Modulation of the Internal Aldimine pK_a's of 1-Aminocyclopropane-1-carboxylate Synthase and Aspartate Aminotransferase by Specific Active Site Residues[†]

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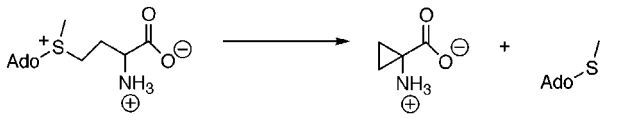
Received December 21, 2001; Revised Manuscript Received January 25, 2002

ABSTRACT: The active sites of the homologous pyridoxal phosphate- (PLP-) dependent enzymes 1-aminocyclopropane-1-carboxylate (ACC) synthase and aspartate aminotransferase (AATase) are almost entirely conserved, yet the pK_a's of the two internal aldimines are 9.3 and 7.0, respectively, to complement the substrate pK_a's (*S*-adenosylmethionine pK_a = 7.8 and aspartate pK_a = 9.9). This complementation is required for maximum enzymatic activity in the physiological pH range. The most prominent structural difference in the active site is that Ile232 of ACC synthase is replaced by alanine in AATase. The I232A mutation was introduced into ACC synthase with a resulting 1.1 unit decrease (from 9.3 to 8.2) in the aldimine pK_a, thus identifying Ile232 as a major determinant of the high pK_a of ACC synthase. The mutation also resulted in reduced k_{cat} (0.5 vs 11 s^{-1}) and k_{cat}/K_m values (5.0×10^4 vs 1.2×10^6 M^{-1} s^{-1}). The effect of the mutation is interpreted as the result of shortening of the Tyr233−PLP hydrogen bond. Addition of the Y233F mutation to the I232A ACC synthase to generate the double mutant I232A/Y233F raised the pK_a from 8.2 to 8.8, because the Y233F mutation eliminates the hydrogen bond between that residue and PLP. The introduction of the retro mutation A224I into AATase raised the aldimine pK_a of that enzyme from 6.96 to 7.16 and resulted in a decrease in single-turnover k_{max} (108 vs 900 s^{-1} for aspartate) and k_{max}/K_m^{app} (7.5×10^4 vs 3.8×10^5 M^{-1} s^{-1}) values. The distance from the pyridine nitrogen of the cofactor to a conserved aspartate residue is 2.6 Å in AATase and 3.8 Å in ACC synthase. The D230E mutation introduced into ACC synthase to close this distance increases the aldimine pK_a from 9.3 to 10.0, as would be predicted from a shortened hydrogen bond.

The group of PLP-dependent enzymes designated subgroup I aminotransferases by sequence analysis (*1*) and aminotransferase subclass I by structural analysis (*2*) has five members. All but one of these catalyze transamination, the interconversion of α-amino and α-keto acids. The exception is 1-aminocyclopropane-1-carboxylate (ACC)[1] synthase (*S*-adenosyl-L-methionine methylthioadenosine lyase, EC 4.4.1.14), which catalyzes a 1,3-elimination of 5′-methylthioadenosine (MTA) from *S*-adenosyl-L-methionine (SAM) (Scheme 1). The recently solved crystal structure of ACC synthase (*3*) shows remarkable structural similarity of this enzyme to aspartate aminotransferase (aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1, AATase), its closest relative in the group. The greatest correspondence is in the region around the cofactor binding site. The active site residues are conserved between the two enzymes with the exception of isoleucine 232, which is alanine 224 in AATase (Scheme 2).

Scheme 1: ACC Synthase Catalyzes the α,γ-Elimination of MTA from SAM



Despite the conservation of residues around the PLP, the enzyme-bound cofactors of each enzyme have strikingly different UV spectra at neutral pH (Figure 1), a result of the different pK_a's of the enzyme−PLP Schiff base [7.0 for AATase (*4*) and 9.3 for ACC synthase (*5*)]. The unprotonated Schiff bases absorb maximally at ∼360 nm (AATase) or ∼385 nm (ACC synthase), while the protonated forms of both enzymes absorb maximally at ∼420−430 nm (Figure 1). The spectra are therefore pH dependent, and the pK_a of the aldimine can be determined by spectrophotometric titration. Plots of k_{cat}/K_m vs pH for these enzymes exhibit two pK_a's, one of which matches the spectrophotometrically determined pK_a and is therefore attributed to the internal aldimine (*6*). The second corresponds to the substrate amino group. The pH of optimum activity lies between the two pK_a's, i.e., when the enzyme aldimine is protonated but the substrate amino group is not (ACC synthase) or vice versa (AATase). This situation is optimal because the initial

[†] This work was supported by NIH Grant GM35393. A.C.E. was supported by a National Science Foundation Graduate Research Fellowship.

