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Hydrogen Tunneling in a Prokaryotic Lipoxygenase

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Supporting Information

ABSTRACT: A bacterial lipoxygenase (LOX) shows a deuterium kinetic isotope effect (KIE) that is similar in magnitude and temperature dependence to the very large KIE of eukaryotic LOXs. This occurs despite the evolutionary distance, an ∼25% smaller catalytic domain, and an increase in E_a of ∼11 kcal/mol. Site-specific mutagenesis leads to a protein variant with an E_a similar to that of the prototypic plant LOX, providing possible insight into the origin of evolutionary differences. These findings, which extend the phenomenon of hydrogen tunneling to a prokaryotic LOX, are discussed in the context of a role for protein size and/or flexibility in enzymatic hydrogen tunneling.

Lipoxygenases (LOXs) make up a widespread family of enzymes that convert unsaturated fatty acids to allyl hydroperoxides and other oxidized products. The first step of their reaction is a net H-atom abstraction by a metal hydroxide, usually iron but occasionally manganese,[1] that occurs between two double bonds to generate a radical intermediate.[2] The H-atom abstraction step is of significant interest because of the large, nearly temperature-independent deuterium kinetic isotope effects (KIEs) that have been reported for enzymes from soybean[3] and humans.[4] The range of KIEs (30−100) is well outside the semiclassical limits and has led to models that treat the reaction as a nonadiabatic, proton-coupled electron transfer:[5,6] accordingly, the proton behaves as a wave packet rather than a particle, passing from the substrate to the product via a transition state defined by thermally activated motions of the protein heavy atoms.

One compelling question for LOXs, as well as other C−H bond activating enzymes, is how each protein achieves the close donor−acceptor distances needed for efficient tunneling. In particular, the overall protein size and flexibility are expected to play important roles in this phenomenon. In recent years, LOXs from cyanobacteria that have diverged significantly from their plant and animal homologues and are notably smaller in size have been described.[7−9] The enzyme characterized herein from *Nostoc* sp. is one-half of a fusion protein that has been shown to convert α-linolenic acid to an allylic epoxide in a two-step reaction.[10] *In vitro* assays of the C-terminal domain (residues 344−773, NspLOX) further demonstrate conversion of α-linolenic acid and linoleic acid (LA) to hydroperoxides at similar rates.[8,11] In the study presented here, the expression of NspLOX in *Escherichia coli* is shown to yield a stable protein with ∼10% of the activity of the LOX from soybeans (SLO-1). KIEs and their temperature dependence are reported for wild-type (WT) NspLOX and a single-site mutant. These studies extend the evidence for tunneling in LOX to a prokaryotic enzyme, providing a window into the impact of altered protein structure and size on hydrogen tunneling mechanisms.

Kinetic Parameters for NspLOX. Reaction rates for the oxidation of h_{31}-LA and uniformly deuterated LA are shown along with KIEs in Figure 1. The study of LA allows direct

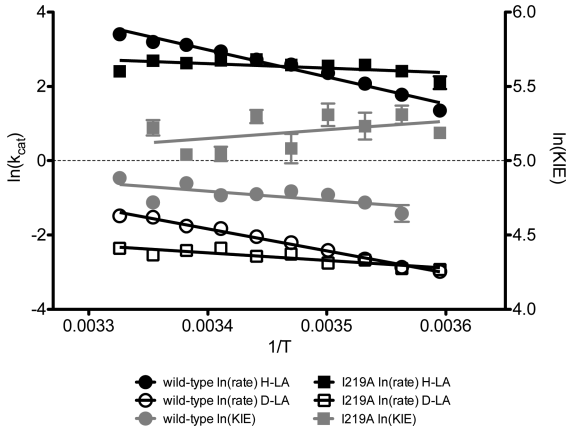

Figure 1. Temperature-dependent rates and KIEs for WT NspLOX and its I219A variant.

