Comparative kinetic isotope effects on first- and second-order rate constants of soybean lipoxygenase variants uncover a substrate-binding network

Shenshen Hu,1,4,* Adam R. Offenbacher,1,3,4 Edbert D. Lu1,4,* and Judith P. Klinman1,2,4,*

From the 1Department of Chemistry, University of California, Berkeley, California 94720; 2Department of Molecular and Cell Biology, University of California, Berkeley, California 94720; 3Department of Chemistry, East Carolina University, Greenville, North Carolina 27858; 4California Institute for Quantitative Biosciences (QB3), University of California, Berkeley, California 94720

Running title: Kinetic isotope effects identify substrate binding networks

ABSTRACT

Lipoxygenases are widespread enzymes found in virtually all eukaryotes, including fungi, and, more recently, also in prokaryotes. These enzymes act on long-chain polyunsaturated fatty acid substrates (C18 to C20), raising questions regarding how the substrate threads its way from solvent to the active site. Herein, we report a comparison of the temperature dependence of isotope effects on first- and second-order rate constants among single-site variants of the prototypic plant enzyme soybean lipoxygenase-1 (SLO) substituted at amino acid residues inferred to impact substrate binding. We created 10 protein variants including four amino acid positions, Val-750, Ile-552, Ile-839, and Trp-500, located within a previously proposed substrate portal. The conversion of these bulky hydrophobic side chains to smaller side chains is concluded to increase the mobility of flanking helices, giving rise to increased off rates for substrate dissociation from the enzyme. In this manner, we identified a specific "binding network" that can regulate movement of the substrate from the solvent to the active site. Taken together with our previous findings on C-H and O2 activation of SLO, these results support the emergence of multiple complementary networks within a single protein scaffold that modulate different steps along the enzymatic reaction coordinate.

Introduction

Understanding how enzymes work, especially with their vast rate enhancements of up to 1026-fold over uncatalyzed reactions, has been a continuous endeavor for enzymologists (1). Ever since the introduction of the Michaelis-Menten equation over 100 years ago, enzyme kinetic analysis has played a critical role in revealing the catalytic mechanisms of enzymes (2). Beginning in the 1970s, the integration of kinetic isotope effects (KIEs) into the study of enzymes expanded the scope and insight derived from the classic Michaelis-Menten equation (3,4). Some of the major applications of KIEs have been to determine the nature of rate determining steps and to infer the nature of the chemical processes occurring within the enzyme active site (4-9). Most recently, in combination with various biophysical probes, patterns in KIEs at varied pressure and temperature have been utilized to investigate the link of protein motions to the chemical steps of catalysis (10-13).

Lipoxygenases are found to be widespread in plants, animals, fungi, and prokaryotes; the plant enzymes initiate the production of a spectrum of
fatty acid hydroperoxides with key physiological roles in germination, growth, and senescence as well as a defense response to pathogens (14), (e.g. (15)). One such enzyme, soybean lipoxygenase (SLO) has emerged as a paradigmatic system for investigating enzymatic C-H activation reactions (16-20). Extensive kinetic studies of SLO under steady state conditions have revealed a classic “ping-pong” mechanism in which a proton and electron are first irreversibly removed from one of its physiologically relevant substrates, linoleic acid (LA), by a non-heme iron center Fe(III)-OH, followed by the trapping of the substrate-derived radical by molecular oxygen (21). The C-H activation step has been concluded to dominate \( k_{\text{cat}} \) at all temperatures, supported by the large (ca.~80) and nearly temperature-independent \( D_{k_{\text{cat}}} \) values for wild type (WT) SLO (16,17). Further, it has been possible to link the properties of \( k_{\text{cat}} \) to both local and global motions within the protein by comparing the properties of WT enzyme to a suite of mutations targeted at active site hydrophobic side chains (L546, L754, I553). The temperature-dependence of \( D_{k_{\text{cat}}} \) has been shown to be particularly informative (17-20,22-24), indicating a more temperature dependent \( D_{k_{\text{cat}}} \) as the side chain bulk at each of these position is reduced. A mechanistic interpretation invokes a role for global conformational sampling in WT-SLO that leads to a precise alignment between the H donor (substrate) and acceptor (Fe(III)-OH). This property becomes compromised upon the decrease in volume at key active site residues, facilitating an onset of distance sampling along the H donor and acceptor coordinate to recapture the tunneling-ready distance characteristic of WT-SLO, and an almost constant \( D_{k_{\text{cat}}} \) (17-19,23-25). The temperature-dependence of \( k_{\text{cat}} \) has also been identified as a possible indicator of perturbation-induced changes in global conformational sampling, in particular any change in the distribution of catalytically active protein substates (22,26). Note that the aforementioned kinetic investigations of SLO and their corresponding implications have been mainly focused on the first-order rate constants \( (k_{\text{cat}}) \) and related kinetic isotope effect value \( (D_{k_{\text{cat}}}) \), that fully represent the chemical transformation chemical step, Scheme 1, where \( k_{\text{cat}} = k_{\text{chem}} \) (27).