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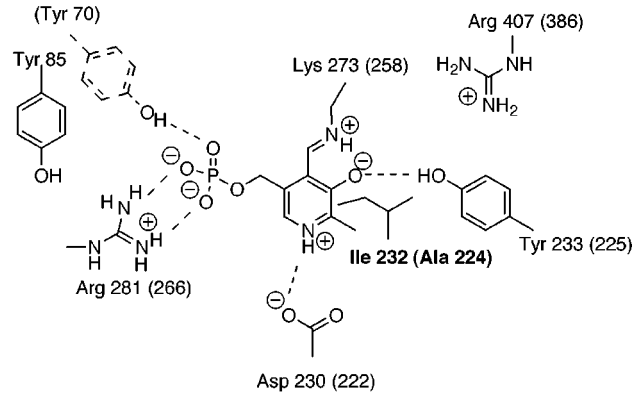
[1] Abbreviations: AATase, aspartate aminotransferase; ACC, 1-aminocyclopropane-1-carboxylate; AMPSO, 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MTA, 5′-deoxy-5′-methylthioadenosine; PLP, pyridoxal 5′-phosphate; SAM, *S*-adenosyl-L-methionine; TAPS, *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid; WT, wild type.

10.1021/bi016084l CCC: $22.00
Published on Web 02/23/2002

Scheme 2: Partial Comparison of AATase and ACC Synthase Active Sites[a]



[a] All active site residues shown are conserved between these two enzymes except the highlighted Ile232 (ACC synthase), which is Ala224 in AATase. The AATase residue numbers are in parentheses. Tyr70 of AATase (dashed) occupies a significantly different position in the structure from the homologous ACC synthase residue, Tyr85.

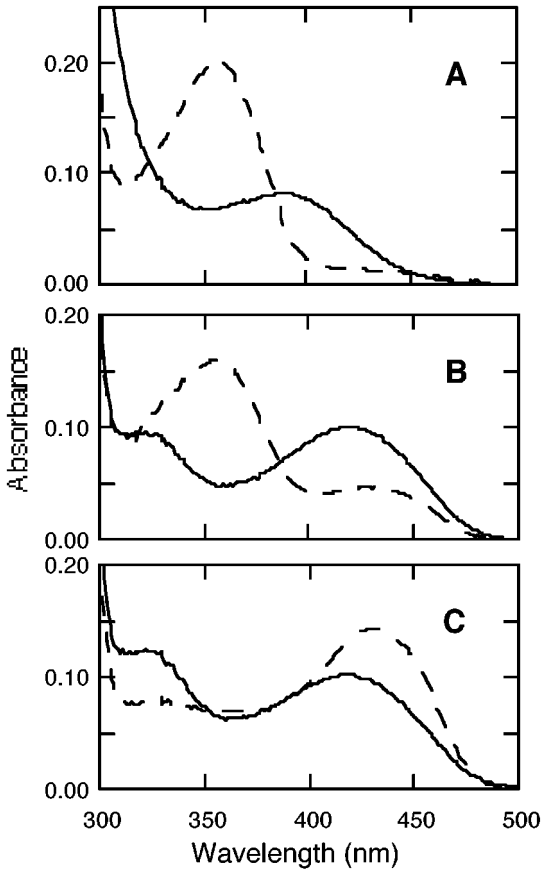


FIGURE 1: pH dependence of the absorption spectra of WT ACC synthase (solid line) and AATase (dashed line). The spectra were taken at pH 10.4 (A), 7.5 (B), and 6.1 (C). The wavelengths of maximum absorbance for ACC synthase are 420 nm (low pH) and 385 nm (high pH), and those for AATase are 430 nm (low pH) and 360 nm (high pH).

transaldmination step of the transamination reaction requires that the aldimine electrophile be protonated while the amino acid nucleophile is in the free base form. Although the reverse is true in the situation described for AATase, the necessary proton distribution requires only a single proton transfer from the substrate to the aldimine. In each case, the enzyme pK_a must be modulated to be far enough away from that of the substrate so that an optimum pH near the physiological pH value is realized.

Although some factors have been identified as having a role in the control of the aldimine pK_a, the source of the large difference between the pK_a's of these enzymes is not fully understood. We address here the role of the residues in contact with the PLP cofactor in controlling this pK_a, focusing primarily on the variant residue Ile232 (ACC synthase)/Ala224 (AATase) (Scheme 2) and its effect on the interactions of the cofactor with other residues.

EXPERIMENTAL PROCEDURES

Reagents. SAM was purchased from Calbiochem and used without further purification. This commercial preparation is ~60% (*S*,*S*)- and 40% (*R*,*S*)-SAM as judged by HPLC.

Mutagenesis and Protein Purification. All mutant plasmids were generated by standard PCR site-directed mutagenesis and the sequences confirmed by DNA sequencing (UC Berkeley DNA Sequencing Facility). The primer sequences for mutagenesis were 5′-ATT AGC GAT GAA GCT TAC TCC GGA ACA GCT TTT AGC-3′ and 5′-GCA AAA AGC TGT TCG GGA GTA AGC TTC ATC GCT AAT-3′ (ACC synthase I232A), 5′-GCG ATG AAG CTT TCT CCG GAA CAG CTT TT-3′ and 5′-GCT GTT CCG GAG AAA GCT TCA TCG CT-3′ (ACC synthase I232A/Y233F), 5′-CAC CTC ATT AGC GAA GAG ATC TAC TCC GGC ACA GC-3′ and 5′-GCT GTG CCG GAG TAG ATC TCT TCG CTA ATG AGG TG-3′ (ACC synthase D230E), and 5′-CCG CTG TTT GAC TTC ATT TAC CAG GGT TTT GC-3′ and 5′-CGG GCA AAA CCC TGG TAA ATG AAG TCA AAC AGC-3′ (AATase A224I). The WT and mutant enzymes were overexpressed and purified as previously described for the WT enzymes (*5*).

