Supporting Information

Methods

**NspLOX growth and purification.** The C-terminal lipoxygenase domain of the all8020 gene from Nostoc sp. PCC 7120, with an additional N-terminal His$_6$ tag, was expressed from pET-17b (previously described) in BL21(DE3) CodonPlus-RIL and using Studier’s autoinduction method. The media used 2xYT as the base, 1x metal supplementation, and 50 mg/L ampicillin. For each growth, a 500 mL culture was inoculated with 2 mL of an overnight LB culture (100 mg/L ampicillin and 34 mg/L chloramphenicol). After a 4-h incubation at 37°C and 300 rpm shaking the incubator temperature was reduced to 20°C and the culture incubated overnight. Cells were harvested by centrifugation and stored at -80°C.

The cell pellet was resuspended in BugBuster supplemented with 20 mM sodium phosphate (pH 7.4), 200 µM magnesium sulfate, 1 mM AEBSF, and 25 U/mL benzonase. The suspension was stirred for 15 min at room temperature and then 30 min at 4°C. Cell debris were pelleted by centrifugation (4°C, 20 000 rpm, 35 min).

The clarified cell lysate was loaded on to a GE Healthcare His GraviTrap column. The column was washed with 5 mL of 50 mM sodium phosphate (pH 8.0), 500 mM NaCl, 10% glycerol (Buffer A), 7 mL of Buffer A + 500 mM sodium chloride + 70 mM glycine, 5 mL of Buffer A, 5 mL of Buffer A + 20 mM imidazole. Bound protein was eluted with four 1-mL aliquots of Buffer A + 250 mM imidazole, with the majority of the protein eluting in fraction 2. The purified protein was dialyzed into 100 mM sodium phosphate (pH 8.0), 10 mM sodium chloride, frozen on liquid nitrogen and stored at -80°C. Protein so prepared was assayed for iron content by ICP-AES (Table S3).

**Kinetic assays.** Reactions were initiated by the addition of enzyme to a solution of linoleic acid in 100 mM sodium phosphate, 10 mM sodium chloride and monitored at 234 nm (ε$_{234}$ = 23 400 M$^{-1}$ cm$^{-1}$). The lag phase characteristic of lipoxygenase auto-activation was observed and the rate in the linear portion recorded. For $h_{31}$-linoleic acid reactions, protein concentrations were calculated from the dilution factor. For $d_{31}$-linoleic acid reactions, the protein concentration in each assay was calculated from the A$_{234}$ ($ε_{234}$ = 230 000 M$^{-1}$ cm$^{-1}$) observed before substrate turnover began.

Enzyme diluted to working concentration (< 1 µM) lost approximately 10% of its activity after 5 min. To compensate, the concentrated enzyme stock (> 300 µM) was diluted to the working concentration immediately before addition to the reaction. A new working stock was made for each run. The concentrated enzyme stock retained its activity for at least 2 h when kept on ice.

**Parameter calculations.** Kinetic parameters and their associated errors were determined using Prism and RStudio. Standard errors of mean values are presented. Experimental KIEs were calculated using the bootstrap method and are bias adjusted. Arrhenius prefactor ratios were treated using Fieller’s Theorem. Reaction rates decreased above 27.5°C and 25°C for WT and
I219A, respectively, and deviated from the trend below 7.5°C for WT with H-LA, and those data are omitted. Rates are corrected for metal content (Table S4).

**Structural homology model.** A structural homology model of NspLOX was constructed using Prime with default settings. Two prokaryotic LOXs, *P. homomalla* allene oxide synthase (30% identity in the LOX domain, PDB 3DY5) and *P. homomalla* 8R-lipoxygenase (26% identity in the LOX domain, PDB 2FNQ) were selected as templates. An active-site overlay of the two template structures with SLO-1 is shown in Figure S6. The resultant structural homology model for NspLOX is shown in an overlay with the full-length catalytic core of SLO-1, Figure S5 and the active site of SLO-1, Figure S7.

**Table S1.** Michaelis constants for WT NspLOX.

| Temperature | $K_{m}$ for H-LA (µM) | $K_{m}$ for D-LA (µM) |
|-------------|------------------------|------------------------|
| 5°C         | 0.32±0.06              | 0.39±0.07              |
| 10°C        | 0.53±0.08              | 0.27±0.04              |
| 15°C        | 0.59±0.07              | 0.54±0.09              |
| 20°C        | 0.72±0.06              | 0.42±0.05              |
| 25°C        | 1.14±0.11              | 0.70±0.09              |
| 30°C        | 1.08±0.10              | 1.14±0.14              |
**Table S2.** Complete thermodynamic parameters for WT and I219A NspLOX with H-LA and D-LA. SLO values are included for comparison and were previously reported. H-LA/D-LA parameters were calculated before rounding and for that reason, may not match precisely. Ranges within parenthesis are skewed standard error ranges. Arrhenius parameters for SLO were recalculated using nonlinear least squares fitting from Knapp, et al. Published values are included in square brackets, if different.

