Mechanistic Studies of 1-Aminocyclopropane-1-carboxylate Deaminase (ACCD): Characterization of an Unusual PLP-dependent Reaction

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Supporting Information

Methods

Description of reaction mixtures for ^{13}C-KIE studies and purification of residual ACC: Large scale reactions (performed in duplicate) were conducted by dissolving ~ 950 mg of ACC to a final concentration of 50 mM in 100 mM potassium phosphate (pH 7.5). To initiate the reaction, wt ACCD enzyme was added and the reaction mixture was slowly stirred at 25 °C. The progress of the reaction was periodically assessed using the coupled LDH assay to determine the concentration of reaction product, α-KB. The enzyme activity decreased over ~ 24 h, so aliquots of fresh enzyme were periodically added as turnover became sluggish. As the reaction neared completion, a 0.5 mL aliquot was removed for determining the fraction of reaction and the remainder of the reaction mixture was cooled to 4 °C and filtered using a YM-10 membrane (Millipore, Billerica, MA.) to remove the enzyme. The filtered sample was loaded onto Dowex-50 cation exchange resin (100 mL) to recover the residual ACC starting material. The resin had been previously equilibrated with 250 mL of 2 M HCl and washed with H_2O (~ 300 mL) until the pH of the column eluate was neutral. After loading the reaction onto the

column, the resin was washed with an additional 500 mL of H_2O to elute the reaction product (α-KB) and phosphate buffer. ACC was then eluted with 300 mL of 6% NH_4OH in H_2O (pH 11.5) and the solvent was removed by rotary evaporation and lyophilization. The column eluate was repeatedly dissolved in ~250 mL H_2O and lyophilized to remove the majority of the NH_3. For each large scale reaction, a separate commercial batch of ACC was used. A 50 mg sample of ACC was saved from each commercial batch for determination of the ^{13}C content of unreacted starting material. The sample was dissolved in 200 mL (the approximate volume of the large-scale reactions) of 100 mM potassium phosphate (pH 7.5) and was purified by cation exchange chromatography in a manner identical to that described for the recovery of residual ACC from the large scale reactions.

Measuring ^{13}C-enrichment (R/R_O) in ACC using ^{13}C-NMR spectroscopy: Following purification, the unreacted and residual ACC samples were dissolved separately in 660 μL of an H_2O:D_2O mixture (90:10% v/v) and analyzed by ^{13}C-NMR spectroscopy to determine the relative ^{13}C enrichment at each carbon atom of ACC. NMR spectra were collected on a Varian Unity 500 MHz NMR spectrometer at the NMR core facility at the University of Texas, Austin. A total of ten ^{13}C-NMR spectra were recorded for each sample. Each spectrum was composed of 256 scans, separated by a delay time of 80 sec between scans to ensure full relaxation of each of the ^{13}C nuclei of ACC between individual instrument pulses. For integration of ^{13}C-NMR peaks, a zeroth order baseline correction was made in the vicinity of each signal, and the peak areas were determined by integrating a 10 Hz window centered around the chemical shift of each peak. The same integration parameters were then applied to each of the nine other

spectra in that particular set. The relative ^{13}C enrichment of the carboxylate carbon of ACC was used as an internal standard to normalize the relative ^{13}C enrichment of C_α and C_β within each sample. This relative measurement of the ^{13}C content of C_α and C_β is hereafter referred to as R for residual ACC samples recovered from large scale wt ACCD reactions, or as R_O for unreacted ACC samples. From the averaged values of R and R_O determined from the ten NMR spectra, the ratio R/R_O and its associated standard error $\Delta(R/R_O)$ was calculated for the C_α and C_β atoms. It should be emphasized that for each large-scale reaction, the R and R_O measurements are derived from the same commercial batch of substrate.

