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Heavy-Enzyme Kinetic Isotope Effects on Proton Transfer in Alanine Racemase

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Supporting Information

ABSTRACT: The catalytic effects of perdeuterating the pyridoxal phosphate-dependent enzyme alanine racemase from *Geobacillus stearothermophilus* are reported. The mass of the heavy perdeuterated form is ~5.5% greater than that of the protiated form, causing kinetic isotope effects (KIEs) of ~1.3 on k_{cat} and k_{cat}/K_M for both L- and D-alanine. These values increase when Cα-deuterated alanine is used as the substrate. The heavy-enzyme KIEs of ~3 on k_{cat}/K_M with deuterated substrates are greater than the product of the individual heavy-enzyme and primary substrate KIEs. This breakdown of the rule of the geometric mean is likely due to coupled motion between the protein and the proton-transfer reaction coordinate in the rate-limiting step. These data implicate a direct role for protein vibrational motions in barrier crossing for proton-transfer steps in alanine racemase.

The role of protein motions in enzyme catalysis has been under debate over the past decade or more.[1−14] Certainly, loop and domain motions on the micro- to millisecond time scale are important in closing off active sites from bulk solvent, providing catalytically productive environments. The controversial aspect is the role of protein motions in overcoming barriers to chemical transformations in closed active sites. Once the productive enzyme−substrate complex is formed, energetic barriers from 12 to 18 kcal/mol must be traversed to form the enzyme−product complex.

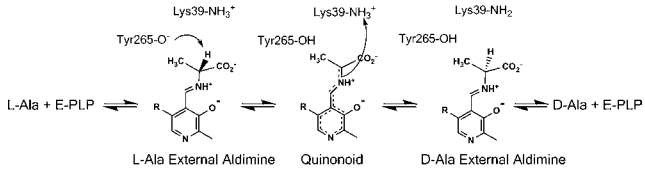
The catalytic effects of isotopically substituted (i.e., light vs heavy) enzymes can in principle address the involvement of protein motions in barrier crossing because of the theoretically addressable and minimal nature of the catalyst alteration.[15] Heavy-enzyme kinetic isotope effects (KIEs) were first measured over 40 years ago on *Escherichia coli* alkaline phosphatase under conditions where hydrolysis of the phosphoenzyme intermediate is rate-limiting.[16,17] The K_M values for the protiated and deuterated enzymes are identical and show the same temperature dependence. The k_{cat} values, on the other hand, are greater for the protiated enzyme by a factor of 1.8, yet the temperature dependence of k_{cat} is the same for both. These results were not originally interpreted in terms of protein motions and catalysis.

More recently, Schramm and co-workers measured the catalytic effects of isotopically substituted enzymes for the explicit purpose of probing the participation of protein vibrational motions in chemical barrier crossing.[18,19] Their work on purine nucleoside phosphorylase shows that the rate of nucleoside phosphorolysis is decreased by 20−27% as a result of the 10% increase in mass of the heavy enzyme.[18] Substrate KIEs for inosine were unaltered by the increased mass of the heavy enzyme, as were the steady-state kinetic parameters because of rate-limiting product release. Their work on HIV protease under conditions where chemistry is rate-limiting showed heavy-enzyme KIEs of 1.2 and 1.9 on k_{cat} and k_{cat}/K_M, respectively, due to an approximately 12% increase in enzyme mass.[19] These results imply that protein motions facilitate chemical barrier crossing in enzyme active sites.

The experiments of Schramm and co-workers employed enzymes in which the transition states probed primarily involve motions of heavy atoms, although simple hydrogen transfers between heteroatoms are involved. This may be the reason that the substrate KIEs for purine nucleoside phosphorylase are essentially identical for light and heavy enzymes even though protein motions facilitate barrier crossing. We hypothesized that enzyme-catalyzed reactions in which proton transfer is the central, rate-limiting step may show a coupling between enzyme vibrational motions and the motion of the hydrogen in the transition state, leading to nonequivalent substrate KIEs with the isotopic enzymes.