Despite the emerging physical pictures that increasingly provide a resolved role for (local and global) motions that lead to productive reaction barrier crossings via hydrogen tunneling, a physical understanding of how SLO acquires and binds its long chain fatty acid substrates has remained enigmatic (28,29). One of the major reasons is a hitherto inability to obtain X-ray structures of an enzyme-substrate complex (either with the natural substrate linoleic acid or a substrate analogue). A straightforward interpretation of kinetic isotope effects on the second order rate constant \( (D_{k_{\text{cat}}}/K_{\text{m}}) \) can be challenging in comparison with \( D_{k_{\text{cat}}} \), due to the often complicating features of both substrate binding and subsequent catalytic steps contributing to \( k_{\text{cat}} \). As depicted in Scheme 1 for SLO, substrate binding can be conceptualized as an initial diffusional encounter (represented by \( k_{\text{on}} \) and \( k_{\text{off}} \)), followed by substrate moving into and reorganizing within the long substrate binding channel (represented by \( k_{i} \) and \( k_{i} \)). \( k_{\text{cat}}/K_{\text{m}} \) reflects all steps before the first irreversible step and, thus, includes the initial substrate binding (E+S \( \rightleftharpoons E\cdot S \) ), movement/reorientation of substrate after the initial substrate binding (E:S \( \rightleftharpoons E\cdot S' \)) and the chemical step (E:S' \( \rightarrow E\cdot P \)). Since the chemical step alone limits \( k_{\text{cat}} \), \( k_{i} \) will be slower than \( k_{i} \) (leading to the stable E:S’ complex in Scheme 1). Under the boundary conditions for \( k_{\text{cat}}/K_{\text{m}} \) and \( k_{\text{cat}} \) illustrated in Scheme 1, the observed off rate for substrate release, \( k_{\text{off}} \), becomes equal to \( k_{i} \). The isotope effect on \( k_{\text{cat}}/K_{\text{m}} \), \( D_{k_{\text{cat}}}/K_{\text{m}} \), is shown in its generic form in Eq (1) and can be seen to be a function of \( D_{k_{\text{cat}}} \), \( k_{\text{chem}} \) and \( k_{\text{off}} \), in which \( k_{\text{chem}} \) and \( k_{\text{off}} \) refer to the rate constants for the chemical step and the release of bound substrate back into solution, respectively (27,30).

\[
D(k_{\text{cat}}/K_{\text{m}}) = (D_{k_{\text{cat}}} + k_{\text{chem}}/k_{\text{off}})/(1+k_{\text{chem}}/k_{\text{off}}) \quad (\text{Eq 1})
\]

The \( D_{k_{\text{cat}}}/K_{\text{m}} \) values of WT SLO have been found to vary with temperature, changing from values much smaller than \( D_{k_{\text{cat}}} \) at low temperature (<32°C) to a value approximating \( D_{k_{\text{cat}}} \) as the temperature is increased (27). As demonstrated by accompanying studies of viscosity, solvent isotope effects and a pH profile at low temperature (<32°C), the substrate diffusion to the active site, a subsequent rearrangement of pre-associated substrate, and the chemical C-H activation are all partially rate limiting for proto-LA (H-LA), whereas the C-H activation becomes fully rate-
Kinetic isotope effects identify substrate binding networks

limiting for the much slower perdeutero-LA (D-LA). Significantly, the addition of substrate mimics such as oleic acid (OA) and oleyl sulfate (OS) lead to an increase in $\frac{d_{\text{cat}}}{K_{\text{cat}}}$ at 5°C, approaching the value of $d_{\text{cat}}$; this result has implicated a regulatory site that causes the substrate to undergo much rapid dissociation (31). Similarly, in the absence of allosteric effectors, the C-H activation step also begins to fully dominate the $k_{\text{cat}}/K_{\text{m}}$ values for both H-LA and D-LA (>32°C) (27). These different behaviors of the magnitude and temperature sensitivity of $d_{\text{cat}}$ and $k_{\text{cat}}/K_{\text{m}}$ are rich in mechanistic information. However it is extremely difficult to tease out a meaningful physical model based solely on the data for WT SLO.

In the present study, results are provided that extend the investigation of $d_{\text{cat}}/K_{\text{m}}$ to a series of SLO variants that were predicted to have a possible impact on the efficiency of substrate release and/or binding to enzyme. As shown herein, while the kinetic features of certain SLO variants are essentially the same as WT, for a range of mutants the $d_{\text{cat}}/K_{\text{m}}$ value has become insensitive to changes in temperature. Four mutants, V750A, I552A, I839A and W500F, are of particular interest, due to their generation of greatly elevated values for $k_{\text{cat}}$ relative to WT. These four residues are located proximal to two helices previously proposed to be important structural elements within the substrate portal (32,33). These new data provide strong support for the previously defined substrate entry point, as well as identifying a new long range network that modulates the movement of substrate to or from the solvent interface. The data highlight the valuable information content inherent to KIEs reflected in $k_{\text{cat}}/K_{\text{m}}$, and offer a new tool for understanding substrate binding pathways.

Results

WT SLO and ten other variants, either bordering the proposed substrate binding site (W500F, I538A, I552A, I553A, L546A, V750A, L754A, I553A and I839A) (28) or at the predicted substrate entrance portal (E256A) (34), were chosen for the current investigation. The values and temperature dependence of $k_{\text{cat}}$ and $d_{\text{cat}}$ were obtained through steady state kinetics assay with protio-linoleic acid (H-LA) and perdeutero-linoleic acid (D-LA) between 10°C and 45°C using a continuous spectroscopic assay, in which the formation of the product hydroperoxide is monitored at 234 nm (Experimental Procedure). Uncertainty in the precise substrate concentration in individual UV-Vis measurements of H-LA and D-LA can lead to errors in fitted $k_{\text{cat}}/K_{\text{m}}$ value and $d_{\text{cat}}/K_{\text{m}}$ values. To overcome this limitation, a competitive assay (Experimental Procedures) involving HPLC was used to measure the $d_{\text{cat}}/K_{\text{m}}$ at different temperatures. The final $k_{\text{cat}}$, $d_{\text{cat}}$ and $d_{\text{cat}}/K_{\text{m}}$ values at the lowest (10°C) and close to highest temperature (40°C) for WT and ten mutants are summarized in Table 1.

