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Genetic engineering approaches to enzyme design and mechanism

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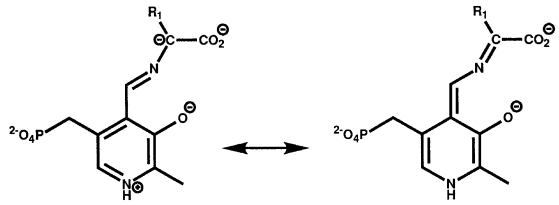
ABSTRACT: Aspartate aminotransferase (AATase) and aminocyclopropane carboxylate synthase (ACC synthase) are pyridoxal phosphate (PLP)-dependent enzymes whose common junction of mechanistic divergence is after the formation of a C_α carbanion from the amino acid substrate bound to PLP as a Schiff base (aldimine). AATase catalyzes the reversible interconversion of α-amino acids and α-keto acids, while ACC synthase effects the irreversible decomposition of *S*-adenosylmethionine (SAM) to 1-aminocyclopropane-1-carboxylate (ACC) and 5′-methylthioadenosine (MTA). ACC is subsequently converted to ethylene, the plant ripening and senescence hormone, by ACC oxidase, the next enzyme in the pathway. AATase and ACC synthase exhibit many similar phenomenological characteristics that result from different detailed mechanistic origins. The k_{cat}/K_M *versus* pH profiles for both enzymes are similar (AATase, acidic pK_a = 6.9, basic pK_a = 9.6; ACC synthase, acidic pK_a = 7.5, basic pK_a = 8.9); however the acidic pK_a of AATase reflects the ionization of an enzyme proton from the internal Schiff base, and the basic one is that of the α-amino group of the substrate, while the opposite situation obtains for ACC synthase, *i.e.* the apparent pK_a of 7.4 is due to the α-amino group of SAM, whereas that of 9 reflects the Schiff base pK_a. The mechanistic imperative underlying this reversal is dictated by the reaction mechanism and the low pK_a of the α-amino group of SAM. The low pK_a of SAM requires that the enzyme pK_a be moved upward in order to have sufficient quantities of the reacting species at neutral pH. It is shown by viscosity variation experiments with wild-type and active site mutant controls of both enzymes that the reaction of SAM with ACC synthase is 100% diffusion controlled ($k_{cat}/K_M = 1.2 \times 10^6$ l mol^{-1} s^{-1}) while the corresponding reaction for the combination of L-aspartate with AATase is insensitive to viscosity, and is therefore chemically not diffusion limited. Tyr225 (AATase) or Tyr233 (ACC synthase) forms a hydrogen bond with the PLP in both enzymes, but that formed with the former enzyme is stronger and accounts for the lower pK_a of the Schiff base. © 1998 John Wiley & Sons, Ltd.

KEYWORDS: enzyme design; enzyme mechanism; genetic engineering

INTRODUCTION

PLP is the organic cofactor enlisted by a varied group of enzymes that catalyzes most of the mechanistically challenging transformations of α-amino acids.[1] PLP enables such reactions as transamination, α- and β-decarboxylation, racemization and side-chain elimination and replacement chemistry to occur by stabilizing the C_α carbanion of the amino acid by resonance delocalization into the pyridinium nitrogen atom (Scheme 1).



Scheme 1. Resonance stabilization of the C_α carbanion bound to PLP.

Only three amino acids are completely conserved in this family of enzymes: the lysine that forms the internal aldimine, the arginine that ion pairs with the α-carboxylate of the amino acid and one glycine.[2] Christen's group has identified a subset of PLP-dependent enzymes, termed the alpha class, that 'catalyzes transformations of amino acids in which the covalency changes are limited to the same carbon atom that carries the amino group forming the aldimine linkage to the coenzyme. This carbon atom is the alpha carbon in most cases.'[2] This group of enzymes shares substantial identity or similarity of 12 active site residues. This paper details how slight variations in active site geometry that are mandated by evolutionary considerations, control the pK_as of the active site internal aldimine for two enzymes of the alpha class: AATase and ACC synthase (Scheme 2).