Spectrophotometric pK_a Determinations. ACC synthase (~15 μM) was dissolved in buffer (5 mM TAPS, 0.5 M KCl, 20% glycerol) in a total volume of 1 mL, and the initial pH of 5.5 was adjusted with 0.5 M TAPS, pH 8.4 (final pH = 5.5−7.5), 0.5 M AMPSO, pH 10.7 (final pH = 7.5−9.0), or 0.5 M CAPS, pH 11.7 (final pH = 9−11). The spectra were recorded from 250 to 550 nm on a UVIKON 860 double-beam spectrophotometer (KONTRON Instruments). Higher pH solutions were filtered through a 0.22 μm syringe filter to remove precipitated enzyme. The pH values were measured with a Corning 320 pH meter equipped with a Corning semimicro combination electrode. Data were imported into Kaleidagraph (Synergy Software) and normalized for protein concentration from the absorbance at 280 nm. The absorbance data for the wavelengths of maximum absorbance difference between high and low pH (375 and 435 nm) were plotted as a function of pH, and the pK_a value was determined by fitting these data to eqs 1 and 2, respectively:

$$A = \frac{A_1 - A_2}{1 + 10^{(pK_a - pH)}} + A_2 \quad (1)$$

$$A = \frac{A_1 - A_2}{1 + 10^{(pH - pK_a)}} + A_2 \quad (2)$$

where A_1 and A_2 are the high and low absorbance limits, respectively.