Inspection of $d_{\text{cat}}/K_{\text{m}}$ and $d_{\text{cat}}$ as a function of temperatures indicates three unique trends. The representative SLO in trend I is WT (Figure 1A), where $d_{\text{cat}}/K_{\text{m}}$ values rise from being much smaller than $k_{\text{cat}}$ at 10°C towards $k_{\text{cat}}$ with the increasing temperature. The data generated from the current study is in alignment with previous reports from this laboratory and Holman and co-workers (27,35). E256A and R707A behave essentially the same as WT in terms of the temperature effects on $d_{\text{cat}}$ and $d_{\text{cat}}/K_{\text{m}}$ (Table 1). The variant I553A also appears to conform to Trend 1, with the caveat that the isotope effect on $k_{\text{chem}}$ has become much more temperature dependent, which will dampen any increase in $d_{\text{cat}}/K_{\text{m}}$ that is due to changes in rate determining steps. A clear divergence of behavior is seen for L546A, L754A and I538A, for which the $d_{\text{cat}}$ values decrease with temperature and the $d_{\text{cat}}/K_{\text{m}}$ is almost temperature independent (Trend II) (see Figure 1B). These are due to a decrease in $d_{\text{cat}}$ that is accomplished by a faster increase in $k_{\text{off}}$ than $k_{\text{chem}}$ at elevated temperature (Eq 1). The final pattern, revealed for V750A, I552A, I839A and W500F, is of special interest as both $d_{\text{cat}}$ and $d_{\text{cat}}/K_{\text{m}}$ are nearly temperature-independent and virtually identical (Trend III), Figure 1C, 1D. In this instance the temperature independence of $d_{\text{cat}}/K_{\text{m}}$ can be directly attributed to a relatively small contribution of $k_{\text{chem}}/k_{\text{off}}$ (Eq 1).

The previous study of WT suggested a shift in the rate-limiting step toward the chemical step, $k_{\text{chem}}$, as the explanation for the elevation of $d_{\text{cat}}/K_{\text{m}}$ with increasing temperature (27). To investigate whether the substrate diffusion step (E+S $\rightleftharpoons$ E-S) remains partially rate-limiting in the Trend II and Trend III behaviors, the viscosity dependence experiments were performed on representative SLOs from Trend II (L546A) and Trend III (V750A, I552A), pH=9.0.
Kinetic isotope effects identify substrate binding networks

at 20°C. The viscosigen effects on WT SLO were also re-investigated as a control. As seen in Figure 2, for WT SLO, the $k_{cat}/K_m$ decreased dramatically as the relatively viscosity increased, indicating a 50% diffusion control, consistent with previous studies (27,35). In sharp contrast, the $k_{cat}/K_m$ values in L546A, V750A and I552A are weakly sensitive or independent of the increasing viscosity, indicating that the diffusion of substrate to enzyme is not significantly rate-limiting for these variants.

The kinetic parameters for WT SLO and the targeted mutants were further analyzed with H-LA as substrate in H2O and D2O (20°C) to assess whether a solvent-dependent step, that is distinct from $k_{cat}$, has been ascribed to $k_i$ and $k_1$ (Scheme 1) (27), would be partially rate limiting. The values reported in Table 2 were obtained under pH conditions where the rates for $k_{cat}$ and $k_{cat}/K_m$ have plateaued, avoiding potential complications from an impact of D2O on pKa (27). The reported data are corrected for the small impact of D2O on solvent viscosity (See Experimental Procedures). WT SLO shows a very small solvent isotope effect (SIE) on $k_{cat}$ (close to unity) and a larger value on $k_{cat}/K_m$, consistent with the previous investigation(27). For comparison, the impact of L546A, I552A, V750A on the SIE for $k_{cat}/K_m$ appears to be within experimental error relative to WT, implying that the change in the kinetic features of $\delta k_{cat}/K_m$ in these three mutants is unrelated to a solvent-dependent step.

In order to gain a more quantitative measure of the observed effects, changes in the commitment factor and $k_{cat}$ (Eq 1) were calculated where relevant (Table 3). We note that decreases in computed $k_{off}$ values for substrate release can be accompanied by even greater decreases in $k_{cat}$ values. For example, in comparison to WT, the active site mutants L546A and L754A demonstrated 60-fold and 675-fold decreases in the $k_{cat}$ in comparison to 180- and 2000-fold reductions for the C-H activation step. Thus, the commitment value, $k_{chem}/k_{off}$, provides a more accurate assessment of the shift in rate limiting steps. As shown in Table 3, all seven mutants in Trend II and Trend III demonstrate a decreased commitment compared to WT, implicating a more efficient substrate release process in relation to changes in the C-H activation step. In particular, Trend III mutants (V750A, I552A, W500F and I839A) show smaller commitment values than Trend II mutants (L546A, L754A, I538A), and an increased $k_{off}$ value relative to WT despite the 2-4 fold decrease in $k_{cat}$.

Discussion

The synergistic analysis of mutagenesis, viscosity, and solvent and substrate isotope effects on $k_{cat}/K_m$ and $k_{cat}$ is able to tease apart the changes in rate limitation arising from the initial substrate binding step (E+S $\rightleftharpoons$ E·S), a subsequent reorientation/isomerization of the substrate (E·S $\rightleftharpoons$ E·S') and the chemical step (ES' $\rightarrow$ EP), Scheme 1. While the contribution from D2O-related steps ($k_i$ and $k_1$ in Scheme 1) is essentially the same in all the mutants, the substrate diffusion step ($k_{cat}$) is significantly less rate-limiting in the single mutants tested (L546A, V750A, and I552A). The comparison between $\delta k_{cat}/K_m$ and $\delta k_{cat}$ further indicates distinct kinetic features underlying the three mutants. In the cases of V750A and I552A, the almost identical $\delta k_{cat}$ and $\delta k_{cat}/K_m$ at low temperature supports the conclusion that the C-H bond cleavage is the primary rate-limiting step for both $k_{cat}$ and $k_{cat}/K_m$. In contrast, the $\delta k_{cat}/K_m$ value of L546A is half of the $\delta k_{cat}$ value at 10°C, clearly showing the presence of at least one additional kinetically limiting step beyond the substrate binding and chemical step.