comparisons to the well-documented reactivity of LA with SLO-1. We observe that the K_m for NspLOX toward LA is very low [0.3−1.1 μM (Table S1 of the Supporting Information)], which, together with the characteristic lag phase seen in initial rate studies of other LOXs prevented the collection of accurate Michaelis−Menten curves at low substrate concentrations. Reaction rates were, therefore, measured under saturating conditions (ambient O_2 and 10 μM LA) at all temperatures. NspLOX has a k_{cat} at 20 °C of 19.0 ± 0.2 s^{-1} with h_{31}-LA and a KIE of 118 ± 3. The Arrhenius prefactor (A_H) and energy of activation (E_a) with h_{31}-LA are quite elevated for a native LOX [$A_H = (2.7 \pm 1.5) \times 10^{10}$ s^{-1}, and $E_a(H) = 12.4 \pm 0.3$ kcal/mol]. The $E_a(D)$ is slightly lower, 11.6 ± 0.3 kcal/mol, which leads to the appearance of a KIE that increases slightly with temperature; however, this difference between the isotopic E_a values is not statistically significant ($P < 0.10$). The KIE therefore arises solely from differences in prefactors ($\Delta\Delta S^{\ddagger}$). While this behavior is similar to that of SLO-1, the magnitude of ΔE_a is reduced in

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Table 1. Kinetic Parameters for WT NspLOX, WT SLO-1, and Their Mutants

	k_{cat} (s^{-1})		KIE				
	20 °C	30 °C	20 °C	30 °C	E_a(H) (kcal/mol)	ΔE_a (kcal/mol)	A_H (s^{-1})
WT NspLOX[a]	19.0 ± 0.2	37.1 ± 0.8[b]	118 ± 3	128 ± 3[b]	12.4 ± 0.3	−0.7 ± 0.3	(2.7 ± 1.5) × 10^{10}
I219A NspLOX[a]	15.0 ± 0.5	16.7 ± 2.4[b]	155 ± 7	164 ± 26[b]	2.7 ± 0.5	1.1 ± 0.5	(1.3 ± 1.1) × 10^{3}
WT SLO-1[c]	275 ± 9	297 ± 12	84 ± 11	81 ± 5	1.6 ± 0.2	1.1 ± 0.2	(4.1 ± 1.3) × 10^{3}
L546A SLO-1[c]	3.9 ± 0.3	4.8 ± 0.6	126 ± 3	109 ± 4	4.0 ± 0.7	1.6 ± 0.6	(4 ± 4) × 10^{3}

[a]Data for WT and I219A collected at 7.5−27.5 and 5−25 °C, respectively. [b]Extrapolated from an Eyring fit (Figure 1) for comparison to SLO-1 at 30 °C. [c]From ref 16.

relation to that of the plant enzyme (Table 1). Additional kinetic parameters are provided in Table S2 of the Supporting Information.

The most striking difference between WT NspLOX and WT SLO-1 is the large enthalpy of activation for the former. *This indicates that the properties that control the KIE are distinct from the net barrier controlling hydrogen transfer.* Nagel and co-workers have recently described anomalously elevated values for E_a and A_H in a thermophilic alcohol dehydrogenase operating under the nonphysiologic condition of low temperature.[12] Their model invokes a reversible trapping of enzyme into inactive or reduced-activity conformational substates. The return of such molecules to the catalytically viable region of the conformational landscape requires thermally activated fluctuations that are the origin of the elevated E_a values.

As an alternative, the elevated E_a for NspLOX may result from the expression of a single-domain protein, in the absence of its *in vivo*-partnered enzyme activity. However, it is fairly common for separate proteins to become fused within prokaryotic operons and for individual domains to gain and lose partners through the course of evolution.[13] As described below, we pursued the mechanistic origin of the elevated E_a value for WT NspLOX using site-specific mutagenesis.

Preparation and Characterization of I219A. NspLOX is smaller than SLO-1, lacking the N-terminal β-barrel found in most LOXs. Additionally, the catalytic domain consists of 430 amino acids in comparison to 562 for SLO-1.[14] Sequences for core portions of the proteins are shown in Figure S1 of the Supporting Information, the sequences being 22% identical and 40% similar in the represented region. Despite the low degree of overall homology, we identified Ile219 in NspLOX as the analogue of the active site Leu546 in SLO-1. Leu546 sits proximal to the active site iron in SLO-1 and has been thought to position the reactive carbon of the substrate for C−H bond activation.[15,16] The positions of Ile219 and Leu546 are within an 86-residue region that is relatively well conserved and seven amino acids N-terminal of a conserved isoleucine. In SLO-1, this is position 553, one helix turn away from Leu546 (Figure S2 of the Supporting Information); structures for SLO-1 mutated at position 553[17] or 546 (unpublished data) indicate that the side chain positions of Leu546 and Ile553 are correlated. Thus, the choice of Ile219 as a mimic of Leu546 in SLO-1 appears well-validated.