|                  | WT-H       | WT-D       | I219A-H    | I219A-D    | WT SLO-H    | L546A SLO-H   |
|------------------|------------|------------|------------|------------|-------------|--------------|
| $k_{cat}$ @ 20° (s$^{-1}$) (measured) | 19.0±0.2   | 0.162±0.004 | 15.0±0.5   | 0.096±0.006 | 275±9       | 3.9±0.3      |
| $k_{cat}$ @ 30° (s$^{-1}$) (extrapolated for NspLOX) | 37.1±0.8   | 0.289±0.001 | 16.7±2.4   | 0.102±0.007 | 297±12      | 4.8±0.6      |
| $E_a$ (kcal/mol) | 12.4±0.3   | 11.6±0.3   | 2.7±0.5    | 3.7±0.6    | 1.6±0.2     | 4.0±0.7      |
| $A_H$ (s$^{-1}$) | (2.7±1.5)$\times10^{10}$ | (6.2±2.8)$\times10^{10}$ | 1300±1100 | 49±47 | 4100±1300 | 4000±4000 |
| $\Delta H^\ddagger$ (kcal/mol) | 11.6±0.3   | 10.9±0.3   | 2.1±0.5    | 3.1±0.6    | 1.0±0.2     | 3.4±0.7      |
| $-T\Delta S^\ddagger$ @ 20° (kcal/mol) | 3.8±0.3   | 7.4±0.3   | 13.5±0.5   | 15.5±0.6   | 12.9±0.2    | 13.0±0.6     |
| KIE @ 20° (measured) | 118±3     | 155±7     | 84±11      | 126±3      |
| KIE @ 30° (extrapolated for NspLOX) | 128±3   | 164±26    | 81±5       | 112±11     |
| KIE average     | 118±3     | 181±7     |            |            |
| $\Delta E_a$(D–H) (kcal/mol) | -0.7±0.3 | 1.1±0.5 | 1.1±0.2 [0.9±0.2] | 1.6±0.6 [1.9±0.6] |
| $A_H/A_D$       | 430 (175-900) | 30 (5-770) | 12±6 [18±5] | 8 (0-44) [4±4] |
| $\Delta H^\ddagger$ (kcal/mol) | -0.7±0.3 | 1.1±0.6 | 1.1±0.2 | 1.5±0.6 |
| $\Delta(T\Delta S^\ddagger)$ @ 20° (kcal/mol) | 3.5±0.3 | 1.9±0.5 | 1.5±0.2 | 1.2±0.6 |
Table S3. Comparison of number of amino acids found in surface loops of NspLOX and SLO. Loops that are more than ten residues shorter in NspLOX are indicated in bold.

| Loop | NspLOX | SLO | change in length |
|------|--------|-----|-----------------|
|      | N-term | C-term | amino acid length | N-term | C-term | amino acid length |
| 1    | 96     | 98   | 3               | 368    | 380   | 13               |
| 2    | 107    | 117  | 11              | 389    | 426   | 38               |
| 3    | 141    | 143  | 3               | 451    | 465   | 15               |
| 4    | 256    | 260  | 5               | 589    | 602   | 14               |
| 5    | 369    | 374  | 6               | 713    | 738   | 26               |
| 6    | 401    | 409  | 9               | 767    | 799   | 33               |
| Total| 69     |      | 115             |

Table S4. NspLOX iron content (as determined by ICP-AES) and correction factors used in determining rates.

| % iron content | Correction factor |
|----------------|-------------------|
| Wild-type      | 80.1±0.3%         | 1.25             |
| I219A          | 67.7±0.6%         | 1.48             |
**Figure S1.** Partial sequence alignment of SLO and NspLOX. Metal ligands (three His and one Asn) are shown in bold. The mutation site (546 in SLO and 219 in NspLOX) is indicated with grey.

