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Kinetic Investigations of the Rate Limiting Step in 12- and 15- Human Lipoxygenase

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ABBREVIATIONS: AA, arachidonic acid (eicosa-5Z,8Z,11Z,14Z-tetraenoic acid); LA, linoleic acid (9Z,12Z-octadecadienoic acid); D-LA, per-deuterated linoleic acid; HPOD, 13(S)-hydroperoxy-9(Z),11(E)-octadecadienoic acid; ICP-MS, inductively coupled plasma mass spectroscopy; MCD, magnetic circular dichroism; RP-HPLC, reverse phase-HPLC; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; LO, lipoxygenase; 12-hLO, human platelet 12-lipoxygenase; 15-hLO, human reticulocyte 15-lipoxygenase-1; 15-rLO, rabbit reticulocyte 15-lipoxygenase; sLO, soybean lipoxygenase; k_{cat}, enzymatic first order rate constant for fatty acid substrate; k_{cat}/K_M, enzymatic second order rate constant for fatty acid substrate; $k_{cat}/K_{M(O_2)}$, enzymatic second order rate constant for molecular oxygen substrate; KIE, kinetic isotope effect; $^D k_{cat}$, kinetic k_H/k_D isotope effect on k_{cat}; $^D k_{cat}/K_M$, kinetic k_H/k_D isotope effect on k_{cat}/K_M; SIE, solvent isotope effect.

ABSTRACT: Mammalian lipoxygenases have been implicated in several inflammatory disorders; however, the details of the kinetic mechanism are still not well understood. In the current paper, human platelet 12-lipoxygenase (12-hLO) and human reticulocyte 15-lipoxygenase-1 (15-hLO) were tested with arachidonic acid (AA) or linoleic acid (LA), respectively, under a variety of changing experimental conditions, such as temperature, dissolved oxygen concentration, and viscosity. The data presented show that 12-hLO and 15-hLO have slower rates of product release (k_{cat}) relative to soybean lipoxygenase-1 (sLO-1), but similar or better rates of substrate capture for the fatty acid (k_{cat}/K_M) or molecular oxygen ($k_{cat}/K_{M(O_2)}$). The kinetic isotope effect (KIE) for 15-hLO was determined to be temperature independent and large ($^D k_{cat} = 40 \pm 8$), over the range of 10-35 °C, indicating that C-H bond cleavage is the sole rate limiting step and proceeds through a tunneling mechanism. The $^D k_{cat}/K_M$ for 15-hLO, however, was temperature dependent, consistent with our previous results [Lewis, E. R., Johansen, E., and Holman, T. R. (1999) *J. Am. Chem. Soc. 121*, 1395-1396], indicating multiple rate limiting steps. This was confirmed by a temperature dependent, k_{cat}/K_M solvent isotope effect (SIE), which indicated a hydrogen bond rearrangement step at low temperature, similar to sLO-1 [Glickman, M. H. and Klinman, J. P. (1995) *Biochemistry 34*, 14077-14092]. The KIE could not be determined for 12-hLO due to its lack of ability to efficiently catalyze LA, but the k_{cat}/K_M SIE was temperature independent, indicating distinct rate limiting steps from both 15-hLO and sLO-1.

Lipoxygenases are found in a large variety of organisms, such as bacteria (*1*), plants (*2, 3*), and mammals (*4*). In plants, lipoxygenase is involved in germination and senescence (*5, 6*). In humans, the products of lipoxygenase are the precursors to leukotrienes and lipoxins, which have been implicated as critical signaling molecules in a variety of inflammatory diseases and cancers (*7-10*). Human 5-lipoxygenase is implicated in bronchial constriction (*11*) and prostrate cancer (*12*), 12-lipoxygenase in immune disorders (*13*) and breast cancer (*14, 15*), while 15-lipoxygenase is implicated in the primary stage of arteriosclerosis (*16*) and colorectal cancer (*17*). The importance of these enzymes in inflammation and cancer regulation have elicited great interest in human lipoxygenase as a potential therapeutic target and emphasize the need for further investigations of the lipoxygenase mechanism.

Historically, soybean lipoxygenase-1 (sLO-1) has been the most extensively studied enzyme of all the lipoxygenases due to its ease of purification and kinetic stability. Investigators have determined that sLO-1 activates the 1,4-diene portion of linoleic acid (LA) for attack by molecular oxygen to produce the hydroperoxide product, 13(S)-hydroperoxy-9(Z),11(E)-octadecadienoic acid (HPOD) (Scheme 1) (*3, 18*). The molecular mechanism of this reaction entails activation of the resting $Fe(II)-OH_2$ enzyme by one equivalent of HPOD to produce the active Fe(III)-OH species (*19, 20*). The Fe(III)-OH then stereo-specifically abstracts the pro-(S) hydrogen atom through a tunneling mechanism, leaving a pentadienyl radical (*21-23*). The mechanism of this abstraction has recently been postulated to proceed through an environmentally coupled mechanism where both position and molecular motion dictate the hydrogen atom abstraction (*24*). Molecular oxygen then regio- and stereo-specifically attacks the (S)-face of the radical through direction by a hydrophobic pocket in the active site (*25*), creating a hydroperoxide radical, which is subsequently reduced to HPOD. The microscopic rate limiting steps for substrate capture (k_{cat}/K_M) by sLO-1 at low temperature (5 ºC) have been determined to be diffusion, hydrogen bond rearrangement and hydrogen atom abstraction (*26*) (Note: The terms substrate capture (k_{cat}/K_M) and product release (k_{cat}) have been defined previously (*27*) and shall be used in the same manner in this report). The hydrogen bond rearrangement step has been proposed by our lab to be due to the hydrogen bond network (Gln_{495}, Gln_{697} and Asn_{694}) that connects the substrate cavity (Gln_{495}) to the iron atom (Asn_{694}), potentially triggering a change in the iron environment (i.e. reactivity) (*23*). As

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temperature increases, however, the abstraction step becomes the sole rate limiting step for substrate capture. The rate of product release (k_{cat}) for sLO-1, however, has been shown to be solely dependent on hydrogen atom abstraction, from 5 ºC to 40 ºC (*28*).

The mammalian enzymes are similar to plant lipoxgenases with regards to their sequence homologies and structure, as seen by comparing the soybean lipoxygenase structures with the rabbit reticulocyte 15-lipoxygenase (15-rLO) structure (*29-35*). However, little is known regarding the molecular mechanism by which the mammalian lipoxygenases generate the hydroperoxide product from arachidonic acid (AA). This is primarily due to their difficulty in purification and their rapid auto-inactivation, which makes steady state kinetics difficult to interpret. Our lab circumvented these complications with human reticulocyte 15-lipoxygenase-1 (15-hLO) by using a competitive kinetic isotope effect (KIE) method, which determined that 15-hLO abstracts the hydrogen atom through a tunneling mechanism ($^{D}k_{cat}/K_{M} =$ 47 ± 7 at 5 µM substrate) (*36*). In addition, we determined that the $^{D}k_{cat}/K_{M}$ was temperature dependent indicating multiple rate limiting steps at low temperature, similar to sLO-1. This was an intriguing result because 15-hLO has significantly lower rates of product release (k_{cat}) than sLO-1. Previously, we postulated that this lowered rate for 15-hLO was due to an increase in the ligand sphere strength of the active site iron (His_{544} ligand for 15-hLO vs. Asn_{694} ligand for sLO-1), which would lower the driving force of the hydrogen atom abstraction and hence lower k_{cat} (*37*). One could then speculate that a slower k_{cat} would create a kinetic bottleneck and make hydrogen atom abstraction the sole rate limiting step for both substrate capture (k_{cat}/K_{M}) and product release (k_{cat}) for 15-hLO, however, this is not the case. 15-hLO manifests multiple rate limiting steps for substrate capture, despite a lowered rate of hydrogen atom abstraction. In the current paper, we have extended this investigation to include noncompetitve kinetic studies of 15-hLO and human platelet 12-lipoxygenase (12-hLO), in order to determine the nature of the multiple rate limiting steps and compare their mechanisms with sLO-1.