AATase catalyzes the reactions shown in Scheme 3. The first step of the sequence (not illustrated) is the

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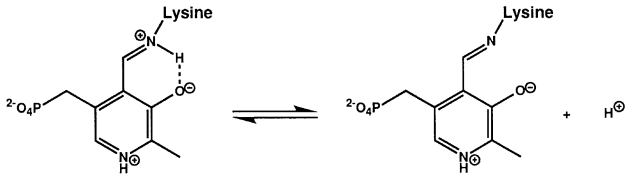
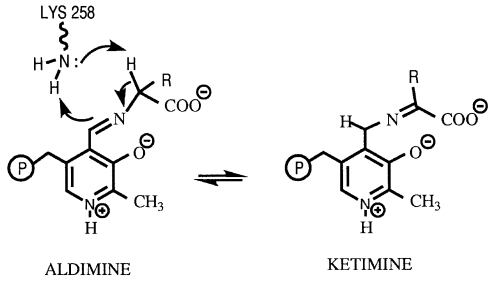


Scheme 2. Dissociation of the internal aldimine proton in PLP-dependent enzymes.

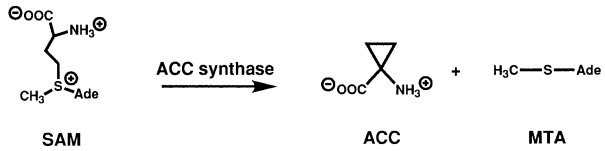


Scheme 4. Catalysis of the 1,3-azallylic rearrangement of the internal aldimine to the ketimine catalyzed by aminotransferases.

reaction of the amino acid with the internal aldimine shown in Scheme 2 to form the external aldimine of Scheme 4 which illustrates the essential chemistry of enzymatic transamination, the 1,3-azallylic rearrangement. The final step is hydrolysis of the ketimine to yield the 2-oxo acid (Scheme 3).

ACC synthase catalyzes the decomposition of *S*-adenosylmethionine (SAM) to ACC and 5′-methylthioadenosine (MTA) (Scheme 5). This is the first enzyme in the two-step pathway that converts SAM to ethylene, an important effector of ripening and senescence in plants.

The x-ray structure of AATase has been available since 1979[3] and that of ACC synthase is nearing completion.[4] Although the two enzymes share only about 15% similarity, a model of the active site of ACC synthase was constructed by a parsed homology method, and the major hypotheses concerning the identities and catalytic roles of putative active site residues were confirmed by site-directed mutagenesis.[5] Some of the active site residues for the two enzymes are shown in Fig. 1.



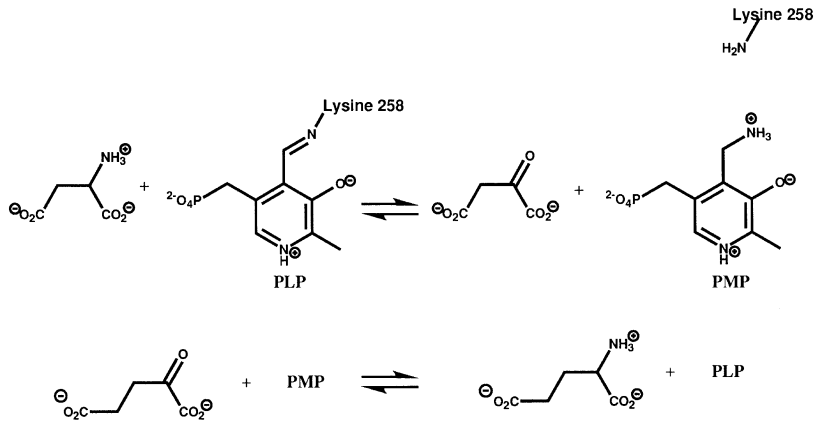
Scheme 5. The reaction catalyzed by ACC synthase.

RESULTS AND DISCUSSION

ACC synthase is an evolutionarily perfect enzyme

Values of the kinetic constants k_{cat} and k_{cat}/K_m for the wild-type and Y225F (AATase) and wild-type and Y233F (ACC synthase) mutants are given in Table 1 [the convention Y*xyz*F denotes that tyrosine (Y) at sequence number *xyz* in the protein is replaced by phenylalanine (F)].

The tyrosine to phenylalanine mutation reduces the value of k_{cat}/K_m by about 20-fold for both enzymes; however, the mechanistic reasons for the reduction are distinctly different. The mutation in AATase results in a 450-fold lowering of k_{cat} that is partially offset by a 30–50-fold reduction in K_m; however, the corresponding mutation in ACC synthase elicits no effect on k_{cat} and a 20-fold increase in K_m. The importance of this



Scheme 3. The two AATase half-reactions. The top line shows the conversion of L-aspartate and enzyme-bound PLP to oxaloacetate and bound PMP. The PLP form of the enzyme is re-established by reaction of the PMP form with 2-oxoglutarate in the second half-reaction.