We chose alanine racemase (AR) to test this hypothesis. AR catalyzes the reversible interconversion of L- and D-alanine using pyridoxal 5′-phosphate as a coenzyme. The stepwise proton-transfer mechanism (delineated using multiple KIEs)[20] is shown in Scheme 1. Previously, we reported free energy

Scheme 1



profiles for AR at pH 6.9 and 8.9 as well as an isotopic free energy profile.[21−23] At high pH, the two proton-transfer transition states in the stepwise mechanism are jointly rate-limiting, and substrate and solvent KIEs are observed in both directions (see the Supporting Information for details).[21]

Heavy, perdeuterated AR (^{D}AR) was expressed in minimal medium in D_2O using deuterated glycerol as the carbon source (see the Supporting Information). Light AR (^{H}AR) was similarly expressed using protium in place of deuterium. The

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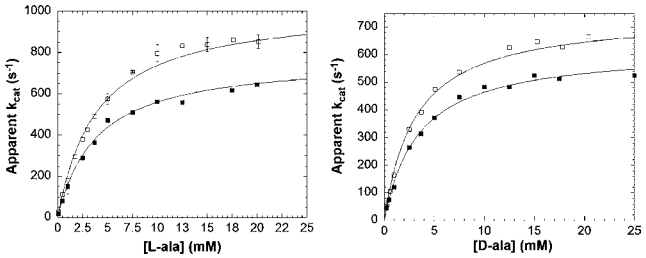

Figure 1. Michaelis–Menten kinetics for ^{H}AR (□) and ^{D}AR (■) with L-alanine (left) and D-alanine (right) (pH 8.9, 25 °C).

Table 1. Heavy-Enzyme KIEs for Alanine and [2-^{2}H]Alanine (pH 8.9, 25 °C)

substrate	$^{HE}k_{cat}$	$^{HE}(k_{cat}/K_m)$
L-Ala	1.32 ± 0.04	1.3 ± 0.1
D-Ala	1.21 ± 0.03	1.3 ± 0.1
[2-^{2}H]-L-Ala	1.67 ± 0.05	3.2 ± 0.4
[2-^{2}H]-D-Ala	1.40 ± 0.04	2.9 ± 0.4

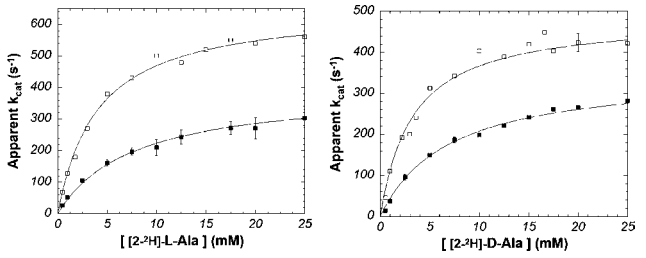

Figure 2. Michaelis–Menten kinetics for ^{H}AR (□) and ^{D}AR (■) with [2-^{2}H]-L-alanine (left) and [2-^{2}H]-D-alanine (right) (pH 8.9, 25 °C).

Table 2. Primary Substrate KIEs for ^{H}AR and ^{D}AR (pH 8.9, 25 °C)

substrate	enzyme	$^{D}k_{cat}$	$^{D}(k_{cat}/K_m)$
L-Ala	^{H}AR	1.56 ± 0.04	1.6 ± 0.2
	^{D}AR	1.98 ± 0.07	3.8 ± 0.4
D-Ala	^{H}AR	1.53 ± 0.04	1.6 ± 0.2
	^{D}AR	1.77 ± 0.04	3.5 ± 0.3

enzymes were purified to near homogeneity by identical procedures. The measured 5.5% increase in mass is identical to the theoretical value for perdeuterated enzyme with all exchangeable deuterons equilibrated with the H_2O solvent.

Michaelis–Menten saturation curves for ^{H}AR and ^{D}AR with protiated L- and D-alanine are presented in Figure 1. Heavy-enzyme KIEs on both k_{cat} and k_{cat}/K_M were detected (Table 1; the reported errors are based on the standard errors from nonlinear regression presented in Table S1 in the Supporting Information and standard error-propagation techniques[24]). They are similar in magnitude (~30%) to those observed by Schramm and co-workers on purine nucleoside phosphorylase and HIV protease.[18,19] Figure 2 shows saturation curves for ^{H}AR and ^{D}AR with Cα-deuterated L- and D-alanine, which effect primary substrate KIEs on k_{cat} and k_{cat}/K_M.[20,23] The heavy-enzyme KIEs are significantly larger with deuterated alanines than with protiated ones. The same kinetic data (Figures 1 and 2 and Table S1) can be alternatively presented as substrate KIEs (Table 2).