MATERIALS AND METHODS

Materials. LA and AA were purchased from Aldrich Chemical Co. and per-deuterated linoleic acid (D-LA) was purified from a mixture of per-deuterated algal fatty acids from Cambridge Isotope Labs, as

previously described (*38*). All other reagents were reagent grade or better and were used without further purification.

RP-HPLC purification of AA, LA, and D-LA. All commercial fatty acids were re-purified using a Higgins Analytical Haisil (25 cm × 4.6 mm) C-18 column. Solution A is 99.9% MeOH and 0.1% acetic acid, while solution B is 99.9% H_2O and 0.1% acetic acid. An isocratic elution of 85% A:15% B was used to purify all commercial fatty acids and they were stored at –80 ºC for a maximum of 6 months.

Expression and Purification of Proteins. 12-hLO and 15-hLO were expressed and purified as described previously (*39*). Briefly, both enzymes contain His-tags and must be purified in one step, then stored in glycerol at –80 ºC, or significant inactivation occurs. Iron content of all lipoxygenase enzymes were determined on a Finnegan inductively coupled plasma mass spectrometer (ICP-MS), using internal standards of cobalt(II)-EDTA, and data were compared with standardized iron solutions. All kinetic measurements were standardized to iron content.

Steady State Kinetic Measurements. Steady state kinetics values were determined by following the formation of the conjugated product at 234 nm ($\varepsilon = 2.5 \times 10^4$ M^{-1} cm^{-1}) with a Perkin-Elmer Lambda 40 spectrophotometer. No photo-degradation of the product was observed. Assays of 12-hLO were carried out in 25 mM Trizma buffer (pH 7.5) and assays of 15-hLO were carried out in 25 mM Hepes buffer (pH 7.5). LA and AA concentrated stock solutions were stored in 95% ethanol, and diluted into buffer so that the total ethanol concentration was less than 1.5%. Fatty acid concentrations were verified by allowing the enzyme reaction to proceed to completion, and quantitating at 234 nm. Enzymatic reactions were initiated by the addition of ≈ 5 nM 12-hLO or ≈ 30 nM 15-hLO (normalized to iron content). Assays were 2 mL in volume with substrate concentrations ranging from 1-25 µM and constantly stirred with a rotating magnetic bar. It should be noted that higher substrate concentrations were avoided to prevent the formation of micelles, which would alter the free substrate concentration (*40*). Initial rates (up to the first 15% of the reaction) for each substrate were fitted to the Michaelis-Menten equation using the KaleidaGraph (Synergy) program on a Macintosh and errors determined. All subsequent kinetic measurements were handled in a similar form.

pH Studies. Kinetic measurements were determined at pH ranges from 7-9 in Trizma and Hepes buffer. The total ionic strength was adjusted in all cases to 25 mM by the addition of NaCl. Hepes buffer was found to inhibit 12-hLO but not 15-hLO, consequently further kinetic studies were performed using Trizma with 12-hLO and Hepes with 15-hLO.

Temperature Dependence. Initial rates of reaction were measured by the above methods. Temperature control of the reaction was achieved by the use of temperature-controlled cuvettes with a stream of nitrogen gas to inhibit condensation. To account for temperature-induced pH differences in Trizma and Hepes, buffers were equilibrated to temperatures from 10-45 ºC and then pH adjusted.

Reaction Rates at Varying O_2 Concentrations. Reaction rates of 12-hLO (AA) and 15-hLO (LA) were determined by measuring oxygen consumption on a Clark oxygen monitor as previously published (*26*). Reactions were carried out as a function of oxygen concentration in 1 mL solutions, which were constantly stirred and equilibrated under air at 25 ºC (258 μM O_2). The reaction was initiated by addition of ≈ 10 nM 12-hLO and ≈ 30 nM 15-hLO (normalized to iron content) via a gastight Hamilton syringe to the reaction chamber. The experiments were repeated at a number of different concentrations of oxygen established by passing mixtures of N_2 and O_2 over stirred solutions in the reaction chamber for 20 minutes. The new oxygen concentration was calibrated against the value of O_2 dissolved in an air-saturated solution at 25 ºC (258 μM O_2). The rate of oxygen consumption was recorded at O_2 concentrations from 300 to 5 μM. The fatty acid substrate concentration was five times the K_M for the respective enzyme, 15 μM AA for 12-hLO and 25 μM LA for 15-hLO.

Noncompetitive Kinetic Isotope Effects. Steady state KIE values were determined for 15-hLO by following the formation of the conjugated product at 234 nm, as discussed above and were carried out in 25 mM Hepes buffer (pH 7.5). For protonated LA, ≈ 45 nM 15-hLO was used for each assay and ≈ 105 nM 15-hLO (normalized to iron content) for the D-LA.

Solvent Isotope Effects. Kinetic measurements were performed with 12-hLO and 15-hLO at temperatures from 10-45 ºC in 25 mM Buffer (Trizma or Hepes, respectively) using H_2O or D_2O, pL 7.5 (pH meter reading was 7.1 for D_2O). SIE results were determined by averaging the values from three separate experiments. Since the LA has a pK_a between 7-8 in buffer solutions not containing detergent

(*26*), the influence of pH on our SIE experiments with 15-hLO in 25 mM Hepes was also examined at pL 7.1, 7.5, and 7.9.

Viscosity Studies. Reactions were carried out at different relative viscosities ($\eta_{rel} = \eta/\eta^0$, η^0 is the viscosity of H_2O at 20 ºC) in order to ascertain if the human lipoxygenase catalysis is diffusion controlled. A number of viscogens were surveyed (ethylene glycol, glycerol, dextrose, maltose, and sucrose) by adding 1 mL of varying concentrations of viscogen to 1 mL substrate buffer solution (2x concentration) and monitored as discussed previously for the steady state kinetics.

RESULTS

Expression and Purification of Lipoxygenases. Both 12-hLO and 15-hLO yielded approximately 50 mg/L from the one-step, His-Bind (Qiagen) column and were greater than 90% pure, as judged by SDS-PAGE (stained with Coomassie brilliant blue, data not shown). As isolated, ICP-MS indicated that the human enzymes contained 0.35 moles of iron per mole enzyme, on average, and all kinetic values were corrected for iron content.

pH Studies. Changing the pH of the buffer while maintaining a constant ionic strength revealed that 12-hLO and 15-hLO had highest activity in the pH range 7-8 (data not shown), consistent with previous results for 12-hLO (*41*). Therefore, all further kinetic experiments were performed at physiological pH (7.5).

Kinetic Analysis of 12-hLO and 15-hLO. Both human lipoxygenases exhibited hyperbolic steady-state kinetics from which the rate constants k_{cat} (Figure 1) and k_{cat}/K_M (Figure 2) were determined (Table 1). It should be noted that severe auto-inactivation was observed at high substrate concentrations for both enzymes if the substrate was not HPLC purified and stored at –80 ºC. This suggests that the reaction products of substrate oxidation by molecular oxygen may lead to auto-inactivation of human lipoxygenases. 12-hLO displayed an increasing k_{cat} value, from 2.5 ± 0.1 s^{-1} at 15 ºC to 46 ± 4 s^{-1} at the maximum temperature tested, 45 ºC. These results were higher than those previously recorded, most likely due to our correction for iron content (*41*). For 15-hLO, the k_{cat} value of 10.2 ± 1.6 s^{-1} (20 ºC) agreed with our previously reported results for 15-hLO without a His-tag and demonstrates the His-tag

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has no effect on catalysis (*40*). 15-hLO showed a maximum velocity between 20-35 ºC, then decreased with increasing temperature. In order to investigate this decrease in k_{cat} for 15-hLO at higher temperatures, we performed a series of experiments to evaluate auto-inactivation of both human lipoxygenases at 15 ºC, 30 ºC, and 45 ºC. We added three sequential additions of HPLC purified substrate to the enzyme and compared their rates as the reaction was allowed to go to completion (data not shown). These experiments indicated that 15-hLO underwent auto-inactivation with increasing temperature and thus lowered the k_{cat} for 15-hLO, consistent with previous results (*42, 43*). 12-hLO was not observed to decrease in rate, but rather the k_{cat} increased at high temperatures, indicating little auto-inactivation with HPLC purified AA. The k_{cat}/K_M value (Figure 2) for both enzymes increased with increasing temperature and leveled off above 30 ºC (average value above 30 ºC for 12-hLO was 96.2 ± 0.5 µM^{-1} s^{-1} and for 15-hLO was 3.9 ± 1.2 µM^{-1} s^{-1}).