The commitment factors calculated from the $\delta k_{cat}/K_m$ and $\delta k_{cat}$ values are able to quantify the shifting of the rate-limiting step in $k_{cat}/K_m$ for SLO variants (Table 3). The commitment value in WT was calculated as 2.1 at low temperature, where the rate-limitation is comprised of both C-H activation and substrate binding/release steps; the three mutants that demonstrate Trend I kinetic features (E256A, R707A, and I553A) all show increased commitment factors, a result of $k_{off}$ decreasing more than $k_{chem}$. Conversely, the commitment factors in the Trend II and Trend III mutants all decrease (by 2-20 times in comparison to the WT). The smaller commitment factors in Trend II and Trend III relative to WT are consistent with the viscosity dependence results that indicate a decrease in rate limitation from the substrate diffusion step (cf. Figure 2 for the representative Trend II mutant L546A and Trend III mutants V750A and I552A). Note that the commitment factors of the Trend III mutants are in general smaller than the Trend II mutants (Table 3), implying that the chemical step
partially rate-limiting in Trend II mutants. The configuration(s) (E·S_catalytically competent and tight bending initially formed ‘inactive’ complex to a that the reorientation of the substrate from its release and C-H activation. Given the absence of a vicosigen effect for L546A (Figure 2), we propose that the reorientation of the substrate from its initially formed ‘inactive’ complex to a catalytically competent and tight bending configuration(s) (E·S ⇌ E·S’) may have become partially rate-limiting in Trend II mutants. The previously generated double mutant of SLO (L546A and L754A) demonstrated the importance of a substrate reorientation steps, likely a consequence of the very significant increase in the active site cavity that gives rise to unfavorable initial binding of the substrate.(39,40). When taken together, the kinetic parameters of the double mutant that include a severely impaired \( k_{\text{chem}} \), an almost temperature-independent \( \frac{\Delta k_{\text{cat}}}{\Delta K_m} \) value around 30-40 and a huge \( \frac{\Delta k_{\text{cat}}}{\Delta K_m} \) value are also indicative of a reduced \( k_{\text{off}} \) (30,41).

The fact that the Trend III mutants, V750A, I552A, W500F and I839A, demonstrate a large increase in \( k_{\text{off}} \) values (Table 3) is of particular interest. As discussed above, residues at these four positions lie along the postulated substrate binding pocket (28). Through a detailed examination of the WT SLO structure, we observe a large number of hydrophobic interaction between these four side chains on helix 2 (residues 255-275) and 11 (residues 535-545)(cf. Figure 3A): side chains V750 and I552 are in van der Waals contact with L262 and S263, correspondingly (Figure 3B); the side chain I839 is anchored in the middle of L255 and I538; W500 is adjacent to residue A542, which is also located on helix 11 (Figure 3C). The early studies on SLO had proposed that substrate enters the binding site through a gap between the helix 11 and helix 2. As shown from the present studies, reducing the residue size at V750, I552, I839 or W500 causes a ‘loosening up’ of the substrate portal, facilitating substrate binding (Figure 2) and release (Table 3). It is worth noting that the single mutant at the substrate entrance (E256A on helix 2) does not increase the \( k_{\text{off}} \) value, highlighting the key roles for more deeply buried residues in controlling both \( k_{\text{off}} \) and the precision of substrate binding. The role of I538 on helix 11 remains somewhat enigmatic since conversion to Ala reduces \( k_{\text{chem}} \) by only 40% while \( k_{\text{off}} \) remains the same as WT; in this instance the bulk of the site chains at positions 500 and 839 may play a dominant role in the preservation of structure.

The kinetic observations for Trend III mutants are congruent with the finding of a previously described allosteric effect for WT SLO (31,42,43). In particular, the addition of OA (mixed inhibitor) or OS (allosteric effector only), results in alleviation of the substrate binding/reorientation steps as partial rate-determining steps [i.e. \( \frac{\Delta k_{\text{cat}}}{\Delta K_m} \) \( \equiv \frac{\Delta k_{\text{cat}}}{\Delta \text{chem}} \) at low substrate concentration and reduced
Kinetic isotope effects identify substrate binding networks

Kinetic analysis supports a \( k_{\text{off}} \) value that is approximately 60-fold faster in the presence of allosteric effector OS. HDX-MS analysis of the interactions of OS, which binds tightly (\( K_D \sim 0.6 \mu M \)) to an allosteric site and not in the active site substrate cavity of WT SLO, identified peptides within the N-terminal PLAT domain that had altered HDX properties. The implied conformational change is accompanied by a resolved network of peptides that exhibit enhanced peptidyl flexibility emanating from the PLAT domain to the substrate binding portal (including helices 2 and 11). Thus, enhanced peptide flexibility within helices 2 and 11 also provides a structural basis for increased binding and release rates that arise from the addition of an allosteric effector.

Another recent temperature-dependent hydrogen-deuterium exchange study, comparing WT to I553X and L546A SLO variants, has implicated a role for V750 and I552 within a catalytically-linked protein network for communication between the HDX-identified thermally activated remote loop 317-334, and the active site (Figure 3A,B). Site-specific mutations within this remote loop have been shown to directly influence the enthalpic barrier for the chemical C-H bond activation step. This current study together with the previous investigations of SLO, indicates the existence of distinct protein networks that modulate substrate binding vs. the subsequent bond cleavage steps. These networks consist of residues throughout separate regions of the protein scaffold, intersecting at V750 and I552 (Figure 3).

Conclusion

The observed kinetic changes for ten SLO mutants highlight the information content inherent in \( k_{\text{cat}}/K_m \) regarding the mode of substrate binding in SLO. The origins of the more rate-limiting chemical steps for \( k_{\text{cat}}/K_m \) in these mutants are seen to arise alternately from: (i) impairment of the precise substrate orientation in the binding site that leads to decreased rate constants of the initial chemical step, and (ii) increased flexibility of the substrate portal that increases the substrate-releasing step(s) in the case of V750A, I552A, I839A and W500F. Significantly, these studies are able to define a network within SLO that proceeds from the surface substrate portal to the iron cofactor at the active site, though at the current level of understanding we are unable to describe the details of the substrate movement and positioning within its binding channel. This work, that is based on classical enzymatic kinetic analyses, both deepens our understanding of where substrate binds and establishes a molecular framework for further structure function investigations. These studies are especially compelling, given the decades of effort that have failed to yield direct X-ray structural information regarding the E-S complex of SLO.

Experimental Procedures

General Information. All reagents were purchased from commercial sources and used without further purification unless otherwise indicated. Linoleic acid (>99% purity) (H-LA) was purchased from Sigma-Aldrich and purified again before usage. Perdeuterio-Linoleic Acid (d\textsubscript{31}-LA) for the competitive KIE measurements was isolated from the algal fatty acid mixture (Cambridge Isotope Laboratories, methyl esters U-D 97-98%, DLM-2491-0).