Determining the fraction of reaction (*F*): To determine the fraction of reaction (F), the concentration of α-KB present in the quenched reaction mixture at the end of the large scale reaction (C_1) was compared to the concentration of α-KB (C_2) present in a sample of the same reaction mixture that had been allowed to reach 100% conversion by the addition of excess ACCD. C_1 and C_2 were measured via an end-point assay that coupled α-KB reduction to NADH oxidation using lactate dehydrogenase (LDH). A 1 mL solution containing 150 μM NADH, 4 units of LDH, and 100 mM sodium phosphate buffer (pH 7.0) was transferred to a cuvette and the absorbance at 340 nm was recorded over several minutes. A 1 μL aliquot of the large scale reaction mixture (or of the reaction driven to 100% completion) was then added and the change in absorbance at 340 nm was allowed to reach its equilibrium level. The change in absorbance at 340 nm (ε_{340} of NADH = 6220 $M^{-1}cm^{-1}$) was used to calculate the concentration of α-KB. A total of 10 replicate measurements of both C1 and C2 was made for each large scale reaction.

The fraction of reaction was then calculated as $F = C_1/C_2$, and the standard errors of the C_1 and C_2 measurements were propagated to calculate ΔF.

Calculation of $^{13}(k_{cat}/K_m)$ and its associated standard error: From the R/R_O and F values determined above, the $^{13}(k_{cat}/K_m)$ at both C_α and C_β of ACC (KIE_{calc}) were calculated with eq S.1. The standard errors on KIE_{calc} that result from uncertainties in the fraction of reaction (ΔKIE_F) and ^{13}C enrichment (ΔKIE_R) measurements were then calculated with eq S.2 and S.3, respectively, using the experimentally determined standard errors on R/R_O and F ($\Delta R/R_O$ and ΔF, respectively).[1] The standard error on KIE_{calc} was then calculated with Eq S.4 and is reported in the main text.

Eq S.1:

$$KIE_{calc} = \ln(1-F)/\ln[(1-F)R/R_o]$$

Eq S.2:

$$\Delta KIE_F = -\ln(R/R_o)\,\Delta F/[(1-F)\ln^2[(1-F)R/R_o]]$$

Eq S.3:

$$\Delta KIE_R = -\ln(1-F)\,\Delta\,(R/R_o)/[(R/R_o)\ln^2[(1-F)R/R_o]]$$

Eq S.4:

$$\Delta KIE_{calc} = KIE_{calc}[(\Delta KIE_F/KIE_{calc})^2 + (\Delta KIE_R/KIE_{calc})^2]^{1/2}$$

Results

wt ACCD (H_2O)			wt ACCD (D_2O)		
pH	**k_{cat} (s^{-1})**	**K_m (mM)**	**pD**	**k_{cat} (s^{-1})**	**K_m (mM)**
6.55	1.62(6)	21(2)	7.13	0.32(1)	23(2)
6.65	1.82(3)	18(2)	7.45	0.26(1)	5.4(6)
6.83	1.61(3)	6.7(4)	7.94	0.25(1)	1.7(2)
7.14	1.69(2)	3.0(1)	8.23	0.21(1)	0.7(1)
7.41	1.48(2)	2.2(1)	8.48	0.226(4)	0.46(4)
7.69	1.32(2)	1.2(1)	8.81	0.21(1)	0.46(1)
7.95	1.35(2)	0.96(7)	9.09	0.212(5)	0.37(5)
8.00	1.38(2)	1.2(1)	9.41	0.203(4)	0.73(8)
8.21	1.29(3)	0.85(9)	9.73	0.17(1)	1.4(4)
8.49	1.11(5)	1.2(2)			
8.82	1.02(3)	2.3(4)			
9.12	0.94(4)	6.2(9)			
9.41	0.97(6)	10(2)			
9.71	0.83(3)	13(1)			

