Supporting Information

Heavy Enzyme Kinetic Isotope Effects on Proton Transfer in Alanine Racemase

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Experimental

Materials. Salts were from either Fisher or Aldrich. D_2O and deuterated glycerol were from Cambridge Isotope Laboratories. NaOD, [2-^{2}H]-L-Alanine, and [2-^{2}H]-D-Alanine were from CDN Isotopes.

Alanine Racemase (AR) expression and purification. BL21(DE3) *E. coli* conditioned to grow in D_2O was transformed with pET23a containing the AR gene from *G. stearothermophilus* such that a 6 × His tag was introduced on the N terminus. Cells from a single colony were grown in perdeuterated minimal medium as a seed culture for bioreactor growth. A 2 mL seed culture was used to inoculate 0.5 L of perdeuterated minimal medium in D_2O in a New Brunswick Bioflo 110 fermentor. The pD was adjusted to 7.2, which was maintained during growth by addition of 20 % (w/v) NaOD. The pO_2 was maintained above 10 %, initially by increasing stirring and later by increasing air flow. The composition of the perdeuterated minimal media during the batch phase was: 0.5 % (w/v) perdeuterated glycerol, 6.8 g/L $(ND_4)_2SO_4$, 1.5 g/L KD_2PO_4, 5.2 g/L Na_2DPO_4, 0.5 g/L $(ND_4)_2$ citrate, 0.16 g/L $MgSO_4$, 17 mg/L $FeCl_3$, 0.5 mL of trace metal stock solution (0.5 g/L $CaCl_2$, 0.2 g/L $ZnSO_4$, 0.15 g/L $CuSO_4$, 0.16 g/L $MnSO_4$, 0.2 g/L $CoCl_2$) and 20 g/L EDTA. All protons were previously exchanged for deuterons by rotary evaporation from D_2O.

After consumption of glycerol in the batch phase, the feed-batch phase was initiated by adding fresh solution containing 1 % (w/v) perdeuterated glycerol, 0.2 % (w/v) Mg_2SO_4, 0.3 % (w/v) $(ND_4)_2SO_4$. Expression of AR was initiated at the beginning of the feed-batch phase by addition of IPTG to 0.1 mM. At the end of the growth, the final OD_{600} was ~6, and ~7 g of cell paste were obtained after centrifugation.

Cells were harvested and resuspended in 50 mM KP_i, 300 mM KCl, 20 μM PLP, 0.5 mg/L lysozyme, pH 7.8. The cells were disrupted by sonication. The soluble fraction was incubated at 65 °C for 30 min. The suspension was centrifuged to remove denatured proteins and the supernatant was purified using a 20 mL Ni-NTA column with a 200 mL 10 mM to 0.5 M imidazole gradient in 100 mM KP_i, 300 mM KCl, 10 μM PLP, pH 8. Pure protein fractions, as judged by SDS-PAGE, were pooled and dialyzed against 50 mM K_2HPO_4, 100 mM KCl, pH 8, 10 μM PLP. Approximately 25 mg of purified

perdeuterated AR were obtained. The light enzyme was expressed and purified using identical procedures except deuterium was substituted by protium.

ESI-MS spectra of AR expressed in protiated and perdeuterated media show the major peaks at 44,627 and 47,078 Da, respectively. The theoretical molecular masses for the protiated and perdeuterated AR are 44,616 and 47,056 Da, respectively. The mass of perdeuterated AR (^{D}AR) is ~5.5% greater than that of protiated AR (^{H}AR), which is identical to the theoretical value with all exchangeable deuterons equilibrated with the H_2O solvent. In reality, this result is likely a coincidence due to some of the buried exchangeable deuterons not equilibrating with the H_2O solvent used in purification and assays, and nonexchangeable hydrogens being incompletely deuterated.

AR kinetics. Initial rates of D-alanine and L-alanine racemization by AR were measured using an Olis DSM20 circular dichroism spectrophotometer. The conditions were 50 mM potassium borate, 100 mM KCl, pH 8.9, and 25 °C. The wavelength (215 nm) was chosen such that the CD was linearly proportional to alanine concentration. For kinetics with initial concentrations of alanine higher than 30 mM, 220 nm was used, while 205 nm was used for initial concentrations of alanine lower than 1 mM. Competitive inhibition by 2-methylalanine (2-aminoisobutyrate) was studied using L-alanine as substrate in 50 mM potassium borate, 100 mM KCl, pH 8.9, and 25 °C at 215 nm. Kinetic data were fitted by nonlinear regression using Kaleidagraph 3.6. The error values reported in the tables S1 and S2 are standard errors from the nonlinear regression. The error values reported in tables 1 and 2 of the main text were derived from those reported in tables S1 and S2 using standard error propagation techniques. [1]

