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Hydrostatic Pressure Studies Distinguish Global from Local Protein Motions in C–H Activation by Soybean Lipoxygenase-1

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Abstract: *The proposed contributions of distinct classes of local versus global protein motions during enzymatic bond making/breaking processes has been difficult to verify. We employed soybean lipoxygenase-1 as a model system to investigate the impact of high pressure at variable temperatures on the hydrogen-tunneling properties of the wild-type protein and three single-site mutants. For all variants, pressure dramatically elevates the enthalpies of activation for the C–H activation. In contrast, the primary kinetic isotope effects (KIEs) for C–H activation and their corresponding temperature dependencies remain unchanged up to ca. 700 bar. The differential impact of elevated hydrostatic pressure on the temperature dependencies of rate constants versus substrate KIEs provides direct evidence for two distinct classes of protein motions: local, isotope-dependent donor–acceptor distance-sampling modes, and a more global, isotope-independent search for productive protein conformational sub-states.*

There has been increasing recognition that a hierarchy of protein motions can affect catalytic rate enhancement in enzymatic reactions, with these motions occurring throughout the entire protein on time scales that vary from femtoseconds to milliseconds.[1] Although loop closure directly over the active site has long been implicated in catalysis,[2] the importance of a highly tuned conformational landscape in optimizing active-site chemistry is also increasingly apparent.[3] Biophysical probes, aided by computational work, are showing progress in identifying functionally relevant motions;[1a,4] however, our ability to design experimental methods that can distinguish the impact of global and local protein motions on isolated chemical steps remains a challenging and compelling issue.[5] Soybean lipoxygenase-1 (SLO-1, Figure 1), a prototype for the study of enzymatic C–H activation through hydrogen-tunneling, is providing a unique window into the subtle influence of protein motions on enzyme catalysis.[6,7] In the present study, we focused on understanding the underlying interaction between two distinct classes of catalysis-linked protein motions in H-transfer reactions: local distance sampling that is dependent on substrate labeling with isotopes, and global conformational landscapes that are independent of this labeling.[3,5] We systematically explored the combined impact of temperature and pressure on the full set of kinetic parameters for wild-type (WT) SLO-1 and a range of mutants with established kinetic properties at ambient pressure. Surprisingly, pressure was found to primarily influence the isotope-independent motions in SLO-1, leaving local, isotope-dependent motions virtually unperturbed.

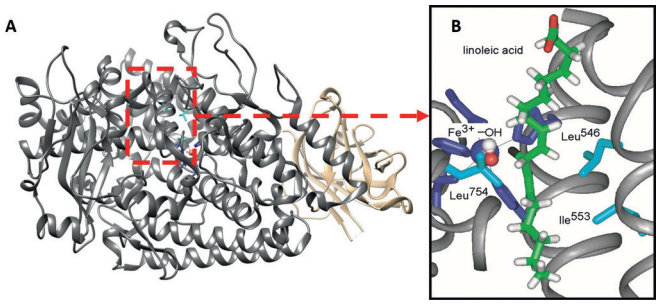

Figure 1. A) X-ray structure of SLO-1 (PDB ID: 1F8N), with active-site region highlighted by a red box and the N terminus shown in tan. B) X-ray structure of the active site of SLO-1, with LA modeled into the active site. The side chains L546, L754, and I553 are shown in cyan. Figure taken from Ref. [6b].

A large number of controls were first conducted to establish an artifact-free steady-state kinetic assay (Section 2.1 in the Supporting Information). The pressure dependence of SLO-1 activity with the substrates linoleic acid (H-LA) and perdeutero linoleic acid (D-LA) was then measured between 1 bar and 1034 bar at five temperatures between 15 °C and 35 °C. Figure 2 A,B shows three dimensional pressure–temperature effects on the rate constants for H-LA ($k_{cat\text{-}H}$) and the primary kinetic isotope effects (KIEs) on k_{cat} ($^{D}k_{cat}$) for WT SLO. In general, the effect of high pressure on the rate constants for both H- and D-LA increases with temperature (Figure 2 A), leading to an almost negligible effect on the $^{D}k_{cat}$ values (Figure 2 B). An alternate representative of pressure effects is plotted for $k_{cat\text{-}H}$ and $^{D}k_{cat}$ at each experimental temperature (Figure S3 in the Supporting Information).