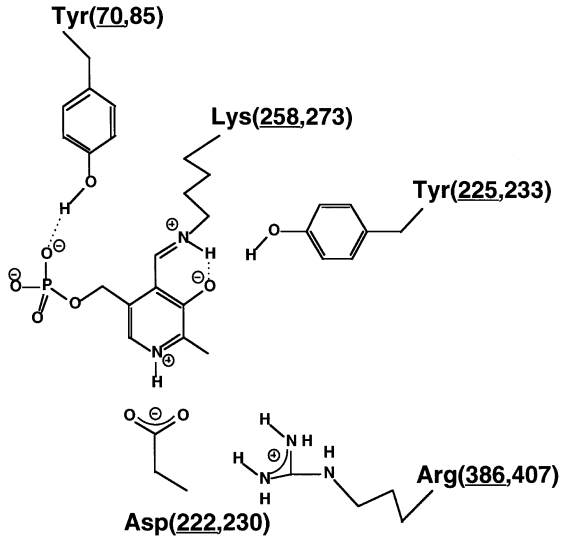


Figure 1. Partial comparison of the amino acid sequences of the active site residues of AATase and of ACC synthase. AATase residues are underlined. Dissociation of the aldimine proton (Scheme 2) exposes the oxyanion of the cofactor, which is stabilized by hydrogen bonding to the adjacent tyrosine.

Table 1. Differential effects of active site tyrosine mutations on AATase and ACC synthase[a]

	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($l\ mol^{-1}\ s^{-1}$)
AATase (WT)	170	1.7	1×10^5
AATase (Y225F)	0.4	0.08	5000
ACC synthase (WT)	9	0.01	8×10^5
ACC synthase (Y233F)	10	0.3	3×10^4

[a] Data from Refs. 6–8.

differential display in k_{cat} and K_m is discussed further below.

It was recently established that the rates of the 1,3-azallylic rearrangement (Scheme 4) and of dissociation of oxaloacetate (OAA) from the AATase–OAA complex are each partly rate determining for AATase, and that the rate of association of amino acid with the PLP form with the enzyme is rapid and at equilibrium compared with the subsequent steps in the reaction, *i.e.* the rate of amino acid association with the enzyme is not diffusion controlled.[6] The reaction of ACC synthase with SAM is, by contrast, approximately 100% diffusion controlled as assessed by the viscocity variation method.[9,10] The rate of association of SAM with ACC synthase is inversely proportional to the first power of the viscocity for two different viscosogens-sucrose and glycerol.

Thus ACC synthase is an evolutionarily perfect enzyme as defined by Albery and Knowles[11] in the sense that there is no pressure to improve the chemical catalytic efficiency of an enzyme whose reaction rate is limited by the physics of diffusive processes. The extent to which a reaction is diffusion controlled is a function only of the rate constant ratio k_{-1}/k_2:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\rightarrow} E + P \qquad (1)$$

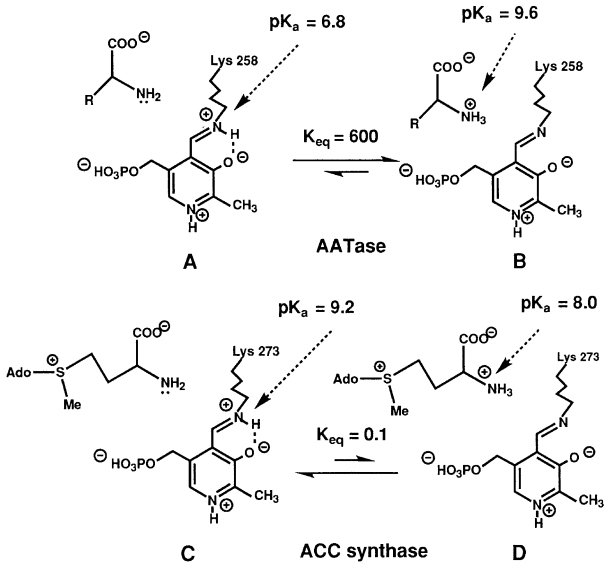
where k_1, k_{-1} and k_2 are the second-order rate constant for association of enzyme with substrate, the first-order dissociation rate constant for the ES complex and the *net* first-order rate constant for conversion of ES to E + P, respectively. The value of $k_{cat}/K_m = k_1k_2/(k_{-1} + k_2)$ from which it is seen that $k_{cat}/K_m \rightarrow k_1$ as $k_{-1}/k_2 \rightarrow 0$. The values of k_{cat}/K_m for both wild-type enzymes are 10^5–10^6 $lmol^{-1}\ s^{-1}$ (Table 1), but $k_{-1} < 2\ s^{-1}$ for the ES complex of ACC synthase but $>500\ s^{-1}$ for that of AATase.[6,7] These differences reflect the higher free energy required to dissociate the larger molecule, SAM, from its complex with ACC synthase compared with that required to dislodge aspartate from AATase.

