amino acids (pK_a = 16),[6c] it still provides remarkable stabilization and the origin stems from solvent effects, stabilizing the carbanion, in contrast to the PLP(H^+)−Ala adduct. In the enzyme AlaR, the external aldimine substrate is further stabilized by 8 kcal/mol to a free energy of 5.3 kcal/mol (Table 1), in accord with experiment (>4.2 kcal/mol).[12] The estimated pK_a of PLP−Ala is thus about 11.3 in the active site of AlaR, by using the experimental pK_a of Tyr265, which is 7.4.[13]

The second major finding, which is depicted in Figure 1, is the change in the deprotonation mechanism and a progressive reduction of the kinetic barrier to deprotonation, $\Delta G^{\ddagger}$. The reaction between the alanine zwitterion and the phenolate ion (or hydroxide ion) does not yield a stable carbanion intermediate in water, and the estimated free energy barrier is 33 kcal/mol. This is consistent with that observed for glycine zwitterion by OH^- (30.4 kcal/mol).[6b] (The depression in pmf at zero of the reaction coordinate is an artifact of anchoring a mirror image of the first half of the proton abstraction in water, which has been shifted by 2 Å for all three reactions.)

The formation of the external aldimine changes the reaction mechanism from a "concerted" process to a stepwise reaction by forming a planar iminium ion that is stabilized by solvation (or a quinonoid intermediate in the PLP(H^+) adduct). Concomitantly, the free energy barrier is lowered by 6.4 kcal/mol (Table 1), in accord with experiment (ca. 5 kcal/mol).[5c,12] In the active site, AlaR further reduces $\Delta G^{\ddagger}$ by 7.7 kcal/mol from QM/MM free energy simulations. Combining all factors from solvation, PLP cofactor, and enzymatic stabilization, we estimate that the overall transition state stabilization for the deprotonation of alanine by AlaR is 14.1 kcal/mol, in reasonable agreement with experiment (17.6 kcal/mol).[12] The overall AlaR enzyme catalysis, that is, the barrier reduction by AlaR relative to that of the uncatalyzed deprotonation of Ala in water, has nearly equal contributions from the PLP cofactor (6.4 kcal/mol) and enzyme−substrate interactions (7.7 kcal/mol), demonstrating that the external aldimine with an unprotonated pyridine is still an effective cofactor for lowering carbon acidity. This is consistent with the observation by Richard and Amyes and co-workers that the acidity of α-amino acids is dramatically enhanced by acetone to form iminium ions.[6]

Clearly, there is major difference between PLP(H^+) and PLP in their ability of enhancing the α-proton acidity. The former forms a stable quinonoid intermediate, whereas, in the latter case, the quinonoid intermediate is not produced and the deprotonation intermediate is less stable. Toney and co-workers suggested that this is advantageous in the AlaR enzyme because it would decrease the reprotonation barrier in the racemization reaction relative to the competitive deamination process, thereby increasing the racemization selectivity.[12] The present results, although do not directly address the issue of dynamic scales of protein reorganization, are consistent with this hypothesis.

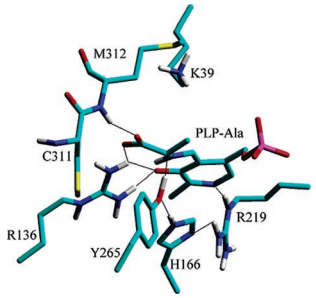

Figure 2. Hydrogen bonding interactions near the aldimine adduct in the active site of alanine racemase. The image depicts the transition state for the L-Ala proton abstraction.

Finally, we have identified that several amino acid residues make specific hydrogen bonding interactions to the external aldimine deprotonation transition state. Most significant are Arg219, which donates a hydrogen bond to pyridine, and Arg136, which is hydrogen bonded to the oxy anion of PLP, the carboxylate group of the substrate, and the basic residue Tyr265′ (Figure 2). Details of these analyses will be presented later.

The present studies show that enhancement of carbon acidity of α-amino acids by PLP with the unusual, unprotonated pyridine is due to solvation effects, in contrast to the intrinsic electron-withdrawing stabilization by the pyridinium ion to form a quinonoid intermediate. Alanine racemase further lowers the α-proton acidity and provides an overall 14−17 kcal/mol transition state stabilization. A consequence of the unusual form of PLP cofactor in AlaR is to raise the free energy of the intermediate.

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Supporting Information Available: Optimized reaction-specific AM1 (AM1-SRP) parameters (12 pages, print/PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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