E295D			Y268F		
pH	**k_{cat} (s^{-1})**	**K_m (mM)**	**pH**	**k_{cat} (s^{-1})**	**K_m (mM)**
6.72	0.103(6)	27(4)	7.00	0.0078(3)	7.0(5)
7.02	0.134(3)	13(1)	7.22	0.0059(1)	5.1(5)
7.27	0.138(3)	7.1(5)	7.33	0.0040(1)	5.1(3)
7.50	0.139(3)	5.0(4)	7.5	0.0040(1)	4.2(4)
7.62	0.147(2)	3.6(2)	7.77	0.0120(2)	4.1(3)
7.88	0.147(3)	2.4(2)	8.12	0.0186(3)	4.0(2)
8.21	0.126(2)	1.7(1)	8.44	0.0261(4)	3.4(2)
8.50	0.124(3)	2.4(2)	9.00	0.0261(6)	3.2(.3)
8.77	0.130(4)	4.0(4)	9.30	0.026(1)	4.9(6)
9.12	0.136(6)	12(1)	9.57	0.029(1)	10(1)
9.50	0.140(4)	36(2)			

Table S1. Summary of fits of steady state kinetic data to the Michaelis-Menten Equation. Standard errors are shown in parentheses.

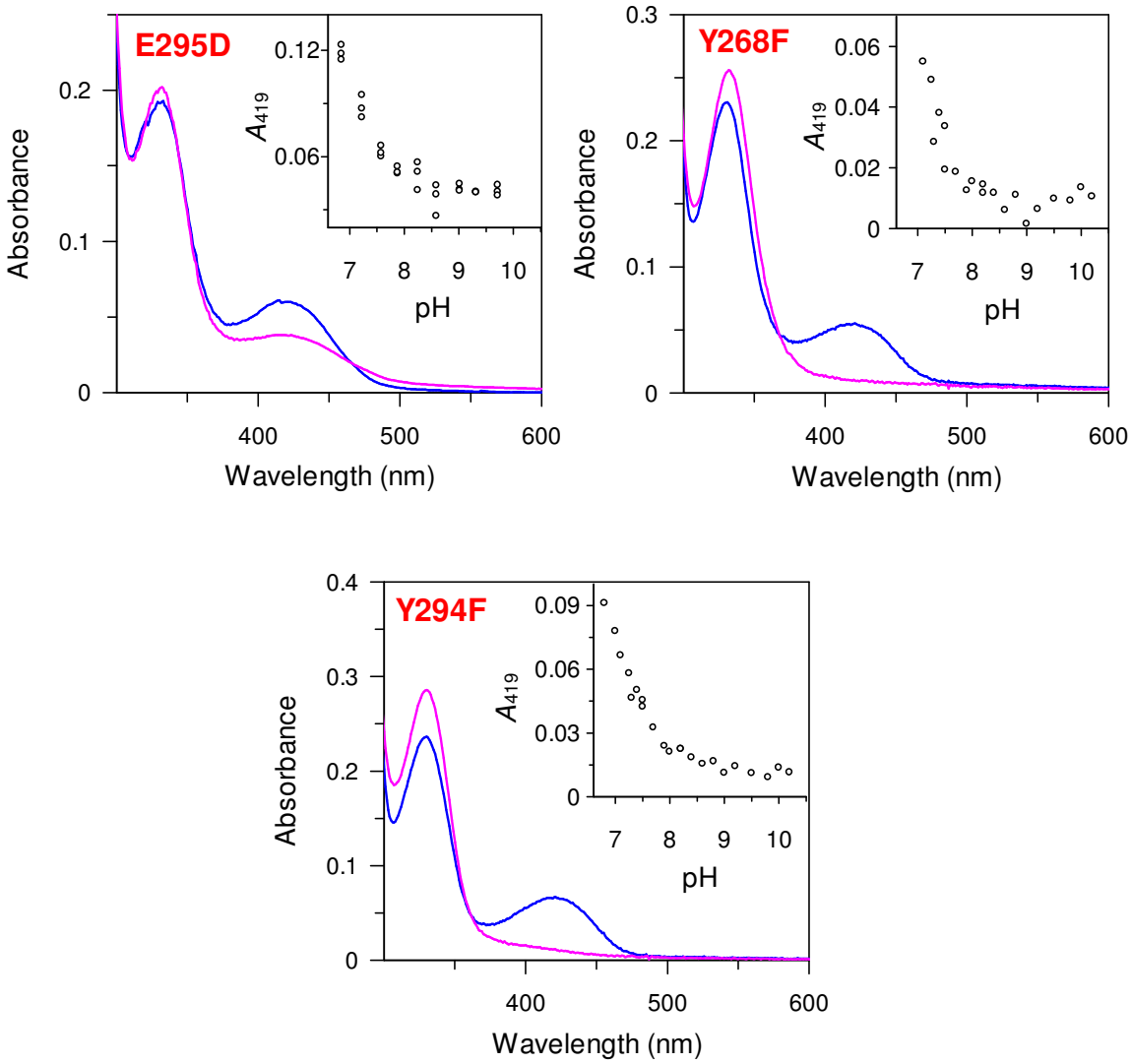

Figure S1: pH-Dependence of internal aldimine absorption for the E295D, Y268F, and Y294F mutant enzymes.

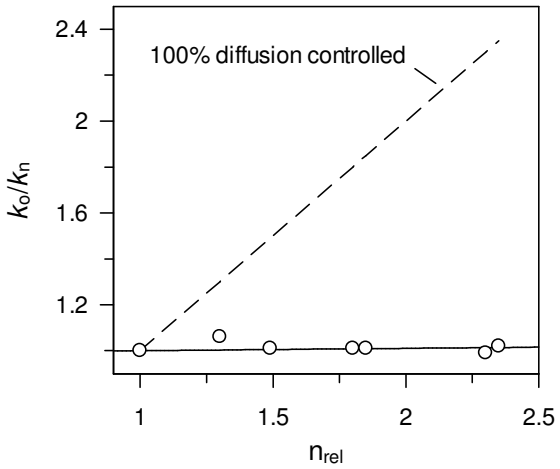

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Figure S2: Viscosity effects on k_{cat} for wt ACCD. The data were fit with eq 4 as described in the Materials and Methods**.**

Model	$SSR_{(LOF)}$	$d_{f(LOF)}$	F	F_c
T_1	0.00124	4	1.20	2.93
T_2	0.000327	3	0.42	3.16
T_1S	0.000204	3	0.26	3.16
	$SSR_{(PE)}$	$d_{f(PE)}$		
Pure Error	0.00463	18		

Table S2. Lack-of-fit *F*-tests for fits of the proton inventory data to various forms of the Gross-Butler equation (see eq 5 in Materials and Methods). The definition of each model (T_1, T_2, and T_1S) is shown in Table 2 of the main text. Lack-of-fit *F*-values were calculated as: $F = (SSR_{(LOF)}/d_{f(LOF)})/(SSR_{(PE)}/d_{f(PE)})$ as described by Draper and Smith,[2] where *SSR* are sum square residuals and d_f are the degrees of freedom for the lack-of-fit (LOF) and pure error (PE) estimates. F_c is the critical *F*-value at the 5% significance level.