The larger increase in primary substrate KIEs on k_{cat}/K_M versus k_{cat} when comparing ^{H}AR and ^{D}AR raises concern regarding a potential change in substrate affinity. This was addressed by measuring the competitive inhibition constant (K_I) for 2-methylalanine, which binds to AR to form an unreactive external aldimine intermediate. Under conditions identical to those used in the KIE measurements, the K_I for ^{H}AR was 24 ± 1 mM, while that for ^{D}AR was 27 ± 2 mM (Table S2). These values are identical within experimental error and support the conclusion that the changes in K_M are due to effects other than simply lower substrate affinity for ^{D}AR. The increase in the heavy-enzyme K_M for the deuterated substrate likely originates in the substrate binding isotope effect of ~1.26 on external aldimine formation with ^{H}AR[21] and its enhancement by enzyme deuteration. The binding isotope effect is due to hyperconjugation of the Cα–H bond with the electrophilic π system of the coenzyme, and it has been independently observed with another PLP-dependent enzyme, aspartate aminotransferase.[25]

Considering the kinetic results either as heavy-enzyme KIEs (Table 1) or as substrate KIEs (Table 2) leads to the same critical conclusion: isotope effects due to enzyme deuteration or substrate deuteration are not independent of each other. This violates the rule of the geometric mean, a principle of isotope effect theory, which holds that isotope effects are independent of each other.[26,27] The parsimonious explanation for the violation of the rule of the geometric mean observed here is that the different isotopic species (enzyme and substrate) are coupled in a rate-limiting event. In the present case, the enzyme is vibrationally coupled to the motion of the proton undergoing transfer from Cα of the external aldimine intermediate to an enzymic base in the rate-limiting step. Another interpretation of these data, which we consider less viable, is discussed in the Supporting Information.

The present results are in accord with the concepts developed by Schwartz and co-workers.[1,28−34] They advocate the idea that protein motions on the femto- to picosecond time scale can be directly involved in barrier crossing through transient formation of high-energy active-site structures highly favorable to reaction (i.e., that allow bond making/breaking to occur with a low energetic barrier). Slower protein motions are also important for the formation of these transient active-site structures via larger-amplitude fluctuations (i.e., conformational changes) on longer time scales, but the higher-frequency motions can potentially be more strongly coupled vibrationally to the reaction coordinate.

This picture is satisfying on many levels and does not demean the important role of electrostatics. The unimolecular enzyme–substrate complex is formed from multiple relatively strong interactions between the two molecules. Hydrogen-bonding, hydrophobic, and electrostatic interactions would prevent the active-site-bound substrate from becoming vibrationally hot (as required for barrier crossing if protein motions are not involved) relative to the protein structure by facilitating rapid vibrational energy redistribution. The Schwartz ideas avoid this conundrum since much of the activation energy is distributed into protein vibrations. These coordinately act to provide a relatively high energy active-site structure that allows bond making/breaking to occur with a low barrier (i.e., the substrate does not have to become vibrationally hot in the midst of a relatively cold protein). This is analogous to the picture painted by Hynes and co-workers for S_N2 reactions in water, where a substantial portion of the activation energy goes into preorganization of the polar solvent shell to a high-energy

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structure that allows nucleophilic substitution to occur with a low intrinsic barrier within it.[35−38]