Reaction Rates at Varying O_2 Concentrations. The kinetic parameters for both enzymes were analyzed at saturating fatty acid substrate conditions (5 times the K_M) at 20 ºC (Figure 3). The $K_{M(O_2)}$ was determined to be 7.0 ± 1.4 µM and 4.2 ± 1.1 µM for 12-hLO and for 15-hLO, respectively (the lowest attainable concentration of O_2 was 5 µM, which leads to a large uncertainty in both of these K_M determinations). The k_{cat} was determined to be 13.1 ± 0.4 s^{-1} for 12-hLO and 5.2 ± 0.2 s^{-1} for 15-hLO, which are in good agreement with k_{cat} values determined spectrophotometrically. A similar correspondence between k_{cat} determined spectrophotometrically and by the Clarke electrode has been previously reported for sLO-1 (*22*).

Noncompetitive Kinetic Isotope Effect. Previously, we reported a large competitive $^D k_{cat}/K_M$ result at 5 µM LA for 15-hLO (*36*). We expanded these results by using a noncompetitive kinetic method, previously devised by Klinman and coworkers for sLO-1 (*44*). Since appropriately deuterated AA was not available, D-LA was used. LA was a comparable substrate to AA for 15-hLO (*45*), however, 12-hLO was only 4% as active with LA as AA (*41*). Consequently, noncompetitive KIE experiments were undertaken only with 15-hLO (Figure 4). The $^D k_{cat}$ value for 15-hLO was largely temperature independent over the experimental range from 10-35 ºC ($^D k_{cat} = 40 ± 8$). This KIE result was much larger than semiclassical theory predicts (KIE = 7-10) and was consistent with a tunneling mechanism

for hydrogen atom abstraction (*46*). The temperature independence of $^Dk_{cat}$ indicated that the product release was fully rate limited by hydrogen atom abstraction. $^Dk_{cat}/K_M$, however, was not temperature independent and increased with increasing temperature to 83 ± 35 (35 ºC). It should be noted that the $^Dk_{cat}/K_M$ value at 35˚ C is larger than the $^Dk_{cat}$ value at 35˚ C (49 ± 7). This is unusual since the two values should coincide at high temperatures if the hydrogen atom abstraction is the sole rate limiting step. This difference is most likely due to the large degree of auto-inactivation at these temperatures for 15-hLO, which instills a high degree of error. In fact, when the error values are taken into account, the two values at 35˚ C overlap.

The temperature dependence of $^Dk_{cat}$ (Figure 5) for both hydrogen and deuterium atom abstraction were fitted with the empirical Arrhenius equation (k = A exp$^{-E_{act}/RT}$), where R represents the gas constant, T is absolute temperature, E_{act} is the activation energy, and A is the Arrhenius prefactor (Table 2). 15-hLO exhibits a weak temperature dependence (E_{act} = 7.7 kcal/mol) and a moderate Arrhenius prefactor ($A_H \approx 3 \times 10^6$ s^{-1}) with LA. Additionally, the ΔE_{act} is near unity (ΔE_{act} is the difference between the protio and deutero E_{act} values) and the KIE is close to temperature independent, leading to an A_H/A_D value greater than one (8 ± 14). The large degree of error makes the A_H/A_D value difficult to interpret.

Solvent Isotope Effects. The kinetic parameters k_{cat} (Figure 6a) and k_{cat}/K_M (Figure 6b) for both lipoxygenases were analyzed with substrate in H_2O or D_2O over the temperature range from 10-45 ºC. The k_{cat} SIE for 12-hLO was 1.5 ± 0.01, indicating a solvent dependent step that was partially rate limiting at 15 ºC. As the temperature increased, the k_{cat} SIE for 12-hLO decreased to unity, indicating no rate-limiting solvent dependent steps at higher temperature. The k_{cat} SIE for 15-hLO, however, was temperature independent (1.0 ± 0.2, average value from 15 to 45 ºC), which indicated no solvent dependent rate-limiting steps for product release. The k_{cat}/K_M SIE for 12-hLO was temperature independent (1.0 ± 0.2, average value from 15 to 45 ºC), while the value for 15-hLO was 2.0 ± 0.02 (15 ºC) and temperature dependent. The temperature dependence of the k_{cat}/K_M SIE for 15-hLO indicated that there was a solvent dependent step that was partially rate limiting at low temperatures and not rate-

limiting above 30 °C. These interpretations were tempered by the relatively large error values for these reactions, due to the imprecise nature of K_M. This higher error was most likely due to the previously mentioned auto-inactivation and the fact that high concentrations of substrate can lead to substrate inhibition for the human lipoxygenases (*40*). The SIE for 15-hLO was also monitored for a pH effect and found to be constant in the pH range 7.1-7.9 (data not shown).

Viscosity Studies. Viscosity experiments were attempted with ethylene glycol, dextrose, sucrose, maltose, and glycerol to test diffusion; however, no two viscogens produced comparable viscosity dependence for either 12-hLO or 15-hLO ((k_{cat}/K_M)°/(k_{cat}/K_M), where (k_{cat}/K_M)° is the value in 100% water). For 12-hLO, 40% ethylene glycol (η_{rel} = 3) did not change the rate, 30% dextrose (η_{rel} = 3) decreased the rate by ≈ 33%, 30% sucrose (η_{rel} = 3) decreased the rate by ≈ 20%, and maltose and glycerol inhibited the reaction completely. For 15-hLO, 40% ethylene glycol (η_{rel} = 3) decreased the rate by ≈ 27%, 30% dextrose (η_{rel} = 3) decreased the rate by ≈ 48%, 30% sucrose (η_{rel} = 3) decreased the rate by ≈ 47%, maltose inhibited the reaction completely, and 40% glycerol (η_{rel} = 3) decreased the rate by ≈ 67%. Since the viscogens are obviously affecting the enzymatic rate in other ways besides inducing the desired diffusion effect, we were unable to make any conclusions. It should be noted that it was previously shown that glycerol affects coordination of the iron active site of sLO-1 and thus may have a similar structural effect on the human enzymes (*47*).

DISCUSSION

In the current paper, we investigated the kinetic mechanism of both 15-hLO and 12-hLO utilizing a variety of steady-state kinetic methods in order to compare their properties directly with the well-studied sLO-1 enzyme. The steady state data obtained by varying the fatty acid substrate concentration demonstrated that the rate of product release (k_{cat}) for both 12-hLO and 15-hLO is significantly slower at 30˚ C than that of sLO-1, 17-fold and 29-fold, respectively (Table 1). The rate of substrate capture (k_{cat}/K_M), however, is more comparable to sLO-1, with 15-hLO being 3-fold slower and 12-hLO being 5-fold faster at 30 °C. k_{cat}/K_M is comparable among the three enzymes as a consequence of lowered K_M