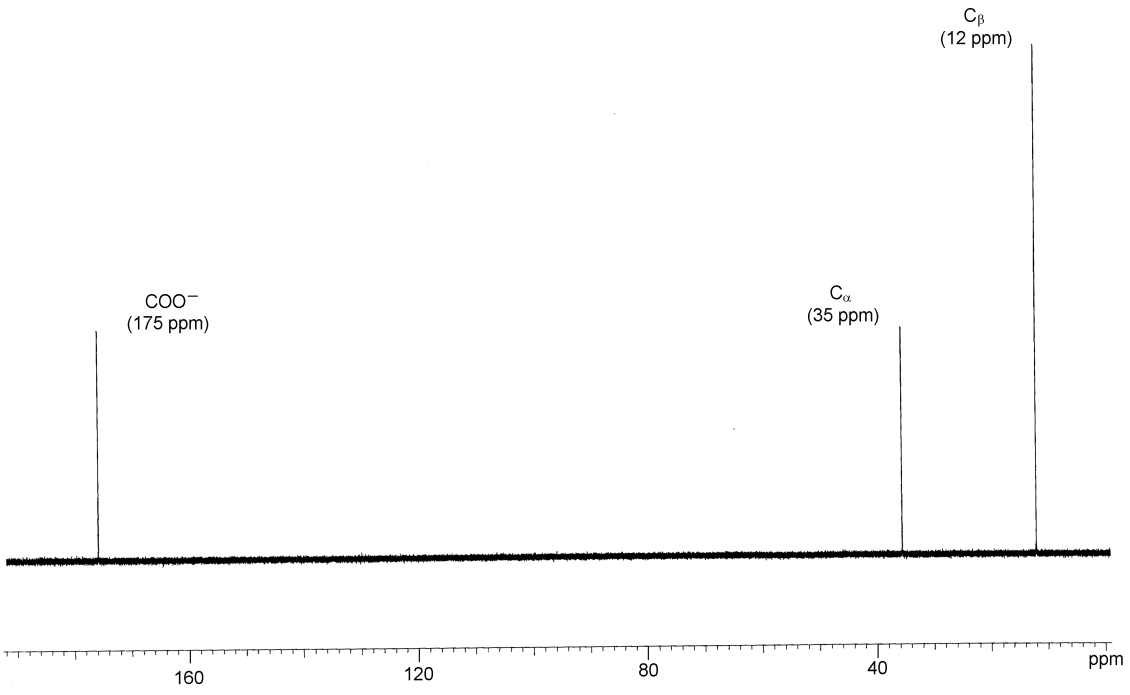

Figure S3. A typical ^{13}C-NMR spectrum of ACC taken under the conditions employed in this study.

Reaction 1

	Unreacted ACC					Residual ACC				
N	COO-	C_α	C_β	$R_{o(C\alpha)}$ = C_α/COO-	$R_{o(C\beta)}$ = C_β/COO-	COO-	C_α	C_β	$R_{(C\alpha)}$ = C_α/COO-	$R_{(C\beta)}$ = C_β/COO-
1	.75	.746	1.32	.994	1.76	.804	.809	1.4	1.01	1.74
2	.775	.755	1.34	.974	1.73	.808	.824	1.42	1.02	1.76
3	.77	.756	1.31	.982	1.70	.81	.832	1.43	1.03	1.77
4	.772	.749	1.31	.970	1.70	.814	.829	1.43	1.02	1.76
5	.735	.748	1.3	1.02	1.77	.839	.846	1.45	1.01	1.73
6	.755	.733	1.32	.971	1.75	.82	.837	1.45	1.02	1.77
7	.75	.741	1.3	.988	1.73	.823	.829	1.46	1.01	1.77
8	.743	.742	1.33	.999	1.79	.851	.839	1.44	0.986	1.69
9	.749	.737	1.3	.984	1.74	.814	.846	1.45	1.04	1.78
10	.757	.741	1.33	.979	1.76	.825	.848	1.44	1.03	1.75
	Average R_o			0.986(4)	1.74(1)	**Average *R***			1.016(5)	1.75(1)