In order to examine these trends in more detail, we introduce the parameter *S*, which represents the ratio of the kinetic parameters at 344, 688, and 1034 bar relative to

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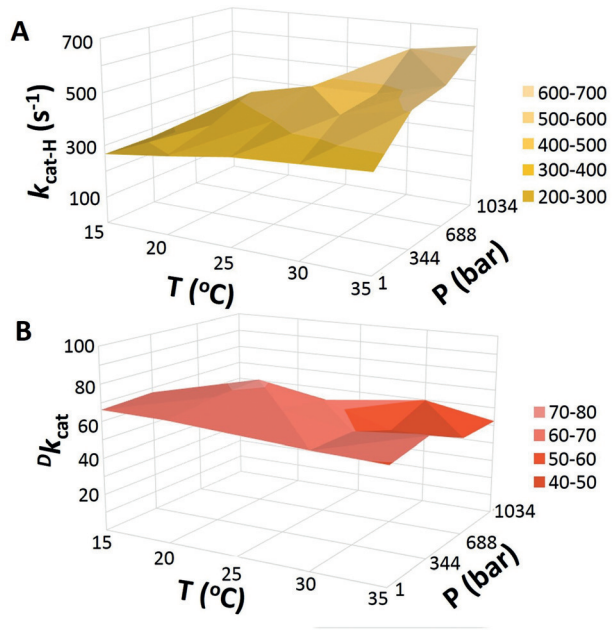

Figure 2. The combined impact of pressure and temperature on kinetic parameters for WT-SLO: k_{cat-H} (A) and $^{D}k_{cat}$ (B).

ambient pressure; this provides a quantitative indicator of the impact of pressure in the experimental temperature range. The unaltered $S(k_{cat-H})$ values at 15 °C for the WT enzyme (Table 1) demonstrate that elevated pressure barely influences the rate constants with H-LA at low temperature. In contrast, at 35 °C, the $S(k_{cat-H})$ value increases to 1.77 at 1,034 bar. The S values with D-LA as substrate are the same as with H-LA at 344 bar, but rise slightly faster above this pressure. We have previously shown that quite small changes in H-transfer donor/acceptor distances can lead to significant rate differences.[6d] Using previously derived expressions,[7a] we attribute the present increases in rate at 1.034 bar to a very small active-site compression of ca. 0.02 Å, which affects H transfer slightly less than D transfer.[8]

Table 1: Impact of pressure on rate constants and KIEs at the extremes of the experimental temperature range.[a]

SLO	T [K]	344 bar S (k_{cat-H})	344 bar S (k_{cat-D})	688 bar S (k_{cat-H})	688 bar S (k_{cat-D})	1034 bar S (k_{cat-H})	1034 bar S (k_{cat-D})
WT	288	1.00 (0.05)	0.98 (0.03	0.97 (0.08)	1.17 (0.02)	0.97 (0.09)	1.22 (0.03)
	308	1.40 (0.18)	1.32 (0.06)	1.50 (0.10)	1.69 (0.07)	1.77 (0.08)	2.10 (0.11)
I553V	288	1.08 (0.09)	1.02 (0.02)	1.10 (0.13)	1.07 (0.01)	1.01 (0.19)	1.25 (0.02)
	308	1.10 (0.02)	1.00 (0.02)	1.43 (0.03)	1.27 (0.02)	1.66 (0.03)	1.82 (0.04)
L546A	288	1.03 (0.02)	1.14 (0.02)	1.20 (0.06)	1.32 (0.04)	1.28 (0.03)	1.56 (0.03)
	308	1.07 (0.02)	1.28 (0.04)	1.42 (0.07)	1.55 (0.04)	1.68 (0.07)	1.95 (0.03)
L754A	208	1.58 (0.10)	N.D.[b]	2.92 (0.14)	N.D.[b]	4.24 (0.24)	N.D.[b]
	308	1.90 (0.13)	N.D.[b]	3.35 (0.20)	N.D.[b]	5.26 (0.44)	N.D.[b]

[a] S is the ratio of kinetic parameters at the pressure listed relative to ambient pressure (Table S3–S6). [b] The k_{cat-D} values were too slow to be accurately determined.