The role of the aldimine pK_a in catalysis

The pH *vs* k_{cat}/K_m profiles are similar for both enzymes. They are bell shaped with ascending pK_as of 6.9 for AATase[8] and 7.5 for ACC synthase.[7] The descending pK_as are 9.6[8] and 8.9,[7] respectively. It is remarkable that these similar curves are generated by opposite ionizations in the two cases. The pK_as in k_{cat}/K_m *versus* pH profiles generally reflect ionization of only free enzyme and free substrate. The *ascending* kinetically determined pK_a of the AATase profile can be assigned with confidence to the ionization shown in Scheme 2 because it is also observed spectrophotometrically both in the wild type enzyme and in an engineered mutant whose pK_a was reduced to 5.8.[12] The alkaline *descending* pK_a of 9.6 was assigned to the α-NH_3^+ of the substrate.[8] The spectrophotometric pK_a of ACC synthase is 9.2,[7] a figure which is within experimental error of the *descending* kinetic pK_a of 8.9,[7] while the *ascending* pK_a is due to the ionization of the α-NH_3^+ group of SAM. Hence the most populated species in the neutral pH range for the two enzymatic reactions are the oppositely configured B (AATase) and C (ACC synthase) of Scheme 6.

Schiff base formation requires that the α-amino group of the amino acid be in the free base form. Its pK_a in AATase is significantly lowered in the ES complex owing to the ion-pairing neutralization by two active site arginine side chains and, correspondingly, the internal aldimine pK_a is raised; thus the proton would migrate from the α-NH_3^+ to the internal aldimine nitrogen atom in the ES complex.[13]

Is there an evolutionary driving force to raise the pK_a



Scheme 6. The dominant protonic configurations of the amino acids and internal aldimines at the pH of maximum activity of AATase and ACC synthase are forms B and C, respectively. See text.

of the internal aldimine of the ACC synthase to a high value? The equation

$$\frac{k_{cat}}{K_m} = \frac{(k_{cat}/K_m)_{lim}}{1 + 10^{(pK_{a_1} - pH)} + 10^{(pH - pK_{a_2})}} \qquad (2)$$

describes the pH dependence of k_{cat}/K_m. Substitution of the observed pK_as of the α-NH_3^+ moiety of SAM and of the internal aldimine nitrogen atom of ACC synthase at pH 8 gives a value of k_{cat}/K_m (obs) that is close to the limiting value; however, if the pK_a of the internal aldimine were 7 in ACC synthase as it is in AATase, k_{cat}/K_m would be reduced by nearly 10-fold. Hence the plant would have to produce 10 times the quantity of enzyme to effect the same SAM $\rightarrow$ ACC reaction flux. We conclude, therefore, that a strong evolutionary mandate drove the pK_a of the internal aldimine of ACC synthase to this higher value, and may now ask what structural accommodations within the active site effect this change.

PLP is confined into its observed position in the AATase structure by a number of contacts.[14,15] Of particular importance for the present consideration is the hydrogen bond from Tyr225 (AATase) or Tyr233 (ACC synthase) to the cofactor (Fig. 1). The hydrogen bond stabilizes the free base form of the internal aldimine, and thus serves to lower the pK_a. The limit of excursion of PLP to the interior of the protein is defined by contact with the side chain of Ala224 of AATase. That residue is replaced by the larger Ile232 in ACC synthase. The x-ray structure of the latter enzyme is not yet completed, but modeling suggests that the PLP must be pushed away from the position that it occupies in AATase, with the likely consequence that the hydrogen bond to the tyrosine is weakened.[7] A weaker hydrogen bond would provide less stabilization to the free base form of the internal aldimine and a correspondingly higher pK_a value for its conjugate acid, the protonated aldimine.

Acknowledgment

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