Reaction 2

	Unreacted ACC					Residual ACC				
N	COO-	C_α	C_β	$R_{o(C\alpha)}$ = C_α/COO-	$R_{o(C\beta)}$ = C_β/COO-	COO-	C_α	C_β	$R_{(C\alpha)}$ = C_α/COO-	$R_{(C\beta)}$ = C_β/COO-
1	.979	.937	1.62	.957	1.655	1.2	1.17	2.03	.975	1.692
2	.977	.908	1.61	.929	1.648	1.21	1.15	2.07	.950	1.717
3	.976	.926	1.62	.949	1.66	1.21	1.19	2.06	.983	1.702
4	.946	.942	1.62	.996	1.712	1.2	1.19	2.07	.992	1.725
5	.97	.906	1.61	.934	1.66	1.21	1.21	2.1	1.00	1.736
6	1.01	.941	1.63	.932	1.614	1.2	1.18	2.07	.983	1.725
7	.99	.954	1.65	.964	1.667	1.23	1.16	2.1	.943	1.707
8	.996	.922	1.61	.926	1.616	1.24	1.19	2.07	.960	1.669
9	.984	.926	1.62	.941	1.646	1.2	1.18	2.06	.983	1.717
10	.999	.948	1.64	.949	1.642	1.22	1.21	2.09	.992	1.713
	Average R_o			.948(7)	1.652(6)	**Average *R***			.976(6)	1.710(6)

Table S3. Summary of raw ^{13}C-NMR integrations and calculation of average R and R_o values for the C_α and C_β atoms in the two replicate reactions. N is the spectrum number. The values in parentheses on the average R and R_o parameters are the standard errors in the final digit.

	Reaction 1		Reaction 2	
Replicate	**C_1**	**C_2**	**C_1**	**C_2**
1	45.19	50.92	42.54	50.48
2	45.14	51.71	42.32	51.39
3	44.99	50.83	42.46	51.74
4	47.14	50.37	42.89	52.39
5	45.60	51.19	43.34	52.43
6	46.01	50.97	44.06	50.68
7	45.95	50.07	43.40	52.66
8	46.99	50.59	44.53	52.79
9	47.61	51.28	43.48	51.41
10	46.52	50.80	42.34	51.33
Average	46.1 ± 0.29	50.9 ± 0.14	43.1 ± 0.24	51.7 ± 0.26
$F = C_1/C_2$	**0.906 ± 0.006**		**0.834 ± 0.006**	

Table S4. Fraction of reaction measurement. The coupled LDH enzyme assay was used to quantify the concentration of α-KB at the end of the each large-scale reaction (C_1) as well as the concentration of α-KB when the reaction was taken to 100% completion by the addition of excess ACCD (C_2). The fraction of reaction, F, and its standard error (ΔF) were then calculated from the averaged values of C_1 and C_2.