Discussion

Extent to which Cα proton transfer transition states are rate-limiting. One could obtain the increases in substrate KIEs observed here for ^{D}AR if the isotope sensitive step(s) were partially rate-limiting for ^{H}AR and became substantially more rate-limiting for ^{D}AR. We can exclude this possiblity based on the fact the KIEs reported here for ^{H}AR are identical within error to the intrinsic KIEs obtained previously from isotopic free energy profiles.[2] They are also identical within error to calculated values for the observed KIEs based on the intrinsic KIEs, reported rate constants, and equations based on a model

where isotope exchange at the quinonoid intermediate is rapid.[2,3] These self-consistent data show that the increases in substrate KIEs reported here are not the result of simply unmasking a partially rate-limiting, isotope sensitive step. Additionally, since KIEs are observed on Cα deprotonation in both directions, and *increase* with ^{D}AR in *both* directions, it would appear that the only possibility is for these two transition states to be jointly rate-limiting in *both* ^{H}AR and ^{D}AR.

The degree to which the transition states for proton transfer to and from Cα limit conversion of substrate to product were calculated to be 65% for proton transfer to and from the *L* face and 30% for proton transfer to and from the *D* face of the external aldimine intermediate. These values were obtained from changes in initial rates calculated via simulation with COPASI.[4,5] Initial rates were first calculated using the rate constants reported by Spies *et al.*,[2] then the energy of the transition state under consideration was reduced by 4 kcal/mol and initial rates were again calculated. Changes in initial rates were calculated for high ($1000\times K_{M;}\, k_{cat}$) and low ($0.001\times K_M$; k_{cat}/K_M) substrate concentrations.

Alternative interpretation of increased substrate KIEs with ^{D}AR. One reviewer raised the concern that the observed effects are due to "the impact of protein deuteration on the distribution of protein among its rapidly equilibrating ground state (that is, the conformational landscape is affected by protein deuteration)." This an excellent point that must be addressed.

One observation weighing against this interpretation is the extreme similarity of crystal structures determined for protiated and deuterated proteins of the same amino acid sequence. The following partial list gives the rmsd for the backbone atoms of several such pairs of structures: myoglobin, 0.18 Å (1VXG, 1CQ2), aldose reductase, 0.09 Å (1UX0, 2QXW), arginase 0.19 Å (2AEB, 2PLL), haloalkane dehalogenase 0.33 Å (2PKY, 2YXP), cytochrome P450cam 0.14 Å (1YRC, 1YRD), carbonic anhydrase 0.11 Å (2AX2, 3KS3). These small differences are within the experimental uncertainties of the structure determinations, and show that the time-averaged structures of protiated and deuterated proteins are identical.

Another important observation is that when heavy enzyme KIEs have been measured either directly on a central chemical step or under steady-state conditions where a central

chemical step is rate-limiting, the observed effect is, so far, always normal (*i.e.*, the light enzyme reacts faster than the heavy enzyme).[6-9] *A priori* there is no reason to presuppose that protein deuteration, if it alters conformational equilibria, should always disfavor the catalytically active conformational states of the protiated enzyme. That is, one might expect deuteration to randomly favor and disfavor catalytically active conformational states of protiated enzymes, depending on the specifics of the vibrational landscape (*i.e.*, enzyme) under consideration. On the other hand, if vibrational motions of the protiated enzyme are coupled to the reaction coordinate then one would expect protein deuteration generally to decrease catalytic activity due to decreases in the frequencies and amplitudes of the motions.

Rokop *et al.*[6] reported on the kinetics of protiated and deuterated *E. coli* alkaline phosphatase under conditions in which phosphoenzyme hydrolysis is rate-limiting.[10] They showed that the k_{cat} and K_M values of the two enzymes have the same temperature dependence. That is, the k_{cat} (and K_M) for protiated and deuterated enzymes have the same activation enthalpy. If the catalytically active conformational states and thereby the transition state structures were different between these enzyme forms, one would expect a difference in activation enthalpies, which is not observed.

Results from a variety of other experimental (*e.g.*, effects of remote mutations on catalysis, hydrogen tunneling in enzymes) as well as computational studies also provide evidence that protein motions are coupled to energetic barrier crossing in enzymes.[11-22] Overall, we feel that the weight of the evidence favors interpretation of the present results as due to protein motions being coupled to barrier crossing, with protein deuteration perturbing the amplitudes and frequencies of these motions thereby disfavoring barrier crossing. In our opinion, this question is not yet definitely answered and should be addressed in future studies of heavy enzyme KIEs.