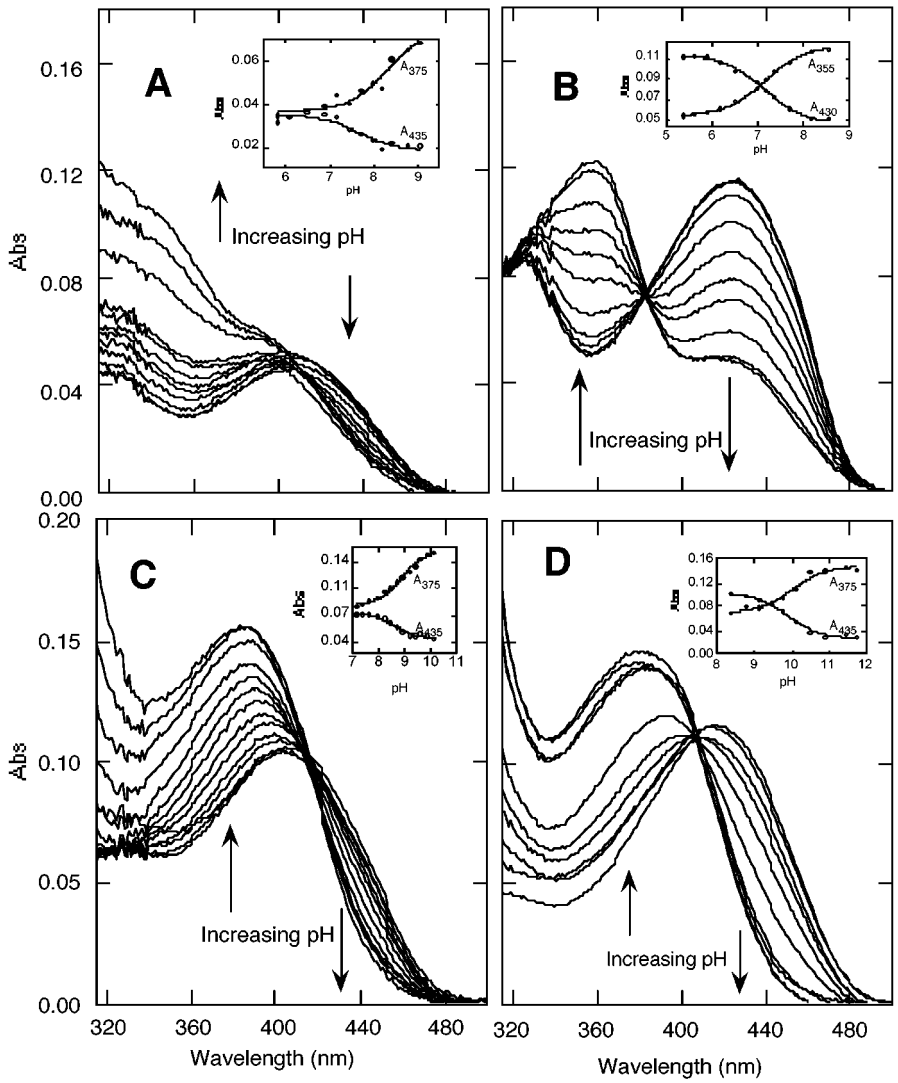


FIGURE 2: pH dependence of the absorption spectra of mutant AATases and ACC synthases. The fitted lines in the insets are least-squares fits to eq 1 (absorbance increasing with pH) and eq 2 (absorbance decreasing with pH). (A) I232A ACCS. The data were recorded at pH 5.85, 6.10, 6.51, 6.90, 7.18, 7.46, 7.73, 7.99, 8.20, 8.41, 8.79, and 9.06. The fitted pK_a's are 8.3 ± 0.3 (375 nm) and 7.7 ± 0.3 (435 nm). The initial enzyme concentration was 15 μM in 5 mM TAPS, 0.5 M KCl, and 20% glycerol, and the spectra were corrected for dilution. The increasing absorbance at shorter wavelengths is due to denaturation of the enzyme (see text). (B) A224I AATase. The data were recorded at pH 5.36, 5.62, 5.90, 6.20, 6.53, 7.00, 7.17, 7.38, 7.69, 8.17, and 8.55. The fitted pK_a's are 7.17 ± 0.02 (355 nm) and 7.15 ± 0.03 (430 nm). The initial enzyme concentration was 15 μM in 5 mM CHES and 0.2 M KCl. (C) I232A/Y233F ACCS. The data were recorded at pH 7.20, 7.37, 7.65, 7.95, 8.24, 8.45, 8.71, 8.94, 9.21, 9.45, 9.61, 9.89, and 10.14. The fitted pK_a's are 8.90 ± 0.07 (375 nm) and 8.62 ± 0.08 (435 nm). The conditions are the same as in (A). (D) D230E ACCS. The data were recorded at pH 8.39, 8.82, 9.16, 9.49, 9.79, 10.15, 10.51, 10.92, 11.45, and 11.75. The fitted pK_a's are 10.0 ± 0.1 (375 nm) and 9.9 ± 0.07 (435 nm). The conditions are the same as in (A).

The AATase pK_a values were determined by the same method except that the starting buffer was 5 mM CHES and 200 mM KCl, pH 11.0, and the pH was adjusted with 100 mM potassium acetate and 200 mM KCl, pH 3.9. The wavelengths of maximum absorbance difference are 355 and 430 nm.

Determination of the Kinetic Parameters for ACC Synthase Mutants. The ACC synthase k_{cat} and k_{cat}/K_m values were evaluated as previously described for the WT enzyme (*7*). The reactions of WT and mutant AATases with aspartate and glutamate were monitored separately under single-turnover conditions with ~10 μM enzyme in 200 mM TAPS, 100 mM KCl, pH 8.4, and varying substrate concentrations. The absorbance changes at 325 nm were followed with an Applied Photophysics Ltd. SF.17MV stopped-flow spectrophotometer.

pH Dependence of the Kinetic Parameters. The kinetics were monitored in a three-component buffer (25 mM MES, 25 mM MOPS, 50 mM 4-hydroxy-*N*-methylpiperidine) that maintains constant ionic strength across the utilized pH range (*5*). The resulting k_{cat}/K_m values were plotted as a function of pH, and the pK_a values were determined by fitting to eq 3 with the Kaleidagraph software package:

$$k_{cat}/K_m = \frac{(k_{cat}/K_m)_{max}}{1 + 10^{(pK_1 - pH)} + 10^{(pH - pK_2)}} \qquad (3)$$

where $(k_{cat}/K_m)_{max}$ is the theoretical maximum value of k_{cat}/K_m.

The Quinonoid Intermediate in the Reaction of ACC Synthase D230E with SAM. A solution of 110 μM ACC synthase D230E in reaction buffer (50 mM potassium phosphate, pH

Table 1: Spectrophotometric and Kinetic Properties of Mutant and Wild-Type ACC Synthases and AATases

enzyme	pK_a[a]	k_{cat}(SAM)[b] (s^{-1})	k_{cat}/K_m(SAM)[b] (M^{-1} s^{-1} × 10^{-4})	k_{max}(Asp)[c] (s^{-1})	k_{max}/K_m^{app}(Asp)[c] (M^{-1} s^{-1} × 10^{-4})	k_{max}(Glu)[c] (s^{-1})	k_{max}/K_m^{app}(Glu)[c] (M^{-1} s^{-1} × 10^{-4})
ACCS (WT)	9.26[d] (0.03)	11.0[d] (0.4)	120[d] (10)				
Y233F	10.2[d] (0.1)	9.6[e] (1.3)	5.1[e] (0.4)				
I232A	8.2 (0.1)	0.5 (0.05)	5.0[f] (0.5)				
I232A/Y233F	8.8 (0.1)	2.2 (0.1)	4.0 (0.4)				
D230E	9.95 (0.10)	0.7 (0.1)	1.3 (0.3)				
AATase (WT)	6.96[g] (0.05)			900 (50)	38 (4)	1400 (100)	0.96 (0.04)
Y225F	8.60[g] (0.02)			ND	ND	ND	ND
A224I	7.16 (0.03)			108 (4)	7.5 (0.2)	126 (2)	0.27 (0.01)
D222E	6.44[h]			64[h,i]	1.2[h,i]	ND	ND

[a] All listed pK_a's were determined spectrophotometrically except that of I232A ACCS, for which the kinetic pK_a is listed because of the larger error associated with the spectrophotometric titration. [b] The commercially available SAM that was used is a mixture of ~60% (*S*,*S*)-SAM and ~40% (*R*,*S*)-SAM, and the kinetic parameters were determined at the pH of maximal activity. [c] The values given are the kinetic parameters for the half-reaction of the PLP form of the enzyme with an amino acid substrate to produce the PMP form of the enzyme and the keto acid products. [d] Reference 5. [e] Reference 7. [f] The theoretical maximum value calculated from eq 3 and the data shown in Figure 3 is 9.4×10^4 M^{-1} s^{-1}. [g] Reference 4. [h] Reference 9. [i] Estimated value (see ref *9* for discussion). Under the conditions used for this mutant (50 mM HEPES, 0.1 M KCl, 100 μM PLP, 10−20 μM AATase, pH 8.0, 25 °C), WT exhibits a k_{max} of 550 s^{-1} and k_{max}/K_m^{app} of 1.2×10^5 M^{-1} s^{-1}.