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values for the human enzymes (where $K_M = 0.30 \pm 0.06$ µM for 12-hLO, $K_M = 2.4 \pm 0.5$ µM for 15-hLO, and $K_M = 27$ µM for sLO-1) (*25*), potentially indicating reduced dissociation constants for the human enzymes with the fatty acid substrate. The temperature effect on these kinetic parameters shows that the k_{cat} of 15-hLO diminishes at temperatures higher than 37° C, while the k_{cat} of 12-hLO increases monotonically, up to 45° C (Figure 1). The decrease in k_{cat} at higher temperatures for 15-hLO was due to the auto-inactivation at high substrate concentrations, as seen in this paper and in other studies (*42, 43, 48, 49*). The k_{cat}/K_M for both 12-hLO and 15-hLO plateaus above 30 °C, which coincides with the physiological temperature (37° C) for both human enzymes (Figure 2). The rate of oxygen incorporation (Figure 3 and Table 1) shows that the $k_{cat}/K_{M(O_2)}$ for the human enzymes is only 2-3 fold less than sLO-1, which can be attributed to the lower $K_{M(O_2)}$ values for 12-hLO (7.0 ± 1.4 µM O_2) and 15-hLO (4.2 ± 1.1 µM O_2) than sLO-1 (48 µM O_2) (*25*). This is significant to their biological activity since dissolved O_2 concentration in tissue has been reported to be between 10-40 µM (*50*), and therefore the $K_{M(O_2)}$ values for 12-hLO and 15-hLO are low enough such that dissolved oxygen is saturating. These combined kinetic data suggest that the human enzymes have evolved such that their overall turnover rate (i.e. k_{cat}) has decreased significantly relative to sLO-1, but their ability to initiate the reaction (i.e. k_{cat}/K_M and $k_{cat}/K_{M(O_2)}$) is comparable or better to that of sLO-1. This point is significant as k_{cat}/K_M is generally considered to represent the rate constant under physiological conditions and suggests that both human enzymes are optimized as physiological catalysts (*51*).

The differences in the general kinetic properties of the three lipoxygenases lead us to investigate the nature of the rate limiting steps for both 12-hLO and 15-hLO and determine how the human enzymes are different from sLO-1 on a microscopic kinetic level. 12-hLO is difficult to study by primary kinetic isotope effects because LA is not a substrate and appropriately deuterated AA is not commercially available. Viscosity dependence could also not be determined because the viscogens tested gave varying results. The only isotopic experiment available was that of comparing kinetic rates in H_2O and D_2O (Figure 6). To this end, we observe that k_{cat} was solvent dependent at low temperature, while k_{cat}/K_M is solvent independent between 10-40° C. This is in sharp contrast to sLO-1, for which k_{cat} is

solvent independent at all temperatures, but k_{cat}/K_M is solvent dependent at low temperature. These results indicate a fundamental change in the microscopic rate constants between the two enzymes, however, its origin is difficult to determine without primary KIE results. Interestingly, sequence analysis between 12-hLO and sLO-1 suggest that they have very similar iron coordination environments: both 12-hLO and sLO-1 have Asn as the sixth ligand, which would imply similar reduction potentials (i.e. similar abstraction chemistry). This, coupled with the fact that 12-hLO has a greater kinetic turnover rate than 15-hLO, suggests that 12-hLO should have similar a mechanism to sLO-1, but this is not the case. We are currently attempting to synthesize deuterated AA with collaborators in order to determine the nature of the hydrogen atom abstraction step for 12-hLO.

The nature of the rate limiting step for 15-hLO, however, can be fully investigated because 15-hLO does oxidize LA. The $^Dk_{cat}$ was temperature independent which indicates that the rate of product release is solely limited by hydrogen atom abstraction, as previously seen for sLO-1 (*26, 44, 46, 52*). The magnitude of the $^Dk_{cat}$ was large ($^Dk_{cat} = 40 \pm 8$), which indicates a tunneling mechanism for hydrogen atom transfer. Previously, we published a large competitive $^Dk_{cat}/K_M$ for 15-hLO which was temperature dependent (*36*). This result is corroborated in the present noncompetitve study in which the $^Dk_{cat}/K_M$ was temperature dependent (Figure 4) and confirms our previous hypothesis that there are multiple rate limiting steps for 15-hLO at low temperature. These multiple rate limiting steps effectively lower the magnitude of the observed $^Dk_{cat}/K_M$ at lower temperature, because the hydrogen atom abstraction, which accounts for the large KIE, is only partially rate limiting and hence only a portion of the true $^Dk_{cat}/K_M$ value is observed. As temperature increases, the hydrogen atom abstraction becomes the sole rate limiting step as seen by the plateau of the $^Dk_{cat}/K_M$ due to the temperature independence of the tunneling mechanism of the abstraction. An unusual observation is that the $^Dk_{cat}/K_M$ value at high temperature is larger than the $^Dk_{cat}$ value. If both $^Dk_{cat}/K_M$ and $^Dk_{cat}$ are solely limited by hydrogen atom abstraction at high temperature, then these values should be the same. We believe this is not observed, due to the auto-inactivation of 15-hLO at high temperature which induces a large error in the noncompetitve $^Dk_{cat}/K_M$ value. The error limits of $^Dk_{cat}$ are within the $^Dk_{cat}/K_M$ error limits at 35 °C, 49 ± 7 and 83 ± 35, respectively. In addition, the $^Dk_{cat}$ value of this study (40 ± 8)

compares well with our previous competitive $^D k_{cat}/K_M$ result (47 ± 7 at 5 μM substrate) (*36*), which is not affected by auto-inactivation.

The $^D k_{cat}/K_M$ data clearly indicate the existence of multiple rate limiting steps at low temperature, however, more data is needed to determine the nature of these steps. Viscosity experiments were attempted to determine if substrate diffusion is rate limiting for substrate capture (k_{cat}/K_M), however no conclusion could be made because all viscogens gave differing results. The SIE was subsequently determined for k_{cat}/K_M in order to establish if a solvent dependent step could be involved as an additional rate limiting step (Figure 6). A significant k_{cat}/K_M SIE (2.02 ± 0.02) for 15-hLO was seen at 15 °C, which diminishes as temperature increases, demonstrating a solvent dependent rate limiting step. The k_{cat} SIE, however, was close to unity and temperature independent, indicating that solvent-dependent steps are not rate limiting for product release (k_{cat}). Both of these results were in complete agreement with the noncompetitve and competitive KIE results and conclusively establish a solvent dependent step as one of the additional rate limiting step in the substrate capture (k_{cat}/K_M) at low temperature. The data also demonstrate that the rate of product release (k_{cat}) is solely limited by hydrogen atom abstraction for 15-hLO.

These results were especially interesting because they mirror the results of sLO-1, where substrate capture (k_{cat}/K_M) displays multiple rate limiting steps but product release (k_{cat}) does not, suggesting similar molecular mechanisms between the two enzymes. We previously demonstrated that the catalytic efficiency of sLO-1 was lowered by disruption of the hydrogen bond network that connects the substrate cavity to the iron coordination (Gln495, Gln697 and Asn694) (Figure 7a) (*23*). We proposed that the hydrogen bond network allowed the substrate binding to affect reactivity by directly changing the coordination distance of Asn694 and hence the reactivity of the iron. This hypothesis is corroborated by the sLO-3/HPOD crystal structure, where the position of Gln514 (Gln495 in sLO-1) is greatly altered by interaction with the bound HPOD (*30*). This hydrogen bond network is also present in 15-rLO (Glu357, Gln548 and His544, Figure 7b) and 15-hLO ($\approx$ 80% sequence homology to 15-rLO) (*53*), which could account for the positive SIE results seen in this investigation for 15-hLO. However, there are differences between the two hydrogen-bond networks of 15-hLO/15-rLO and sLO-1, which complicates

their comparison. His_{544} of 15-hLO and His_{545} of 15-rLO do not change their coordination distance upon substrate binding, as seen by MCD spectroscopy for the Asn_{694} of sLO-1 (*54*). The hydrogen bond network in 15-rLO, however could affect the orientation of the His_{545} ligation by its hydrogen bond to Glu_{357} (Figure 7b) (His_{544} and Glu_{356} in 15-hLO, by sequence alignment). This ligation change could modulate the reactivity of the Fe(III)-OH species due to ligand orbital overlap of His_{544} (15-hLO) or His_{545} (15-rLO) with the iron.