Mutagenesis, Expression, and Purification of the SLO double mutants. Each lipoxygenase was expressed and purified as described previously (20,41). Mutants were prepared following the Stratagene QuikChange Lightning protocol starting from the WT plasmid. The mutant plasmids were isolated and the mutants confirmed by sequencing with three different primers that targeted different regions of the gene: the beginning of the gene, a 500-bp region of the gene containing the mutation site(s) and a region that covers 500-bp up to the end of the gene.

Kinetic Measurement. Steady-state kinetics were performed on a Cary50 spectrophotometer in the single wavelength mode as previously described (20) (Table S1-S4, S6-S11).

Solvent Viscosity Studies. The impact of viscosity on WT SLO, I552A, V750A, L546A was determined at different relative viscosities (\( \eta_r = \eta/\eta^0 \), \( \eta^0 \) is the viscosity of H\textsubscript{2}O at 20°C) as previously described (27). A buffer solutions of 0, 8, 14, 21.5, 26 and 30% by weight glucose, in 0.1 M CHES buffer, pH = 9.0, were prepared with corresponding relative viscosities of 1, 1.25, 1.5, 2.0, 2.5 and 3.0 at 20°C. Enzymatic measurements at 20°C were the same as described above. The enzymatic activities of WT SLO and three single
mutants in 0.1 M CHES (pH = 9.0) were found to be similar to those measured in 0.1 M borate buffer (pH = 9.0).

**Solvent Isotope Effects.** Solvent isotope effects for WT SLO, I552A, L546A were obtained by comparing the kinetic parameters for protio-linoleic acid at 20°C in 0.1 M borate (pH = 9.0) in D₂O (pH meter reading at 8.6) with those in H₂O (pH = 9.0). Since the relative viscosity of heavy water is 1.25, the solvent isotope effects on $k_{cat}/K_M$ needed to be corrected.

**Isolation and Purification of Perdeutero-Linoleic acid (d₃₁-LA) from an Algal Fatty Acid Ester Mixture.** The algal fatty acid ester mixer (100 µL) was first de-esterified with 0.35g NaOH in ethanol (10 mL) /H₂O (3.5 mL). After complete hydrolysis, the mixture was acidified by adding 65 mL H₂O and 1.5 mL acetic acid followed by extraction with CH₂Cl₂ (5 mL × 4 times). The extracted organic layer was dried to an oil, dissolved in methanol and purified via RP-HPLC using Phenomenex semipreparative HPLC column (Luna C18, 100A, 250 mm × 10.00 mm, 5 µM) via isocratic elution (87.9% methanol, 12% H₂O and 0.1% acetic acid) at flow rate of 3 mL/min. The elution containing perdeutero-LA (with protio-LA contamination) were collected, evaporated to oil, and further enzymatically treated with WT SLO to deplete the trace amount of protio-LA contamination (monitored in the spectrophotometer, to ensure at least 10% substrate depletion) and then acid, extracted, and dried as before. The post enzymatic purified perdeutero-LA was dissolved in methanol and purified again via the semipreparative HPLC column via the same condition. The RP-HPLC purified perdeutero-LA was evaporated to dryness, dissolved in methanol and stored at -80°C.

**Competitive Kinetic Isotope effect Measurement.** The competitive KIEs [$\%k_{cat}/K_m$] for WT SLO, I552V, V750A, I538A, I839A and W500F in 0.1 M borate (pH = 9.0) between the temperature 10 - 40°C were determined as previously described with some minor changes (30,44). A known ratio of RP-HPLC-purified protio- and perdeutero-LA (1:8; total concentration of the substrate, 10 µM) was allowed to react with individual SLO variants at the appropriate temperature. The reaction was monitored at 234 nm and stopped with acetic acid quench (final acetic acid concentration in the mixture of ~5%) at less than 3% total substrate consumption. The acidified reaction mixture is extracted with methylene chloride, evaporated to dryness, dissolved in methanol, and injected onto an analytic C18 column (Agilent, Purslit XRs 5 C18, 250 × 4.6 mm) and eluted at 1 mL/min with an isocratic mobile phase of 79.4% methanol, 21.5% H₂O and 0.1% acetic acid. The molar protio/per-deutero 13-HPOD ratios are equated to the corresponding peak area ratios and the competitive KIE ($\%k_{cat}/K_m$) is calculated as $\frac{([P-H]/[P-D])_+}{([P-H]/[P-D])_-}$, where $([P-H]/[P-D])_+$ is the ratio of the integrated peak areas at <3% reaction conversion and $([P-H]/[P-D])_-)$ is the ratio of peak areas at complete conversion.

**Conflict of Interest:** The authors declare that they have no conflicts of interest with the contents of this article.

**References**
1. Wolfenden, R., and Snider, M. J. (2001) The depth of chemical time and the power of enzymes as catalysts. Acc. Chem. Res. 34, 938-945
2. Michaelis, L., and Menten, M. L. (1913) The kinetics of the inversion effect. Biochemistry Z 49, 333-369
3. Cleland, W. W., O'Leary, M. H., and Northrop, D. B. (1977) Isotope effects on enzyme-catalyzed reactions; [proceedings of the Sixth Annual Harry Steenbock Symposium, held in Madison, Wisconsin, on June 4 and 5, 1976], University Park Press, Baltimore
4. Klinman, J. P. (1978) Kinetic isotope effects in enyzymology. Adv. Enzymol. Relat. Areas Mol. Biol. 46, 415-494
5. Klinman, J. P. (1977) Isotope Effects in Hydride Transfer Reactions. in Sixth Steenbock Symposium on Isotope Effects on Enzyme Catalyzed Reactions (Cleland, W. W., O'Leary, M. L., and Northrop, D. B. eds.), University Park Press, Madison, Wisconsin
Kinetic isotope effects identify substrate binding networks