8.5, 10% glycerol) was rapidly mixed 1:1 with 5 μM (*S*,*S*)-SAM in reaction buffer in a 100 μL reaction chamber in the stopped-flow spectrophotometer. The data were imported into Kaleidagraph and fit to eq 4 by nonlinear regression:

$$A = A_{max}\left(\frac{k_1}{k_2 - k_1}\right)(e^{-k_1 t} - e^{-k_2 t}) \quad (4)$$

where A_{max} is the theoretical maximum absorbance (i.e., the absorbance if all of the enzyme is in the quinonoid form) and k_1 and k_2 are the first-order rate constants for formation and decay, respectively, of the quinonoid intermediate.

RESULTS

The I232A Mutation Decreases the Aldimine pK_a of ACC Synthase and Results in a Kinetically Compromised Enzyme. Replacement of the active site residue Ile232 of ACC synthase with alanine, which is found in the corresponding position in AATase, results in a decrease in the pK_a of the enzyme−PLP internal aldimine to a value approaching that of the latter enzyme. Spectrophotometric titration of I232A ACC synthase shows a pK_a of 8.0 (Figure 2A) compared to 9.26 for WT ACC synthase (*5*). The plot of k_{cat}/K_m vs pH exhibits two pK_a's, 7.5 and 8.2 (Figure 3). The former matches that of the substrate α-amino group (*5*), while the latter is equal to that of the enzyme internal aldimine. The corresponding pK_a's in the k_{cat}/K_m profile of the WT ACC synthase are 7.5 and 8.9 (*5*). The small difference between the kinetic and spectrophotometric aldimine pK_a of the WT enzyme is due to irreversible loss of activity at high pH that is not the result of aldimine deprotonation (D. McCarthy, unpublished results). For this reason, the spectrophotometric determination provides the more reliable measure of the pK_a of the aldimine. Thus the Ile → Ala mutation decreases the aldimine pK_a by a full pK unit, marking this residue as one of the major determinants of the aldimine pK_a difference observed between ACC synthase and AATase.

The mutant exhibits a reduced k_{cat} value of 0.5 s^{-1} at pH 8.0 [vs 11.0 s^{-1} for WT (*5*)], but K_m is unchanged, resulting in a k_{cat}/K_m of 5.0×10^4 M^{-1} s^{-1} (Table 1). Although this maximum observed k_{cat}/K_m value is 20-fold below that of the WT enzyme, the theoretical maximum determined from the fit of the data shown in Figure 3 to eq 3 is 9.4×10^4 M^{-1} s^{-1}, 10-fold reduced from WT.

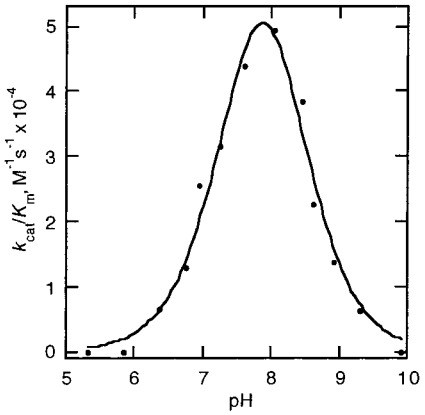


FIGURE 3: The pH dependence of k_{cat}/K_m for I232A ACCS. k_{cat}/K_m values were determined in a three-component buffer (25 mM MES, 25 mM MOPS, 50 mM 4-hydroxy-*N*-methylpiperidine) that maintains constant ionic strength across this pH range. [ACCS I232A] = 25 nM and [SAM] = 2, 5, and 10 μM. The line represents a least-squares fit to eq 3, with the maximum $k_{cat}/K_m = 9.4 \times 10^4$ M^{-1} s^{-1}, pK_1 = 7.5, and pK_2 = 8.2.

The A224I Mutation Increases the Aldimine pK_a of AATase and Reduces Catalytic Activity. Alanine 224 is the AATase active site residue that occupies a position equivalent to Ile232 in ACC synthase (Scheme 2). The introduction of the mutation A224I into AATase results in a small but reproducible pK_a increase from 6.96 in WT AATase to 7.16 (Figure 2B). Although the magnitude of the change is much smaller than that of the reverse I232A mutation in ACC synthase, the shift is in the anticipated direction toward that of WT ACC synthase. The effect on the enzyme activity is also moderate: the k_{max} values for single turnover of both aspartate and glutamate are reduced approximately 9-fold, but the apparent K_m's for both substrates are reduced by nearly half, resulting in k_{max}/K_m^{app} values that are only 4-fold less than that of WT (Table 1).

This mutant also exhibits an absorbance spectrum that is slightly different from that of WT. At low pH, the absorbance at 320 nm is increased and that at 430 nm is reduced relative to WT. At high pH, this mutant is unique in retaining a significant absorbance at 430 nm, suggesting that the enzyme internal aldimine is only two-thirds deprotonated at high pH (Figure 2B).