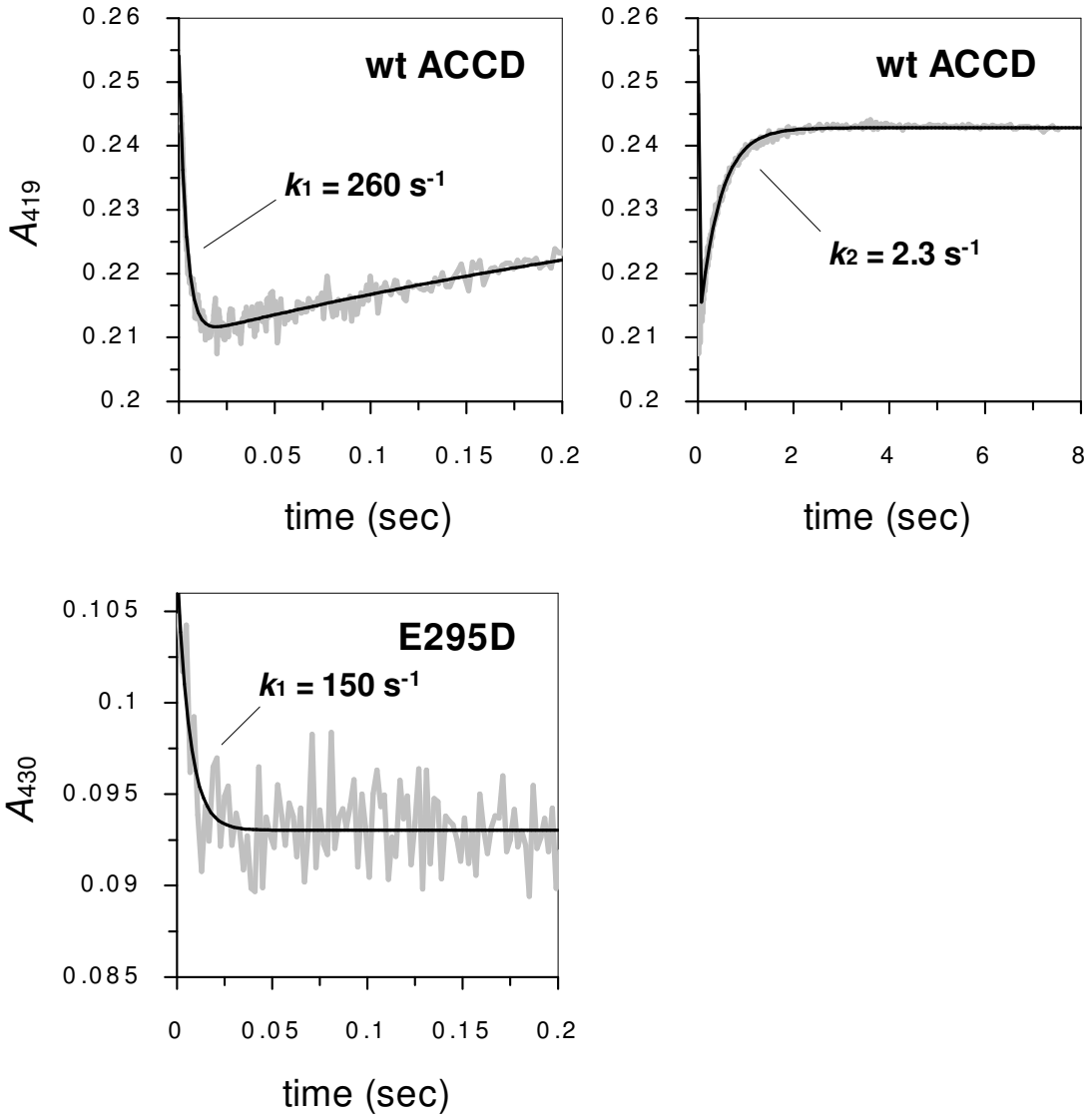

Figure S4. Fits of pre-steady state absorbance changes of wt ACCD and its mutants to exponential equations. The wt ACCD data (followed at 419 nm) were fit with a double exponential equation. The E295D data (followed at 430 nm) were fit with a single exponential equation, and the Y268F and Y294F data (followed at 430 nm) were fit with a triple exponential equation. Fitted parameter values are summarized in Table S4.