In view of the small KIEs and the vibrational coupling proposed here, the present results must be considered within the context of hydrogen tunneling.[39,40] Quantum mechanics/molecular mechanics studies have shown that proton transfers between Cα of alanine and either Lys39 or Tyr265 are very nearly symmetric, yet the intrinsic KIEs are small (1.66 ± 0.09 in the L → D direction and 1.57 ± 0.05 in the D → L direction).[20,21,23,41] Huskey has discussed the role of coupled motions and hydrogen tunneling in the breakdown of the rule of the geometric mean.[26] Although the present results are not conclusive, they suggest that tunneling may occur in the AR-catalyzed reaction and that protein motions may be involved in promoting proton tunneling between Cα and either Tyr265 or Lys39.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, tables of kinetic parameters, and inhibition data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Schwartz, S. D.; Schramm, V. L. *Nat. Chem. Biol.* **2009**, *5*, 551.
(2) Zhang, J.; Klinman, J. P. *J. Am. Chem. Soc.* **2011**, *133*, 17134.
(3) Adamczyk, A. J.; Cao, J.; Kamerlin, S. C.; Warshel, A. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 14115.
(4) Klinman, J. P. *Nat. Chem.* **2010**, *2*, 907.
(5) Kamerlin, S. C.; Sharma, P. K.; Chu, Z. T.; Warshel, A. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 4075.
(6) Kamerlin, S. C.; Warshel, A. *J. Phys. Org. Chem.* **2010**, *23*, 677.
(7) Henzler-Wildman, K. A.; Thai, V.; Lei, M.; Ott, M.; Wolf-Watz, M.; Fenn, T.; Pozharski, E.; Wilson, M. A.; Petsko, G. A.; Karplus, M.; Hubner, C. G.; Kern, D. *Nature* **2007**, *450*, 838.
(8) Henzler-Wildman, K. A.; Lei, M.; Thai, V.; Kerns, S. J.; Karplus, M.; Kern, D. *Nature* **2007**, *450*, 913.
(9) Klinman, J. P. *Philos. Trans. R. Soc. London, Ser. B* **2006**, *361*, 1323.
(10) Hammes-Schiffer, S.; Benkovic, S. J. *Annu. Rev. Biochem.* **2006**, *75*, 519.
(11) Gao, J.; Ma, S.; Major, D. T.; Nam, K.; Pu, J.; Truhlar, D. G. *Chem. Rev.* **2006**, *106*, 3188.
(12) Garcia-Viloca, M.; Gao, J.; Karplus, M.; Truhlar, D. G. *Science* **2004**, *303*, 186.
(13) Benkovic, S. J.; Hammes-Schiffer, S. *Science* **2003**, *301*, 1196.
(14) Bruice, T. C. *Acc. Chem. Res.* **2002**, *35*, 139.
(15) Phillipson, P. E. *J. Mol. Biol.* **1968**, *31*, 319.
(16) Rokop, S.; Gajda, L.; Parmerter, S.; Crespi, H. L.; Katz, J. J. *Biochim. Biophys. Acta* **1969**, *191*, 707.
(17) Han, R.; Coleman, J. E. *Biochemistry* **1995**, *34*, 4238.
(18) Silva, R. G.; Murkin, A. S.; Schramm, V. L. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 18661.
(19) Kipp, D. R.; Silva, R. G.; Schramm, V. L. *J. Am. Chem. Soc.* **2011**, *133*, 19358.
(20) Spies, M. A.; Toney, M. D. *Biochemistry* **2003**, *42*, 5099.
(21) Spies, M. A.; Toney, M. D. *J. Am. Chem. Soc.* **2007**, *129*, 10678.
(22) Spies, M. A.; Woodward, J. J.; Watnik, M. R.; Toney, M. D. *J. Am. Chem. Soc.* **2004**, *126*, 7464.
(23) Sun, S.; Toney, M. D. *Biochemistry* **1999**, *38*, 4058.
(24) Bevington, P. R. *Data Reduction and Error Analysis for the Physical Sciences*; McGraw-Hill: New York, 1969.
(25) Griswold, W. R.; Castro, J. N.; Fisher, A. J.; Toney, M. D. *J. Am. Chem. Soc.* **2012**, *134*, 8436.
(26) Huskey, W. P. *J. Phys. Org. Chem.* **1991**, *4*, 361.
(27) Bigeleisen, J. *J. Chem. Phys.* **1955**, *23*, 2264.
(28) Dametto, M.; Antoniou, D.; Schwartz, S. D. *Mol. Phys.* **2012**, *110*, 531.
(29) Quaytman, S. L.; Schwartz, S. D. *J. Phys. Chem. A* **2009**, *113*, 1892.
(30) Saen-Oon, S.; Quaytman-Machleder, S.; Schramm, V. L.; Schwartz, S. D. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 16543.
(31) Saen-Oon, S.; Schramm, V. L.; Schwartz, S. D. *Z. Phys. Chem.* **2008**, *222*, 1359.
(32) Quaytman, S. L.; Schwartz, S. D. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 12253.
(33) Basner, J. E.; Schwartz, S. D. *J. Am. Chem. Soc.* **2005**, *127*, 13822.
(34) Antoniou, D.; Abolfath, M. R.; Schwartz, S. D. *J. Chem. Phys.* **2004**, *121*, 6442.
(35) Gertner, B. J.; Whitnell, R. M.; Wilson, K. R.; Hynes, J. T. *J. Am. Chem. Soc.* **1991**, *113*, 74.
(36) Gertner, B. J.; Wilson, K. R.; Hynes, J. T. *J. Chem. Phys.* **1989**, *90*, 3537.
(37) Gertner, B. J.; Bergsma, J. P.; Wilson, K. R.; Lee, S. Y.; Hynes, J. T. *J. Chem. Phys.* **1987**, *86*, 1377.
(38) Bergsma, J. P.; Gertner, B. J.; Wilson, K. R.; Hynes, J. T. *J. Chem. Phys.* **1987**, *86*, 1356.
(39) Nagel, Z. D.; Klinman, J. P. *Chem. Rev.* **2006**, *106*, 3095.
(40) Nagel, Z. D.; Klinman, J. P. *Chem. Rev.* **2010**, *110*, PR41.
(41) Major, D. T.; Gao, J. *J. Am. Chem. Soc.* **2006**, *128*, 16345.