The Y233F Mutation in I232A ACC Synthase Results in a pK_a That Nearly Matches That of AATase Y225F and

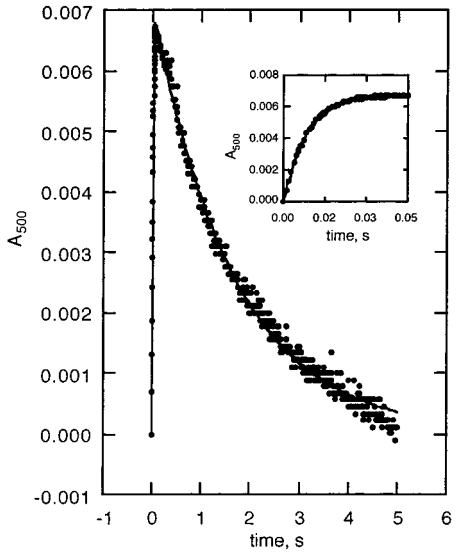


FIGURE 4: Formation and decay of the quinonoid intermediate in the reaction of D230E ACCS with SAM. D230E ACCS (55 μM) was reacted with 2.5 μM (*S*,*S*)-SAM in 50 mM potassium phosphate, pH 8.5, and 10% glycerol. The line represents a least-squares fit to eq 4, with $k_1 = 101$ s^{-1} and $k_2 = 0.59$ s^{-1}. Data were collected at 500 nm at 1 ms intervals for the first 50 ms and at 12.5 ms intervals thereafter using a stopped-flow spectrophotometer (see Experimental Procedures). The inset shows the data from the first 50 ms with an expanded time scale.

Partially Rescues the Reduced k_{cat} of the I232A Construct in ACC Synthase. The double mutant I232A/Y233F ACC synthase exhibits a spectrophotometric pK_a of 8.8 (Figure 2C), which is 0.6 unit above that of I232A ACC synthase and 1.4 units lower than that of Y233F. Kinetically, this mutant enzyme has an elevated K_m of 57 μM and a k_{cat} of 2.2 s^{-1} (4-fold higher than the value of 0.5 s^{-1} in the single mutant I232A), resulting in a k_{cat}/K_m of 4×10^4 M^{-1} s^{-1}, which is comparable to those values recorded for the single mutants I232A (5×10^4 M^{-1} s^{-1}) and Y233F (5.1×10^4 M^{-1} s^{-1}) (Table 1).

The D230E Mutation Results in a 0.7 Unit Increase in the Aldimine pK_a and Stabilizes the Quinonoid Intermediate in the Reaction with SAM. The spectrophotometric pK_a of the D230E mutant of ACC synthase is 9.95 (Figure 2D, Table 1). The activity of this mutant is lower than that of the other active site mutants discussed above, with a k_{cat} value of 0.7 s^{-1} and a k_{cat}/K_m value of 1.3×10^4 M^{-1} s^{-1} at pH 8.5. The Asp → Glu mutation also results in the accumulation of a relatively stable quinonoid intermediate upon reaction with SAM. The rate of formation of the intermediate is 100 s^{-1}, which matches that observed for WT (*8*), but the rate constant for the decay of the intermediate, which is the rate-determining step in the reaction, is much less (0.6 vs 10 s^{-1}); therefore the quinonoid intermediate accumulates (Figure 4).

DISCUSSION

The following discussion considers the roles of residues Asp230, Ile232, and Tyr233 in modulating the aldimine pK_a of ACC synthase, which is significantly higher (9.3) than that of the homologous AATase (7.0). These three residues correspond to Asp222, Ala224, and Tyr225, respectively, of AATase (Scheme 2). The effects of mutations of Tyr225-(233) in both enzymes (*4*, *5*) and of Asp222 in AATase (*9*) have been described. The present focus is primarily on the one member of this triplet that differs between the two enzymes, Ile232/Ala224.