This hypothesis that hydrogen bond rearrangement is a rate limiting step for 15-hLO is surprising since its k_{cat} is 29-fold slower than sLO-1, which could potentially make the abstraction step the sole rate limiting step on k_{cat}/K_M. That this is not the case leads to the intriguing hypothesis that the hydrogen bond rearrangement step is chemically linked with the hydrogen atom abstraction step and hence could constitute a conserved mechanism for both 15-hLO and sLO-1. This requires further investigation of the second coordination sphere of 15-hLO and specific mutations are currently being investigated.

In addition to confirming that 15-hLO has multiple rate limiting steps with regards to substrate capture (k_{cat}/K_M), the noncompetitve KIE experiments have also allowed us to address the nature of the tunneling mechanism of hydrogen atom abstraction. Knapp and Klinman have recently proposed a hydrogen atom transfer promoted by environmental dynamics to explain the large kinetic isotope effects in sLO-1 (*24, 55*). This theory, "environmentally coupled hydrogen tunneling," is a full tunneling model where protein fluctuations, or other motions in the environment, generate a reactive configuration of heavy atoms allowing the hydrogen to transfer via nuclear tunneling (*56*). Certain environmental vibrations affect the tunneling barrier shape and directly links enzyme fluctuations to the reaction coordinate for hydrogen transfer. These vibrations are termed 'active dynamics' and are responsible for the gating which promote hydrogen transfer. This new model of environmentally coupled hydrogen tunneling therefore relates the KIE magnitude to the tunneling barrier width, and the temperature-dependence of the KIE (indicated by the ratio, A_H/A_D) with the extent to which gating modulates hydrogen transfer. Thus, a small A_H/A_D ratio indicates that gating is important in promoting the hydrogen transfer. In the example of sLO-1, Knapp and Klinman determined that the reaction

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coordinate is dominated by the heavy-atom coordinate (i.e. $\Delta G°$ and reorganization energy (λ)) and that gating plays a minor role (*24*). This indicated that the binding site was very constricted, making active dynamics (gating) energetically costly.

If we compare these sLO-1 results with that of 15-hLO, we observe significant differences (Table 2). The $^D k_{cat}$ magnitude has decreased for 15-hLO from that of sLO-1 (40 vs. 81), the E_{act} has increased (7.7 vs. 2.1) and the A_H/A_D has decreased (8 vs. 18). These combined results indicate that the hydrogen transfer coordinate for 15-hLO has a greater gating component than that of sLO-1, which suggests a less constricted binding site, as previously seen for sLO-1 active site mutants (*24*). This conclusion is not surprising, considering that 15-hLO has evolved to catalyze AA, not LA and thus a greater gating component could be introduced to the hydrogen atom transfer for LA. This hypothesis can be tested by comparing the kinetics of deuterated AA with that of deuterated LA and is currently under investigation in conjunction with a collaborator's lab.

To summarize, human lipoxygenases have appeared to retain a high rate of substrate capture (k_{cat}/K_M and $k_{cat}/K_{M(O_2)}$), such that these rates are comparable to or better than sLO-1, at physiological temperature (37 °C). Despite this, their rates of product release (k_{cat}) have slowed relative to sLO-1. 12-hLO has a distinct SIE profile from 15-hLO and sLO-1 which suggests different microscopic rate limiting steps. 15-hLO manifests a solvent dependent rate limiting step during substrate capture (k_{cat}/K_M), which could be due to the hydrogen bond network and suggests that sLO-1 and 15-hLO have a common mechanism which links the hydrogen atom abstraction and the hydrogen bond rearrangement. We propose this common mechanism is due to a hydrogen bond rearrangement of the second coordination sphere (Figure 7), which is similar in both sLO-1 and 15-hLO/15-rLO. Finally, 15-hLO displays a decreased $^D k_{cat}$, increased E_{act} and decreased A_H/A_D, which indicates a significant gating component in the hydrogen atom abstraction suggesting a "loose" fit for LA in the active site. Our group is currently exploring this work further using the deuterated, endogenous substrate, AA, with 12-hLO and 15-hLO in order to address the differences in their microscopic rate constants.

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Table 1. Comparison of Kinetic Parameters for sLO-1[a], 12-hLO[b], and 15-hLO[c]

	k_{cat}[d] (s^{-1})	k_{cat}/K_M[d] $(\mu M^{-1} s^{-1})$	$k_{cat}/K_{M(O_2)}$[e] $(\mu M^{-1} s^{-1})$
sLO-1	297(12)[f]	11(1)[f]	4.4(0.6)[g]
12-hLO	17.8(0.8)	59(12)	1.9(0.4)
15-hLO	10.1(0.6)	4.3(0.9)	1.3(0.3)

[a]Data were collected in Borate Buffer (pH 9.0) with LA.

[b]Data were collected in Trisma Buffer (pH 7.5) with AA.

[c]Data were collected in Hepes Buffer (pH 7.5) with LA.

[d]The rate constants (k_{cat} and k_{cat}/K_M) are reported for 30 ºC.

[e]The rate constants ($k_{cat}/K_{M(O_2)}$) are reported for 20 ºC.

[f]Knapp, M. J., Rickert, K., Klinman, J. P. *J. Am. Chem. Soc.* **2002**, *124*, 3865-3874.

[g]Knapp, M. J, Seebeck, F. P., Klinman, J. P. *J. Am. Chem. Soc.* **2001**, *123*, 2931-2932.

Table 2. Energy of Activation and Arrhenius Prefactor Isotope effects for sLO-1[a] and 15-hLO[b]

	k_{cat}[c] (s^{-1})	KIE[d]	E_{act} (kcal/mol)	ΔE_{act}[e] (kcal/mol)	A_H (s^{-1})	A_H/A_D
sLO-1[f]	297(12)	81(5)	2.1(0.2)	0.9(0.2)	9×10^3 (2×10^3)	18(5)
15-hLO	10.1(0.6)	40(8)	7.7(0.3)	1.0(0.1)	3×10^6 (2×10^6)	8(14)

[a]Data were collected between 5-50 ºC in Borate Buffer (pH 9.0)

[b]Data were collected between 10-35 ºC in Hepes Buffer (pH 7.5)

[c]The rate constant (k_{cat}) is reported for 30 ºC.

[d]KIE = $^D k_{cat}$ = k_{cat}H / k_{cat}D

[e]This is the isotope effect on E_{act}, ΔE_{act} = E_{act}D - E_{act}H

[f]Knapp, M. J., Rickert, K., Klinman, J. P. *J. Am. Chem. Soc.* **2002**, *124*, 3865-3874.

Scheme 1.