Three variants, I553V, L546A and L754A, were then investigated at the same pressure and temperature range as the WT enzyme. The reason that I553V was chosen as the representative of the I553X series is that the most extreme variants, I553A and I553G, led to protein instability under high pressure. As seen in Figure 1 B, L546 and L754 sandwich the reactive carbon atom (position 11) of the substrate into the proper position for reaction with the iron center, whereas I553 is more distal. Compared to the WT, the single mutants L546A and L754A reduce k_{cat-H} by 10^2 and 10^3-fold, respectively, and lead to an increase in the contribution of an H-transfer donor/acceptor distance sampling.[6b] The more distal variant, I553V, exhibits changes in active-site flexibility without a significant effect on the magnitude of k_{cat}.[6c] The fact that these variants show such a wide variation in properties makes them excellent candidates for comparative high-pressure studies. Surprisingly, even with the generation of interior cavities and more flexible active sites, I553V and L546A display pressure-induced trends similar to the WT in their rate constants and $^{D}k_{cat}$, albeit with minor differences in the S values. For L754A, it was only possible to measure k_{cat-H} under high pressure, owing to the very low turnover efficiency with D-LA. Table 1 shows that the k_{cat-H} value for L754A is more sensitive to elevated pressure, as reflected by the maximal approximately 5-fold increase in rate constants above 1k bar, compared to the less than 2-fold acceleration in rate for the other SLO-1 variants. Anisotropic pressure effects are not unexpected, owing to the asymmetric effect of pressure on protein functional compressibility.[9]

The temperature-dependent rate constants (Tables S3–S6 in the Supporting Information) were then fit to the Arrhenius equation to give the activation energies for H-LA ($E_{a(H)}$) and D-LA ($E_{a(D)}$), as well as ΔE_a [$(E_{a(D)}$-$E_{a(H)}$] under varied pressures (Table 2). As shown, increasing the pressure to 688 bar elevates E_a(H) in a regular manner, with a more abrupt change occurring at or above 1 kbar. The Arrhenius prefactor (A_H) rises concomitantly with E_a, as expected for the much greater changes in E_a (Table 2) than k_{cat} (Table 1).[10] In marked contrast to the trends in E_a values, the temperature dependency of the kinetic isotope effect (ΔE_a) remains constant up to 688 bar, in the case of both the weakly temperature-dependent WT SLO ($\Delta E_a \sim 1.0$ kcal mol^{-1}, Table 2, entries 1–3) and the more temperature-dependent I553V and L546A variants ($\Delta E_a \sim 3.0$ kcal mol^{-1}, Table 2, entries 5–7 and entries 9–11). A break in protein behavior above 688 bar is evident from the ΔE_a as well as the E_a values, producing a reduction in the temperature dependence of the KIEs for WT, I553V, and L546A. The concomitant break in E_a and ΔE_a at approximately 1 kbar indicates a discontinuous impact on protein structure that likely involves both a more rigid active site (smaller ΔE_a) and impaired conformational landscape (large E_a). The origin of this effect is currently unknown, but is almost certainly related to a pressure-induced onset of partially unfolded protein.[11]

Table 2: Arrhenius parameters for rate constants and KIEs at each pressure investigated.[a]