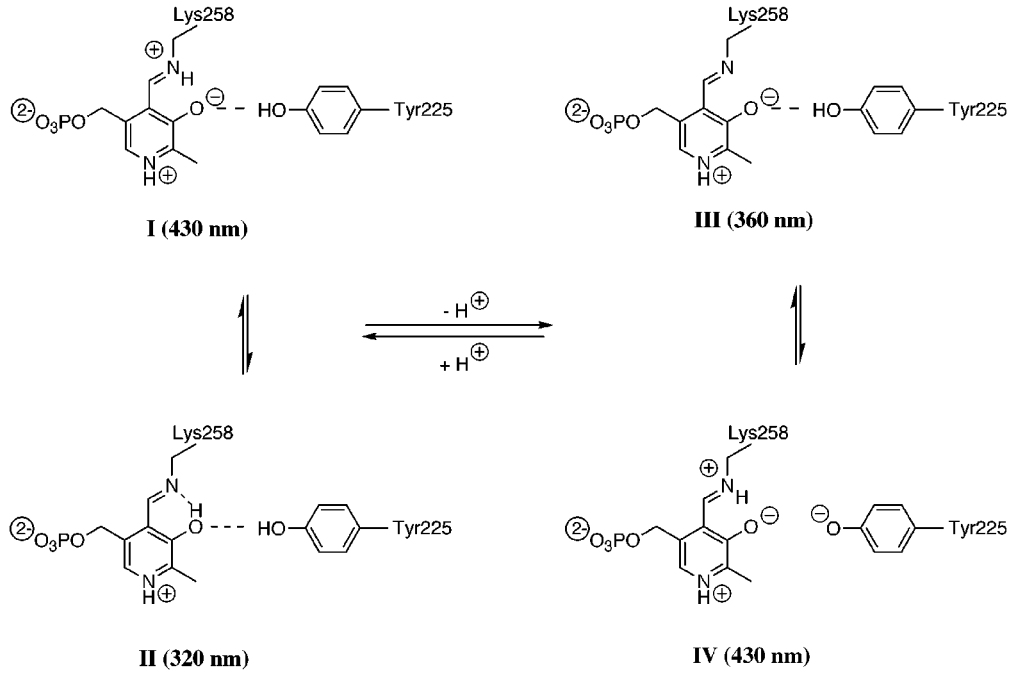
Effects of the I232A Mutation in ACC Synthase and the Reverse A224I Substitution in AATase on the pK_a Values and the Kinetic Parameters. Capitani et al. (*3*) postulated, from the crystal structure of ACC synthase, that the added bulk of Ile232 (vs Ala224 in AATase) might be responsible in part for the large difference in the aldimine pK_a values, because the presence of the larger side chain results in a lengthening of ~0.2 Å of the hydrogen bond between the 3′ oxygen of the cofactor and the side chain of Tyr233 (Tyr225 in AATase) (Scheme 2), although this difference is within the error of the structure resolution. Consistent with this suggestion is the observation that mutation of this isoleucine to alanine, which should allow the hydrogen bond to shorten, does indeed result in a decrease in the aldimine pK_a of ACC synthase, from 9.3 to 8.2 (Table 1). Thus Ile232 is identified as a major determinant of the high aldimine pK_a value of this enzyme.

There is a concomitant decrease in the activity of the enzyme associated with this mutation. The k_{cat}/K_m value for the mutant is more than 20-fold less than that of WT at the optimum pH value. Most of this effect is due to a decrease in k_{cat}, presumably because the substrate−PLP complex is no longer positioned optimally in the active site. The mutant enzyme is also unstable at high pH, as evidenced by the visible denaturation during the spectrophotometric pH titration (Figure 2A). Instability of the deprotonated form of the enzyme has also been observed to a lesser extent in the WT enzyme and may be due to dissociation of the cofactor.

The introduction of isoleucine into AATase at the equivalent position (replacing Ala224) likewise results in a change of the aldimine pK_a in the anticipated direction. In this case, however, the change is small, from 6.96 to 7.16, suggesting that the cofactor may not be displaced significantly to the ACC synthase orientation in the active site to accommodate the large isoleucine side chain. Thus the effect of the isoleucine/alanine exchange is context dependent (*10*). The values of both k_{cat} and K_m are reduced in A224I AATase, resulting in only a small (~4−5-fold) net decrease in k_{cat}/K_m (Table 1).

Spectrophotometric Considerations. Spectrophotometric titrations of subclass I aminotransferases generally exhibit maxima near 430 nm at low pH attributed to the protonated aldimine (*6*, *11*). With increasing pH, this absorbance is replaced, usually with a single isosbestic point, by a peak at 360−390 nm, which is attributed to the deprotonated aldimine. No other species are observed. The titration of the A224I mutant of AATase is anomalous in this respect in that a second peak at 325 nm is observed at low pH, and the 430 nm peak retains ~30% of its intensity at high pH (Figure 2B, Scheme 3). The enolimine tautomer **II** of aldimine **I** absorbs maximally near 320 nm (*6*), so the observed 325 nm absorbance is likely due to this species; however, it is possible that this absorbance arises from a loss of conjugation of the imine with the pyridine ring such as would occur from nucleophilic addition to the Schiff base. The only known A_{430} species is the protonated aldimine; thus the high pH A_{430} form results from deprotonation of an amino acid side chain, most likely Tyr225 (**IV**). Since the spectrophotometric titration exhibits a single isosbestic point, species **I** and **II** and species **III** and **IV** must be in rapid equilibrium. Species

Scheme 3: Forms of the Enzyme-Bound PLP of the A224I Mutant of AATase Cofactor at High and Low pH

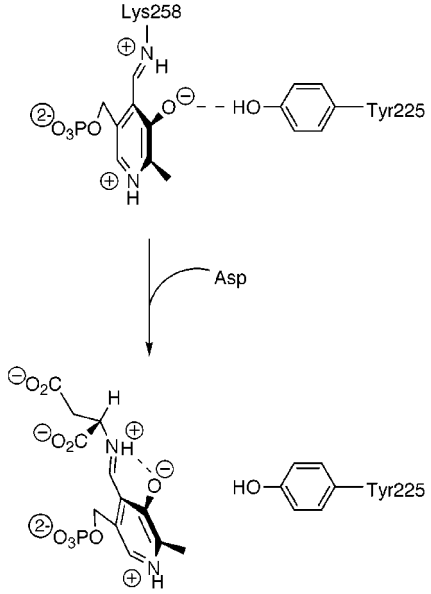


II and **IV** would exist in the A224I mutant because of the weakened hydrogen bond between the 3′ oxygen and Tyr225 effected by the introduced isoleucine. This would increase the basicity of the 3′ oxygen as well as the acidity of Tyr225. The lost hydrogen bond in **IV** vs **III** results in an increased imine pK_a. An increase in aldimine pK_a is also achieved with the Y225F mutation in WT AATase (*4*; Table 1).