Fe(III)-OH

Fe(II)-OH$_2$

Fe(III)-OH

Fe(II)-OH$_2$

O$_2$
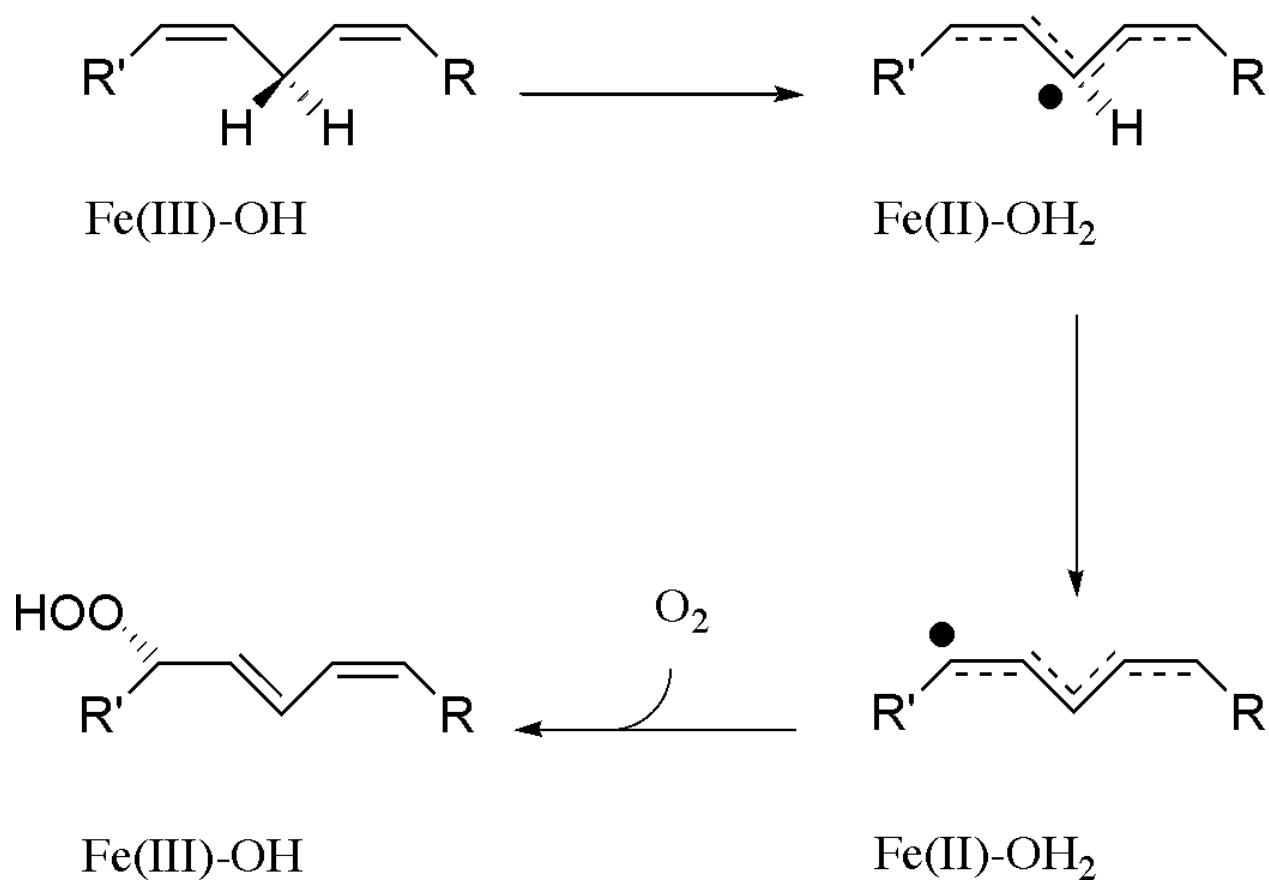
CAPTIONS

Figure 1: Temperature dependence of the observed k_{cat} of 12-hLO (triangles) and of 15-hLO (open circles) in the temperature range 10-45 ºC, pH 7.5.

Figure 2: Temperature dependence of the observed k_{cat}/K_M of 12-hLO (triangles) and of 15-hLO (open circles) in the temperature range 10-45 ºC, pH 7.5. Due to the difference of greater than 1 order of magnitude between the k_{cat}/K_M of 12-hLO and 15-hLO , the y-axis is displayed as logarithmic.

Figure 3: Initial rates of 12-hLO (triangles) and of 15-hLO (open circles) as a function of oxygen concentration at saturating fatty acid substrate (20 ºC, pH 7.5). Rates of oxygen consumption were determined as described in Materials and Methods and were defined as micromoles of dioxygen consumed per micromole lipoxygenase per second (V/E). Data were fit to the Michaelis-Menten equation.

Figure 4: The temperature dependence of the apparent primary k_H/k_D for 15-hLO at pH 7.5, $^D k_{cat}$ (diamonds) and $^D k_{cat}/K_M$ (open squares).

Figure 5: Arrhenius plot of kinetic data for 15-hLO using protio-linoleic acid (circles) and deutero-linoleic acid (open circles). Nonlinear fits to the Arrhenius equation are shown as solid lines.

Figure 6: Solvent isotope effects for the lipoxygenase reaction. Temperature dependence of the observed k_{cat} (a) and k_{cat}/K_M (b) of 12-hLO (triangles) and of 15-hLO (open circles) in the range of 15-45 ºC, pH 7.5.