6. Klinman, J. P. (1978) Primary Hydrogen Isotope Effects. in Transition states of biochemical processes (Gandour, R. D., and Schowen, R. L, eds.), Plenum Press, New York. pp 165-200
7. Cha, Y., Murray, C. J., and Klinman, J. P. (1989) Hydrogen tunneling in enzyme reactions. Science 243, 1325-1330
8. Allemann, R. K., and Scrutton, N. S. (2009) Quantum tunnelling in enzyme-catalysed reactions, Royal Society of Chemistry, Cambridge, UK
9. Klinman, J. P., and Kohen, A. (2013) Hydrogen tunneling links protein dynamics to enzyme catalysis. Ann. Rev. Biochem. 82, 471-496
10. Klinman, J. P. (2013) Importance of protein dynamics during enzymatic C-H bond cleavage catalysis. Biochemistry 52, 2068-2077
11. Pudney, C. R., Guerriero, A., Baxter, N. J., Johannissen, L. O., Waltho, J. P., Hay, S., and Scrutton, N. S. (2013) Fast protein motions are coupled to enzyme H-transfer reactions. J. Am. Chem. Soc. 135, 2512-2517
12. Klinman, J. P. (2015) Dynamically achieved active site precision in enzyme catalysis. Acc. Chem. Res. 48, 449-456
13. Kohen, A. (2015) Role of dynamics in enzyme catalysis: substantial versus semantic controversies. Acc. Chem. Res. 48, 466-473
14. Porta, H., and Rocha-Sosa, M. (2002) Plant lipoxygenases. Physiological and molecular features. Plant Physiol. 130, 15-21
15. Siedow, J. N. (1991) Plant Lipoxygenase: Structure and Function. Annu. Rev. Plant Physiol. Plant Mol. Biol. 42, 145-188
16. Jonsson, T., Glickman, M. H., Sun, S. J., and Klinman, J. P. (1996) Experimental evidence for extensive tunneling of hydrogen in the lipoxygenase reaction: Implications for enzyme catalysis. J. Am. Chem. Soc. 118, 10319-10320
17. Knapp, M. J., Rickert, K., and Klinman, J. P. (2002) Temperature-dependent isotope effects in soybean lipoxygenase-1: Correlating hydrogen tunneling with protein dynamics. J. Am. Chem. Soc. 124, 3865-3874
18. Hatcher, E., Soudackov, A. V., and Hammes-Schiffer, S. (2007) Proton-coupled electron transfer in soybean lipoxygenase: Dynamical behavior and temperature dependence of kinetic isotope effects. J. Am. Chem. Soc. 129, 187-196
19. Meyer, M. P., Tomchick, D. R., and Klinman, J. P. (2008) Enzyme structure and dynamics affect hydrogen tunneling: The impact of a remote side chain (1553) in soybean lipoxygenase-1. Proc. Natl. Acad. Sci. U.S.A. 105, 1146-1151
20. Hu, S. S., Sharma, S. C., Scouras, A. D., Soudackov, A. V., Carr, C. A. M., Hammes-Schiffer, S., Alber, T., and Klinman, J. P. (2014) Extremely Elevated Room-Temperature Kinetic Isotope Effects Quantify the Critical Role of Barrier Width in Enzymatic C-H Activation. J. Am. Chem. Soc. 136, 8157-8160
21. Glickman, M. H., and Klinman, J. P. (1996) Lipoxygenase reaction mechanism: demonstration that hydrogen abstraction from substrate precedes dioxygen binding during catalytic turnover. Biochemistry 35, 12882-12892
22. Hu, S. S., Cattin-Ortola, J., Munos, J. W., and Klinman, J. P. (2016) Hydrostatic Pressure Studies Distinguish Global from Local Protein Motions in C-H Activation by Soybean Lipoxygenase-1. Angew. Chem. Int. Ed. 55, 9361-9364
23. Edwards, S. J., Soudackov, A. V., and Hammes-Schiffer, S. (2010) Impact of Distal Mutation on Hydrogen Transfer Interface and Substrate Conformation in Soybean Lipoxygenase. J. Phys. Chem. B 114, 6653-6660
24. Klinman, J. P., and Offenbacher, A. R. (2018) Understanding Biological Hydrogen Transfer Through the Lens of Temperature Dependent Kinetic Isotope Effects. Acc. Chem. Res. 51, 1966-1974
Kinetic isotope effects identify substrate binding networks

25. Klinman, J. P., Offenbacher, A. R., and Hu, S. (2017) Origins of Enzyme Catalysis: Experimental Findings for C–H Activation, New Models, and Their Relevance to Prevailing Theoretical Constructs. *J. Am. Chem. Soc.* **139**, 18409-18427

26. Offenbacher, A. R., Hu, S. S., Poss, E. M., Carr, C. A. M., Scouras, A. D., Prigozhin, D. M., Iavarone, A. T., Palla, A., Alber, T., Fraser, J. S., and Klinman, J. P. (2017) Hydrogen-Deuterium Exchange of Lipoygenase Uncovers a Relationship between Distal, Solvent Exposed Protein Motions and the Thermal Activation Barrier for Catalytic Proton-Coupled Electron Tunneling. *ACS Cent. Sci.* **3**, 570-579

27. Glickman, M. H., and Klinman, J. P. (1995) Nature of rate-limiting steps in the soybean lipoygenase-1 reaction. *Biochemistry* **34**, 14077-14092

28. Newcomer, M. E., and Brash, A. R. (2015) The structural basis for specificity in lipoygenase catalysis. *Protein Sci.* **24**, 298-309

29. Coffa, G., Schneider, C., and Brash, A. R. (2005) A comprehensive model of positional and stereo control in lipoygenases. *Biochem. Biophys. Res. Commun.* **338**, 87-92

30. Sharma, S. C., and Klinman, J. P. (2015) Kinetic Detection of Orthogonal Protein and Chemical Coordinates in Enzyme Catalysis: Double Mutants of Soybean Lipoygenase. *Biochemistry* **54**, 5447-5456

31. Mogul, R., Johansen, E., and Holman, T. R. (2000) Oleyl sulfate reveals allosteric inhibition of soybean lipoygenase-1 and human 15-lipoygenase. *Biochemistry* **39**, 4801-4807