Entry	Enzyme	Pressure [bar]	E_a(H) [kcal mol^{-1}]	ΔE_a [kcal mol^{-1}][b]	ln(A_H)
1	WT	1	2.63(0.33)	1.19(0.42)	10.2(0.6)
2		344	5.65(0.46)	0.81(0.65)	15.5(0.9)
3		688	6.03(1.17)	1.02(1.12)	15.7(2.3)
4		1034	7.62(0.63)	0.43(0.79)	18.9(1.2)
5	I553V	1	3.06(0.31)	2.82(0.41)	10.5(0.5)
6		344	3.09(0.40)	2.85(0.61)	10.6(0.7)
7		688	4.90(0.46)	2.93(0.69)	13.7(0.8)
8		1034	8.01(0.55)	0.89(1.08)	19.0(1.0)
9	L546A	1	3.90(0.20)	3.02(0.51)	8.7(0.4)
10		344	4.86(0.35)	3.36(0.37)	10.4(0.7)
11		688	5.37(0.48)	2.95(0.52)	11.4(0.8)
12		1034	6.67(0.69)	2.32(0.78)	13.7(1.3)
13	L754A	1	2.79(0.20)	N.D.[c]	3.4(0.1)
14		344	4.31(0.60)	N.D.[c]	6.5(1.0)
15		688	3.64(0.39)	N.D.[c]	6.0(0.7)
16		1034	4.06(0.36)	N.D.[c]	7.1(0.6)

[a] The obtained values for E_a and ΔE_a are slightly different, with larger errors, compared to the previous report,[6] probably due to the narrower temperature window (15–35 °C) compared to a range of 5–50 °C in previous studies. In the present study, protein instability at high pressure precluded measurements below 15 °C or higher than 35 °C.
[b] $\Delta Ea = E_a(D) - E_a(H)$. [c] The E_a(D) values of L754A under varied pressure were experimentally inaccessible.

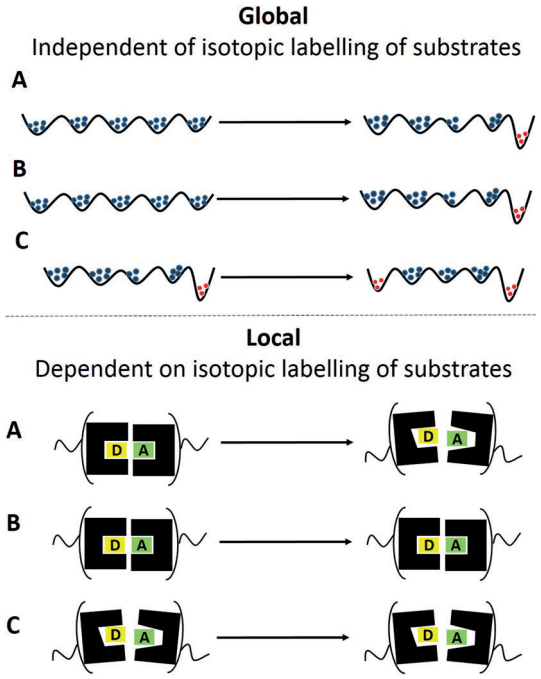

Figure 3. Different patterns that connect changes in local (top) to global (bottom) motions, following the introduction of protein perturbants. In (A), both the global energy landscape and the local distance sampling are altered. In (B) and (C), the effects are uncoupled. The distinction between (B) and (C) is that (B) represents native SLO-1 while (C) represents SLO-1 variants for which protein have already been altered by mutagenesis. D = H-transfer donor, A = H-transfer acceptor.