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Figure 7: A view of the hydrogen bond network of wild-type sLO-1 (a) and 15-rLO (b). The iron atom is colored cyan, the residues that form the hydrogen bonding network are connected by yellow, dashed bonds. The H_{499} of sLO-1 was found to be in two conformations of approximately equal proportions and the water in the 15-rLO structure is not present, presumably due to its exclusion from the active site by the bound inhibitor (inhibitor not shown).
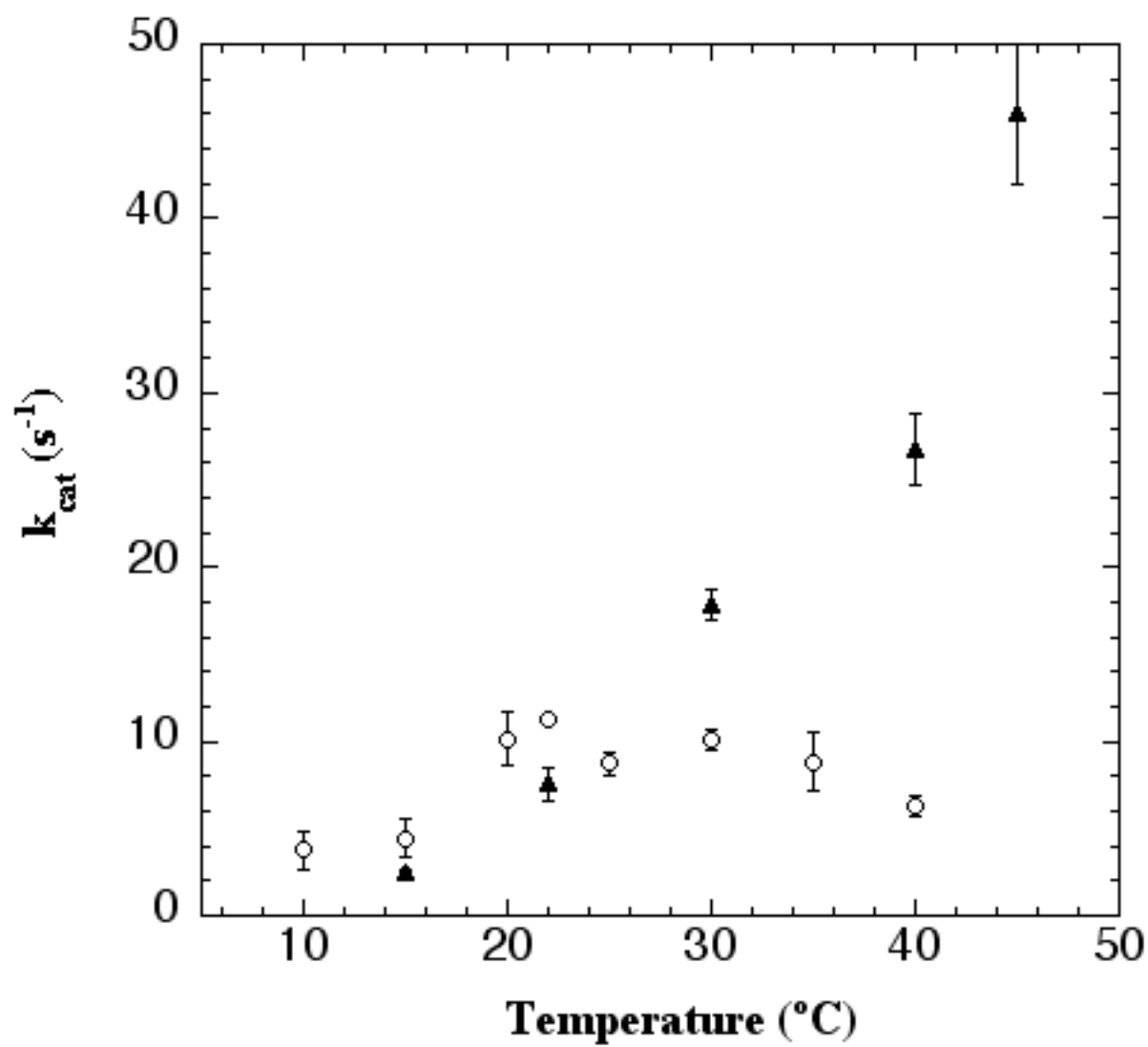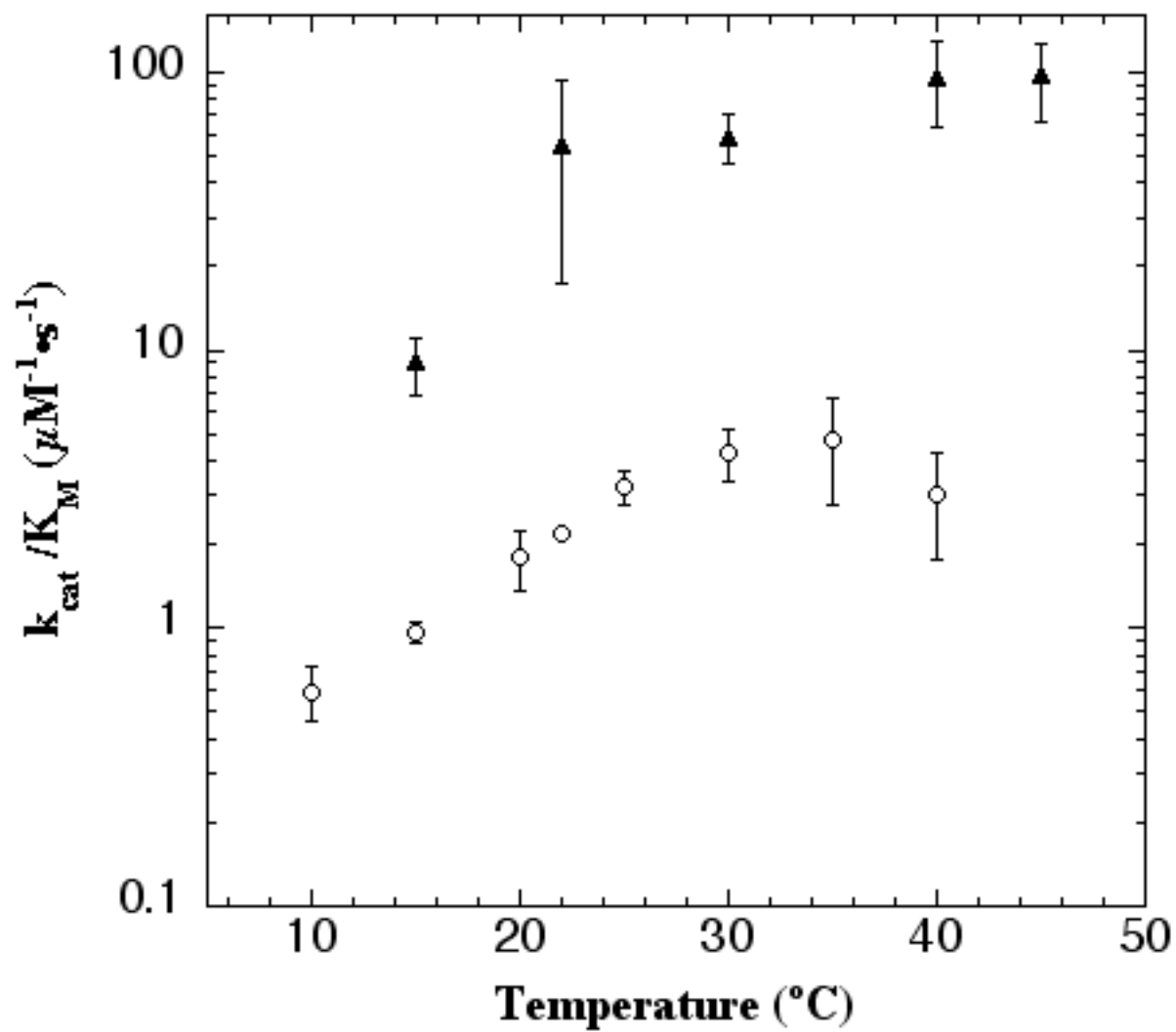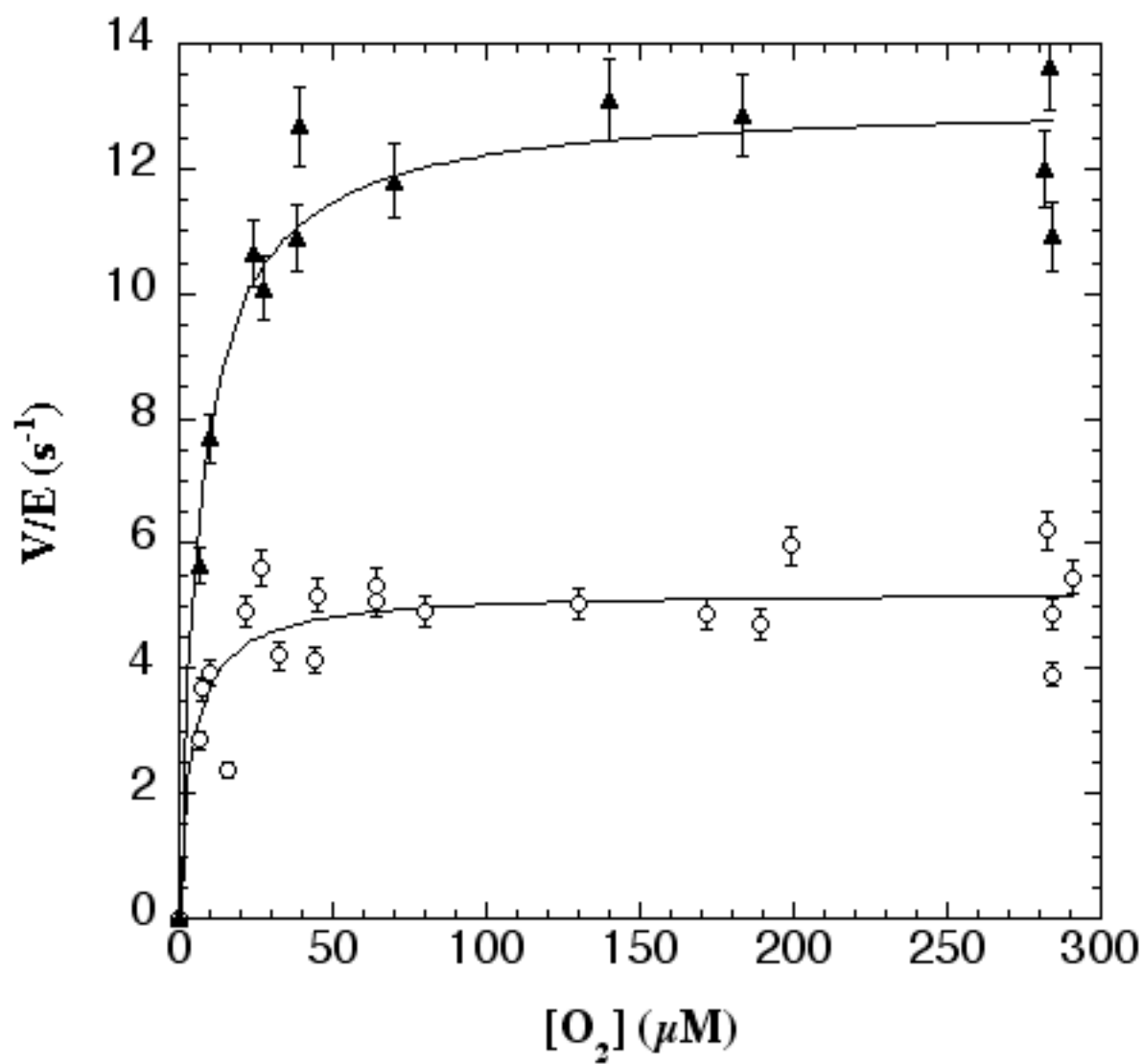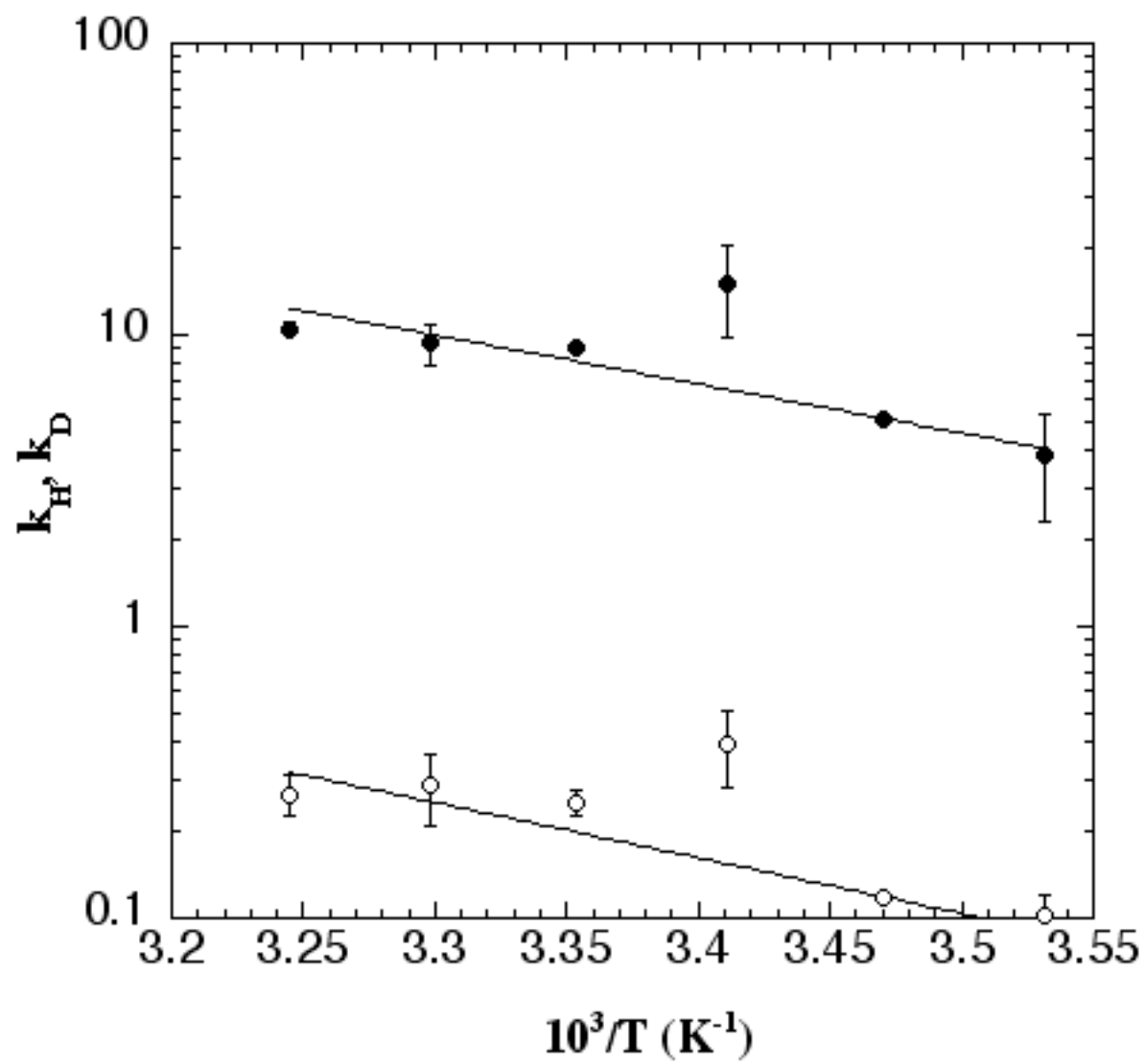
Figure 1.