32. Minor, W., Steczko, J., Stec, B., Otwinowski, Z., Bolin, J. T., Walter, R., and Axelrod, B. (1996) Crystal structure of soybean lipoygenase L-1 at 1.4 angstrom resolution. *Biochemistry* **35**, 10687-10701

33. Bradshaw, M. D., and Gaffney, B. J. (2014) Fluctuations of an exposed pi-helix involved in lipoygenase substrate recognition. *Biochemistry* **53**, 5102-5110

34. Gaffney, B. J., Bradshaw, M. D., Frausto, S. D., Wu, F. Y., Freed, J. H., and Borbat, P. (2012) Locating a Lipid at the Portal to the Lipoygenase Active Site. *Biophys. J.* **103**, 2134-2144

35. Tomchick, D. R., Phan, P., Cymborowski, M., Minor, W., and Holman, T. R. (2001) Structural and functional characterization of second-coordination sphere mutants of soybean lipoygenase-1. *Biochemistry* **40**, 7509-7517

36. Knapp, M. J., Seebeck, F. P., and Klinman, J. P. (2001) Steric control of oxygenation regiochemistry in soybean lipoygenase-1. *J. Am. Chem. Soc.* **123**, 2931-2932

37. Ruddat, V. C., Mogul, R., Chorny, I., Chen, C., Perrin, N., Whitman, S., Kenyon, V., Jacobson, M. P., Bernasconi, C. F., and Holman, T. R. (2004) Tryptophan 500 and arginine 707 define product and substrate active site binding in soybean lipoygenase-1. *Biochemistry* **43**, 13063-13071

38. Phatak, P., Venderley, J., Debrota, J., Li, J. J., and Iyengar, S. S. (2015) Active Site Dynamical Effects in the Hydrogen Transfer Rate-limiting Step in the Catalysis of Linoleic Acid by Soybean Lipoygenase-1 (SLO-1): Primary and Secondary Isotope Contributions. *J. Phys. Chem. B* **119**, 9532-9546

39. Horitani, M., Offenbacher, A. R., Carr, C. A., Yu, T., Hoeke, V., Cutsail, G. E., 3rd, Hammes-Schiffer, S., Klinman, J. P., and Hoffman, B. M. (2017) 13C ENDOR Spectroscopy of Lipoygenase-Substrate Complexes Reveals the Structural Basis for C-H Activation by Tunneling. *J. Am. Chem. Soc.* **139**, 1984-1997

40. Hu, S., Offenbacher, A. R., Thompson, E. M., Gee, C. L., Wilcoxen, J., Carr, C. A. M., Prigozhin, D. M., Yang, V., Alber, T., Britt, R. D., Fraser, J. S., and Klinman, J. P. (2019) Biophysical Characterization of a Disabled Double Mutant of Soybean Lipoygenase: The "Undoing" of Precise Substrate Positioning Relative to Metal Cofactor and an Identified Dynamical Network. *J. Am. Chem. Soc.* **141**, 1555-1567

41. Hu, S. S., Soudackov, A. V., Hammes-Schiffer, S., and Klinman, J. P. (2017) Enhanced Rigidification within a Double Mutant of Soybean Lipoygenase Provides Experimental Support
Kinetic isotope effects identify substrate binding networks

for Vibronically Nonadiabatic Proton-Coupled Electron Transfer Models. *ACS Catal.* 7, 3569-3574

42. Offenbacher, A. R., Iavarone, A. T., and Klinman, J. P. (2018) Hydrogen-deuterium exchange reveals long-range dynamical allostery in soybean lipoxygenase. *J. Biol. Chem.* 293, 1138-1148

43. Wecksler, A. T., Kenyon, V., Garcia, N. K., Deschamps, J. D., van der Donk, W. A., and Holman, T. R. (2009) Kinetic and structural investigations of the allosteric site in human epithelial 15-lipoxygenase-2. *Biochemistry* 48, 8721-8730

44. Lewis, E. R., Johansen, E., and Holman, T. R. (1999) Large competitive kinetic isotope effects in human 15-lipoxygenase catalysis measured by a novel HPLC method. *J. Am. Chem. Soc.* 121, 1395-1396

**FOOTNOTES**

This work was supported by the National Institutes of Health grants to J.P.K. (GM118117) and A.R.O. (GM113432).

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.
### Table 1. The comparison between $^{1}$\(k_{\text{cat}}\) and $^{2}$\(k_{\text{cat}}/K_{\text{m}}\) in SLO variants in 0.1 M borate buffer (pH 9.0)$^{a}$

| Category | Enzyme   | \(k_{\text{cat}}\)-H (s$^{-1}$) | 10°C | 40°C | 10°C | 40°C | 10°C | 40°C |
|----------|----------|-------------------------------|------|------|------|------|------|------|
|          |          |                               |      |      |      |      |      |      |
| Trend I  | WT$^{b}$ | 227 (17)                      | 346 (8) | 66 (5) | 52 (1) | 22 (1) | 48 (3) |
|          | E256A    | 208 (10)                      | 336 (10) | 59 (3) | 55 (2) | 15 (1) | 48 (2) |
|          | R707A    | 114 (8)                       | 181 (4) | 61 (4) | 47 (1) | 13 (1) | 28 (2) |
|          | I553A    | 115 (3)                       | 153 (3) | 148 (5) | 77 (2) | 12 (1) | 57 (2) |
| Trend II | L546A    | 1.91 (0.05)                   | 3.34 (0.04) | 131 (6) | 85 (3) | 64 (3) | 72 (3) |
|          | L754A$^{c}$ | 0.162                        | 0.347 | 106 (8) | 82 (6) | 63 (2) | 68 (3) |
|          |          | (0.009)                       | (0.016) | | | | |
|          | I538A    | 148 (4)                       | 191 (6) | 100 (3) | 65 (2) | 45 (1) | 58 (5) |
| Trend III| V750A    | 179 (6)                       | 173 (7) | 62 (3) | 57 (3) | 50 (3) | 54 (2) |
|          | I552A    | 76 (3)                        | 80 (2) | 66 (3) | 60 (2) | 57 (1) | 52 (1) |
|          | I839A    | 84 (2)                        | 171 (3) | 62 (2) | 72 (2) | 45 (3) | 54 (3) |
|          | W500F    | 174 (6)                       | 250 (4) | 77 (3) | 61 (1) | 55 (2) | 60 (1) |

$^{a}$Kinetic parameters $k_{\text{cat}}$ and $^{2}k_{\text{cat}}$ are obtained by UV-Vis assay. $^{2}k_{\text{cat}}/K_{\text{m}}$ are obtained through competitive assay. $^{b}k_{\text{cat}}$ and $^{2}k_{\text{cat}}$ are from Ref. (25). $^{c}k_{\text{cat}}$ and $^{2}k_{\text{cat}}$ are from Ref. (17).