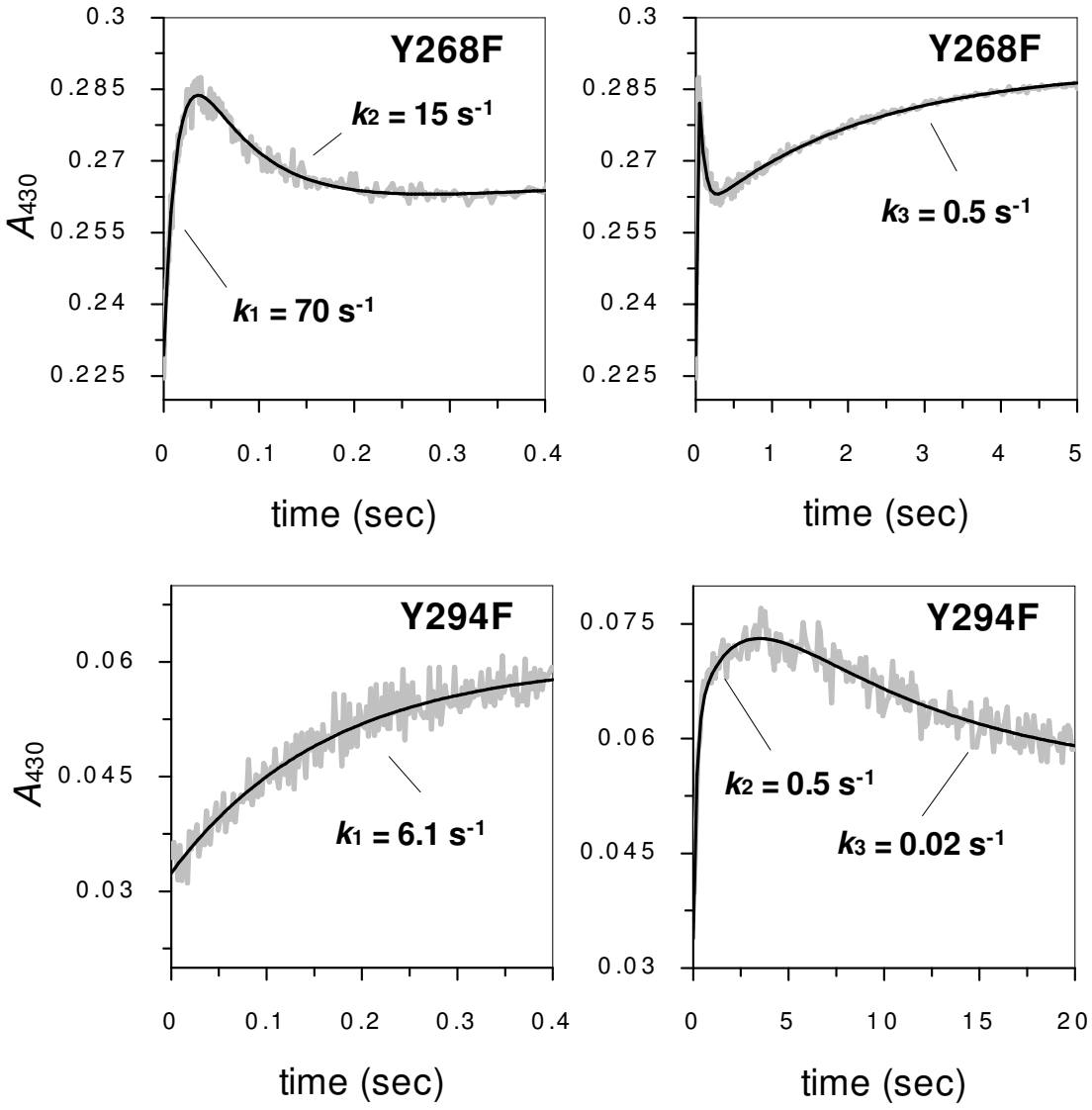

Figure S4 (continued).

Enzyme	A_1	k_1	A_2	k_2	A_3	k_3	C
wt ACCD	-0.044(1)	260(5)	0.033(1)	2.31(3)	-	-	0.254(1)
E295D	-0.015(2)	150(30)	-	-	-	-	0.108(2)
Y268F	0.082(2)	71(2)	-0.053(2)	14.8(4)	0.0310(2)	0.46(1)	0.229(1)
Y249F	0.028(1)	6.1(5)	0.026(3)	0.48(7)	-0.033(2)	0.10(2)	0.034(1)

Table S5. Summary of fitted parameter values obtained from non-linear fits of the stopped-flow data in Figure S4 to exponential equations. Standard errors in the final digit of the parameter estimates are shown in parentheses.

1. Singleton, D. A.; Thomas, A. A., High-precision simultaneous determination of multiple small kinetic isotope effects at natural abundance. *J. Am. Chem. Soc.* **1995,** 117, 9357-9358.
2. Draper, N. R.; Smith, H., *Applied Refression Analysis*. 3rd ed.; John Wiley & sons: New York, 1998; p 706.