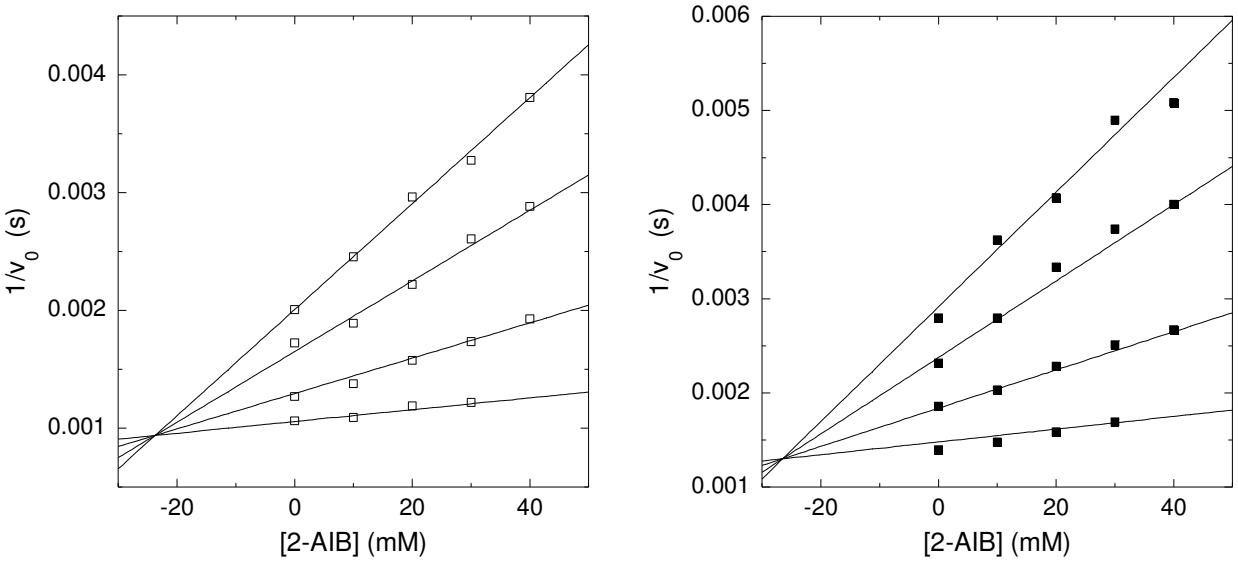
Table S1. Kinetic parameters for ^{H}AR and ^{D}AR in 50 mM potassium borate, 100 mM KCl (pH 8.9, 25 °C). Standard errors from nonlinear regression are given in parentheses.

Enzyme	**Substrate**	**k_{cat} (s^{-1})**	**K_M (mM)**	**$k_{cat}/K_M \cdot 10^{-5}$ ($M^{-1} \cdot s^{-1}$)**
^{H}AR	L-ala	1030 (20)	3.9 (0.3)	2.6 (0.2)
	D-ala	750 (10)	3.1 (0.2)	2.4 (0.2)
	[2-^{2}H]-L-ala	660 (10)	4.0 (0.3)	1.6 (0.1)
	[2-^{2}H]-D-ala	490 (10)	3.3 (0.4)	1.5 (0.2)
^{D}AR	L-ala	780 (20)	3.9 (0.3)	2.0 (0.2)
	D-ala	620 (10)	3.4 (0.2)	1.8 (0.1)
	[2-^{2}H]-L-ala	394 (9)	7.6 (0.6)	0.52 (0.04)
	[2-^{2}H]-D-ala	350 (6)	6.8 (0.4)	0.51 (0.03)

Table S2. 2-Aminoisobutyrate inhibition of ^{H}AR and ^{D}AR with L-alanine as substrate in 50 mM potassium borate, 100 mM KCl (pH 8.9, 25 °C). The data were fitted to the competitive inhibition equation. Standard errors from nonlinear regression are given in parentheses.

Enzyme	**k_{cat} (s^{-1})**	**K_M (mM)**	**K_I (mM)**
^{H}AR	1060 (20)	3.8 (0.2)	24 (1)
^{D}AR	770 (30)	4.1 (0.3)	27 (2)

Figure S1. Dixon plots for ^{H}AR (□) and ^{D}AR (■) inhibition by 2-aminoisobutyrate (pH 8.9, 25 °C). L-alanine was used as substrate at 3.3, 5, 10, 30 mM.



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