The free imine forms (**II** and **III**) of the enzyme are the active ones, so ~30% of the reduced k_{max}/K_m^{app} of the mutant can be attributed to the presence of species **IV** at the pH of optimum activity, which is above the aldimine pK_a. The 40% reduction in apparent K_m effected by the mutation shows that substrate binding is more favorable in the mutant than in WT. The Y225F mutation of AATase similarly elicits a 20-fold reduction in K_m (*4*). The explanation was described as a "tug of war" in which the Tyr225 hydroxyl group competes with the substrate for the cofactor (Scheme 4). Elimination of this hydrogen bond therefore results in a more favorable association with the substrate, because it no longer competes with the tyrosine for PLP. Weakening of the same hydrogen bond via the A224I mutation accomplishes the same result.

Is the Effect of Ile232 Limited to the Tyr−PLP Hydrogen Bond? While it is clear that the I232A mutation in ACC synthase makes the active site more like that of AATase, this construct is an imperfect mimic in that the pK_a of I232A ACC synthase of 8.2 remains higher than that of WT AATase, which is 7.0. Thus the hydrogen bond made with the cofactor from Tyr233 in the I232A construct is probably not as strong as that present in WT AATase. This conclusion is supported by the observation that elimination of that bond by a Tyr → Phe mutation raises the AATase pK_a by 1.6 units (*4*), while that of the I232A/Y233F double mutant is raised only 0.6 unit above that of the single I232A mutant. Further evidence that the I232A mutant of ACC synthase is suboptimal for catalytic function is provided by the finding that this mutation reduces the catalytic activity over WT and that some of this loss is restored by the second mutation

Scheme 4: The Tyr−PLP Hydrogen Bond Is Broken by Substrate Binding[a]



[a] Adapted from ref *4*.

(Table 1). In contrast, the Y225F mutation decreases both the k_{cat} and k_{cat}/K_m values of WT AATase (*4*).

The remaining puzzling observation is that the Y233F mutant of ACC synthase raises the pK_a by 0.9 unit (*5*) above WT, but the introduction of the same mutation into I232A ACC synthase only raises the pK_a by 0.6 unit, contrary to expectation if the hydrogen bond is indeed stronger in the I232A mutant than in WT. This result indicates that other features of the active site contribute substantially to control of the aldimine pK_a. Hayashi and co-workers (*12*, *13*) have proposed that the major determinant of the aldimine pK_a of AATase (and by extension other enzymes in this family) is the imine−pyridine torsion angle and the resulting degree of conjugation between the two. In this model, the role of

Tyr225 would be to impose a geometric constraint by holding the cofactor in a specific position rather than significantly altering the electronic structure of the pyridine ring. The size of the isoleucine side chain might also act to change the geometry of the cofactor, affecting the pK_a in a manner independent of the Tyr233−PLP hydrogen bond. This hypothesis is consistent with the observation that the I232A/Y233F double mutant of ACC synthase has a pK_a remarkably close to that of Y225F AATase (8.8 vs 8.6) rather than Y233F ACC synthase (10.2). At this point, the structure of ACC synthase has not been determined at high enough resolution to investigate this possibility.

What Is the Role of Asp230? The crystal structure of ACC synthase indicates that the distance between Asp230 and the protonated pyridinium nitrogen of the cofactor is 1.2 Å longer (3.8 vs 2.6 Å) than the corresponding distance in AATase (*3*). This difference is initially surprising, as the longer distance interaction should result in a lower ACC synthase pK_a compared to that of AATase. The D230E mutation was introduced in an attempt to reduce this distance, resulting in the anticipated increase in the aldimine pK_a from 9.3 to 10.0. The increase is a result of increased electron density in the ring as a result of the closer association with the carboxylate side chain. It thus appears that the precise pK_a values of the two enzymes are achieved by an interplay of the effects of different amino acids (see below). The analogous D222E mutation in AATase results in a 0.25 unit *decrease* in the aldimine pK_a (*9*), where it is likely that the Glu side chain is too large to be accommodated easily in the position occupied by Asp in the WT enzyme.

In summary, it is demonstrated that Ile232 is a dominant factor in establishing the high aldimine pK_a of ACC synthase over that existing in AATase, which has Ala224 in this position. The mechanism for the isoleucine-induced pK_a elevation is likely an increase in the length of the Tyr233−PLP hydrogen bond over that present in AATase. Mutation of Ile232 to alanine therefore results in a decrease in the aldimine pK_a of ACC synthase. Introduction of isoleucine at the analogous position in AATase weakens the hydrogen bond and raises the pK_a of that enzyme. The effect of Ile232 is in part countered by the longer distance between the pyridine nitrogen of the cofactor and Asp230 in ACC synthase compared to that present in AATase.

ACKNOWLEDGMENT

We thank Dr. Liang Feng for help with the ACC synthase mutant constructs and Dan Malashock and Wendy Shaffer for critically reading the manuscript.

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BI016084L