Analytical size-exclusion chromatography of NspLOX and I219A indicates a mixture of monomers and dimers (Figure S3 of the Supporting Information), with similar specific activities. As a control for a possible impact of enzyme concentration on specific activity, NspLOX was kinetically assayed at 20 °C across a range of protein concentrations that brackets the experimental conditions. The results (Figure S4 of the Supporting Information) indicate that the rate is linear between 3 and 264 μM enzyme.

The rate constant for I219A with h_{31}-LA at 20 °C was 15.0 ± 0.5 s^{-1}, slightly reduced relative to that of WT NspLOX. The deuterium KIE is elevated to 155 ± 7. Unexpectedly, the magnitudes of both E_a and A_H are greatly reduced relative to magnitudes of those of WT NspLOX (Table 1).

Role of Protein Size and Flexibility. The characterization of I219A NspLOX provides considerable insight into the properties of the WT variant. As shown in Table 1, a smaller amino acid at position 219 in NspLOX leads to an enzyme that shows values of E_a, ΔE_a, and A_H almost identical to those of WT SLO-1. We propose that the reduction in the size of a large side chain within the active site of NspLOX introduces a flexibility that is absent from WT NspLOX. This increased flexibility allows the mutant enzyme to sample catalytically viable regions of the conformational landscape more easily (reducing the magnitude of E_a and A_H in a concomitant manner). At the same time, the magnitude of the KIE and its temperature dependence are increased in relation to those of WT NspLOX. The latter is similar to the behavior seen most generally in mutant variants of C−H bond activating enzymes, i.e., an increase in the donor−acceptor tunneling distance that can be compensated for, in part, by a temperature-dependent search for a more suitable tunneling distance.[18]

The fact that WT NspLOX, with a lower k_{cat}, is strictly speaking a less effective catalyst than SLO-1 does not necessarily mean that NspLOX is a functionally defective form of SLO-1. While the values of E_a and particularly ΔE_a are highly informative from a mechanistic point of view, k_{cat} values are not necessarily reflective of evolutionary fitness. This is especially true for enzymes whose purpose is the production of secondary metabolites, the levels of which are tightly regulated and often coupled to environmental conditions. The function of prokaryotic LOXs is not known,[19] except for a report that *Nostoc punctiforme* produces fatty acid hydroperoxides in response to cell wounding.[20]

The key mechanistic property to emerge from the kinetic comparison of NspLOX and SLO-1 is the inferred change in the catalytically relevant conformational landscape. The elevated rate together with a low E_a for C−H bond activation in SLO-1 implies small conformational barriers to the achievement of tunneling ready states. By contrast, the slower rate and much higher E_a of NspLOX can be explained by a reversible trapping of the protein into noncatalytic substates. This raises the question of which changes in protein structure and flexibility may have supported a transition from NspLOX to SLO-1. Our demonstration that insertion of a single, active site mutation into NspLOX reproduces the E_a of SLO-1 suggests one pathway for conferring more conformational fitness. However, this active site alteration in NspLOX cannot improve its rate, presumably by compromising the ability of enzyme to properly constrain and/or position the H-donor and -acceptor atoms with respect to one another. The currently available sequence and structural information

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suggest an alternate route for the tuning of catalytic fitness in SLO-1. The major structural difference between SLO-1 and NspLOX appears to lie in the size of surface loops, which are enlarged in the eukaryotic enzyme (Figure S5 and Table S3 of the Supporting Information). We suggest that a change in surface loops may have provided the catalytically strategic alteration, simultaneously ensuring a catalytically productive placement of the reactive carbon of substrate via retention of a bulky active site side chain while introducing the capability for more protein flexibility through increased surface loop size. Appropriately designed mutagenesis studies should permit further testing of this hypothesis.

■ ASSOCIATED CONTENT

Supporting Information

Experimental methods, detailed kinetics data, and data from controls. 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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