Figure 2.

Figure 3.

Figure 4.
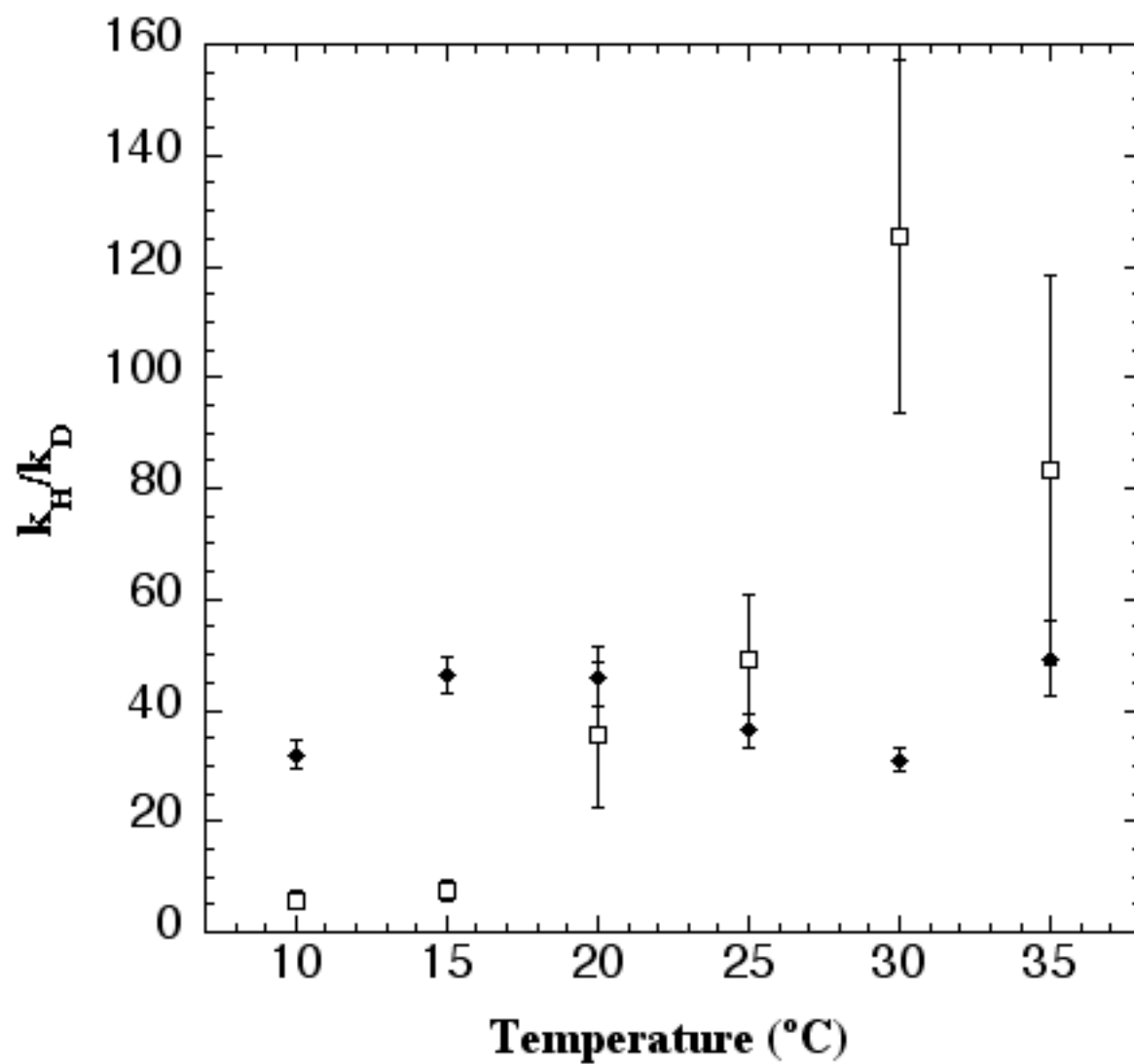
Figure 5.

Figure 6.

Figure 7.