The fact that the values of ΔE_a for WT remain almost identical up to a pressure of 688 bar implies an unaltered force constant for the distance-sampling mode as the protein undergoes compression. The contrasting and significant changes in the overall E_a values in this pressure range could, in principle, have arisen from a variety of factors, including changes in reaction driving force (ΔG^o), reorganization energy (λ), the energy barrier for donor/acceptor distance sampling E_x and/or alterations in the conformational landscape (Supporting Information 2.2). Importantly, our ability to eliminate any significant change to E_x up to 688 bar implies a high degree of insulation of the active site from pressure-induced structural changes. Since the distance sampling term, E_x, which leads to the experimental ΔE_a, is dependent on both the initial H-transfer donor/acceptor distance and the local electrostatic interactions that determine the force constant for distance sampling, we similarly conclude that significant changes in the local electrostatic properties affecting λ and ΔG^o are likely to be quite small. These properties imply that the differential effects of elevated pressure on E_a versus ΔE_a arise from alterations in the global conformational landscape of SLO-1. In support of this conclusion, the variants that impart packing defects and more flexible active sites[6b,c] show significantly elevated E_a values while leaving ΔE_a unaltered with pressure.

The ability to demonstrate a clear cut distinction between local and global effects, as seen herein with high pressure and SLO-1 (Figure 3 B, C), is quite unique, since other perturbants of protein structure/dynamics generate different patterns that connect changes in global conformational sampling with local active-site distance sampling (Figure 3 A). For example, non-physiological temperatures, and/or active-site mutants in thermophilic alcohol dehydrogenase (ht-ADH) and SLO-1 have previously been shown to alter both the overall protein conformational landscape and the active-site packing (Figure 3 A).[10,12,13] Even in earlier hydrostatic pressure studies involving the reductive half reactions of morphinone reductase,[14a] pentaerythritol tetranitrate reductase,[14b] and aromatic amine dehydrogenase,[9] a coupled effect was seen on local and global motions (Section 2.3 in the Supporting Information). We attribute the present result with SLO-1 to its unique structure. As shown in Figure 1 A, SLO-1 is a 94.4 kDa monomer with several unstructured loops on the surface and a buried active site. The surface region may be influenced by pressure through a variety of changed intramolecular interactions, such as disassociation of ion pairs, solvation of newly exposed hydrophilic residues, or an alteration of surface side-chain conformers.[15] Unlike the unstructured surface region of SLO-1, the active site is within a densely packed hydrophobic core that consists mainly of α-helices, which are relatively more resistant than other secondary-structure elements to pressure as a perturbant.[16]

The finding that *both* E_a and A_H are elevated simultaneously without a substantial drop in rate under high pressure is highly analogous to the trends previously reported for ht-ADH[13] and the pair L754A vs. I553A/L754A in SLO-1.[12] A fundamental pattern is emerging for enzymes, in which either non-physiological temperatures (as reported for ht-ADH), mutagenesis (as reported for ht-ADH and SLO-1), or high

pressure (current study) produce an increased population of low-activity or inactive conformers within the conformational landscape. In the current case, we propose that experimental observations of elevated E_a following pressure perturbation can be seen as measures of the (additional) thermal fluctuation required to restore a homeostatic distribution of optimally active protein sub-states from the low-activity or inactive conformers.

In the present study, we engaged high pressure as an alternative means to address the functional role of the protein conformational landscape. Although SLO-1 is thus far unique in its ability to provide a separation of the two classes (local and global) of motion, the findings have general relevance to our understanding of the factors that govern catalysis. The observed elevation of E_a together with an unaltered ΔE_a reveals and corroborates changes in E_a as an indicator of impaired conformational landscapes within enzyme/substrate complexes. Unlike the pressure-induced conformational shifting in protein folding or ligand binding that is often studied by biophysical tools,[11] the differences among sub-conformers in the enzyme/substrate (E·S) conformational landscape is very subtle and difficult to correlate with catalytic efficiency. Our current study validates enzymatic kinetic studies combined with hydrostatic-pressure techniques as a sensitive probe for detecting the shifting in the E·S conformational landscape that is considered critical for optimal catalysis. The pressure–temperature variation in the enzymatic kinetics of WT, I553V, and L546A SLO-1 may further provide a set of experimental parameters that can be used to incorporate global protein conformational sampling into current mathematical models for non-adiabatic deep tunneling of hydrogen.[7] Finally, we suggest that these studies, which validate the concept of remote tuning of catalytic efficiency, should be considered in the future design of biocatalysts.[17]

Acknowledgements

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