### Table 2. Solvent Isotope Effect at 20°C with H-LA.$^{a}$

| Enzyme form | $k_{\text{cat}}$ | $k_{\text{cat}}/K_{\text{m}}$ |
|-------------|------------------|-----------------------------|
| WT          | 1.26 (0.07)      | 1.70 (0.20)                 |
| I552A       | 0.80 (0.02)      | 1.51 (0.11)                 |
| L546A       | 0.83 (0.02)      | 1.56 (0.10)                 |
| V750A       | 0.72 (0.11)      | 2.40 (0.50)                 |

$^{a}$H$_{2}$O buffer: 0.1 borate (pH 9.0) in H$_{2}$O. D$_{2}$O buffer: 0.1 M borate (with a pH meter reading of 8.6) in D$_{2}$O.
Table 3. The commitment ($k_{\text{chem}}/k_{\text{off}}$) and $k_{\text{off}}$ value in SLO variants in 0.1 M Borate Buffer (pH = 9.0) at 10°C.

| SLO      | $k_{\text{chem}}/k_{\text{off}}$ | $k_{\text{off}}$ (s$^{-1}$) |
|----------|----------------------------------|-------------------------------|
| Trend I  |                                  |                               |
| WT       | 2.1 (0.3)                        | 108 (16)                      |
| E256A    | 3.1 (0.3)                        | 66 (7)                        |
| R707A    | 4.0 (0.5)                        | 29 (4)                        |
| I553A    | 12.3 (1.2)                       | 9.30 (0.94)                   |
| Trend II |                                  |                               |
| L546A    | 1.1 (0.1)                        | 1.79 (0.20)                   |
| L754A    | 0.69 (0.13)                      | 0.16 (0.03)                   |
| I538A    | 1.3 (0.1)                        | 118 (8)                       |
| Trend III|                                  |                               |
| V750A    | 0.24 (0.08)                      | 718 (259)                     |
| I552A    | 0.16 (0.05)                      | 472 (167)                     |
| I839A    | 0.45 (0.09)                      | 185 (37)                      |
| W500F    | 0.40 (0.06)                      | 435 (73)                      |

The $k_{\text{chem}}/K_m$ values and $k_{\text{off}}$ values are calculated from $k_{\text{cat}}/K_m$, $k_{\text{cat}}$, and $k_{\text{cat}}$ value through Eq 1. The errors are calculated on the basis of error propagation equations.

Scheme 1. Diagram to illustrate differences between first-order rate constants ($k_{\text{cat}}$) and second-order rate constants ($k_{\text{cat}}/K_m$) within the chemical reaction coordinate of SLO. Given the sole rate limitation of $k_{\text{cat}}$ by the C-H abstraction step, $k_{\text{cat}}$ and $k_{\text{chem}}$ as well as $k_{\text{cat}}$ and $k_{\text{chem}}$ can be used interchangeably. The lack of the contribution of any steps preceding the formation of ES’ to $k_{\text{cat}}$ indicates that $K_i=k_{\text{i}}/k_{-1} \gg 1$. Note that the size of the relative barriers for the conversion of E + S to ES, ES to ES’ and ES’ to EP are for illustrative purposes only, to indicate the multiple partitionings between the forward and reverse steps that contribute to $k_{\text{cat}}/K_m$ but not $k_{\text{cat}}$. The magnitude of $k_{\text{off}}$ in the text and Table 3 refers to the net rate constant for release of the substrate from the stable ES’ complex back to the free substrate and enzyme. For the model shown here, the magnitude of $k_{\text{off}}$ is approximated by $k_{\text{i}}$. 


Figure 1. Results of variable temperature $\Delta k_{cat}/K_m$ (red circle) and $\Delta k_{cat}$ (black square) with WT (A), L546A (B), V750A (C) and I552A (D). The $\Delta k_{cat}/K_m$ values were obtained through competitive assay in 0.1 borate buffer (pH 9.0) while the $\Delta k_{cat}$ values were obtained through steady-state kinetics assays.
Figure 2. Effect of viscosigen WT SLO (cyan inverted triangles), I552A (blue triangles), V750A (black square) and L546A (red circle) in 0.1 M CHES buffer (pH 9.0) with glucose, 20 °C.
**Figure 3.** X-ray structure of SLO (PDB ID: 1F8N), with iron colored in red, Trend I residue (E256, R707, and I553) colored olive green, Trend II residues (L546, L754 and I538) colored light purple, Trend III residues (V750, I552, I839, W500) colored khaki, helix 2 highlighted by yellow, helix 11 highlighted by pink, and previous identified thermal activated remote loop 317-334 highlighted by light blue (25). (A) Bird’s eye view of mutated residues in this study, helix 2, 11, and remote loop 317-334; B) Two networks in SLO intersect at residues V750 and I552. The V750 and I552 are within the network that modulate the chemical steps across the surface loop 317-334, connector S749, Y317, and active site residues I553 and L546. The V750 and I552 are also in contact with residues on the substrate portal helix 2. (C) The residues I839 and W500 are in contact with residues on the substrate portal helix 2 and helix 11. The black arrow in each panel indicates the proposed substrate entrance between helix 2 and 11 (32,33). Q495 and Q697 (colored grey) were proposed to be located around the binding pocket in an earlier study (35).