| SLO   | 479  | WLLAKAYVIVNDSCYHQLMHNLTHAAMEQPFVIATHRHLHLSVLEPIYKLLTYPYRNNNMI   |
|-------|------|-------------------------------------------------------------------|
|       |      | W AK + + ++L H H +E +V+ + R L+ HE + + IP + I                                      |
| NspLOX| 152  | WQYAKRTYIQAEFLSQUEKLHARCHFNEIQVMAIKRRLAPTHPVRAPFINPHLEGIFI          |
| SLO   | 539  | NALARQSEINANGIETTFILPSKYSV------EMSSAVYKNVFTD---QALPADLKRGGV        |
|       |      | N+ A I + G I ++ S+ E+S Y D ++ P DL                                        |
| NspLOX| 212  | NSSAVPKIGSITGFPIASMLTQGSIVDVMKNELSKLKNMPADLPDIPDGLF----              |
| SLO   | 591  | AIKDPSTPHGVRLLIEDYPAADGLEDIAAAKTQVQSVYVPYLLARRDDVKNDESELQHNNW         |
|       |      | TP AA W + +V++ L +D+ + + +Q                                               |
| NspLOX| 268  | -----TP------------------------AAAT--YWELLNNYYEQ---GLLQPFEDLRTVEVNAIQ--- |
| SLO   | 650  | KEAVEKGHDLKDKFDMW------PKLQTLLEDLVEVECLIIWIASALHAAVNEGQYFYGG        |
|       |      | V+ +LK++ + PK + E L + II+ +S LH+ NF QY G                                  |
| NspLOX| 303  | ---VDELFAELKERSLYSGDQFPKYESSE-LKSLILMYIYHSSF1HSWANFKQYDDAG            |

**Figure S2.** Active site of SLO. The iron/hydroxide cofactor is shown in firebrick/red. Leu546 and Leu754 are shown as blue spheres. Ile553 is shown as grey spheres.
Figure S3. FPLC elution profile. NspLOX elution profiles from a size-exclusion column at the assay pH. Wild-type and I219A NspLOX elute at 13.0 mL and 13.1 mL (at 22 °C), respectively, which correspond to masses of 144 kDa and 142 kDa. The shoulder at 14.2 mL in the wild-type, 500 nM, 22°C, profile corresponds to a mass of 94 kDa and is likely the monomer. (Because of their ellipsoid shape, lipoxygenases are expected to elute sooner than their actual mass would predict.) This suggests that NspLOX exists primarily as a mixture of monomer and dimer in solution. The spikes near 1 and 8 mL in the upper figure and near 1, 8, and 18 in the lower figure are pressure artifacts. Chromatograms were normalized to a maximum peak height of 1. The peaks at 19-20 mL show no activity with linoleic acid and are likely contaminants from the FPLC system. The stretched elution profile at 4 °C is due to differences in the volume calibrations of the two FPLC systems used.
**Figure S4.** Dependence of rate on NspLOX concentration. Data were collected at 13 µM NspLOX with both H-LA and D-LA. Concentrations less than 13 µM were assayed using H-LA; concentrations higher than 13 µM were assayed using D-LA. The observed rates were normalized relative to the 13 µM values.

![Graph showing [E] dependence of rate](image)

**Figure S5.** Structure of SLO for comparison to a sequence-derived structure for NspLOX. Loops that are shorter in NspLOX (labeled according to Table S4) are shown as stick models of the main chain trace, with SLO in blue and NspLOX in yellow. Arrows show the N-to-C-terminus direction. The SLO LOX domain (residues 278-839) and the NspLOX model were superimposed using the “super” function in PyMOL. The LOX domain is colored white, the catalytic iron firebrick, the metal-bound hydroxide red, and the N-terminal beta barrel grey.
**Figure S6.** Overlay of the SLO-1 crystal structure (white, PDB 3PZW) with *P. homomalla* allene oxide synthase (blue, PDB 3DY5) and *P. homomalla* 8R-lipoxygenase (yellow, PDB 2FNQ). The analogs to NspLOX Ile219 in SLO (Leu546), 8R-LOX (Ile805), and allene oxide synthase (Leu804) are circled. The iron cofactor and its bound water are shown as spheres. Ligand side chains, Ile553, Leu754 and their analogs in the *P. homomalla* proteins are shown as sticks.

![Figure S6 Overlay of the SLO-1 crystal structure with P. homomalla proteins.](image)

**Figure S7.** Overlay of the SLO-1 crystal structure (white, PDB 3PZW) with the NspLOX homology model (red). The residue mutated in this study (Ile219) is circled.

![Figure S7 Overlay of the SLO-1 crystal structure with NspLOX model.](image)
References

(1) Zheng, Y., Boeglin, W. E., Schneider, C., and Brash, A. R. (2007) A 49-kDa Mini-lipoxygenase from Anabaena sp. PCC 7120 Retains Catalytically Complete Functionality. *J Biol Chem* 283, 5138–5147.

(2) Studier, F. W. (2005) Protein production by auto-induction in high density shaking cultures. *Protein Sci* 41, 207–234.

(3) Motulsky, H. (1995) Confidence Interval of a Ratio of Two Means, in *Intuitive Biostatistics* 1st ed., pp 285–286. Oxford University Press, USA.

(4) Jacobson, M. P., Pincus, D. L., Rapp, C. S., Day, T. J. F., Honig, B., Shaw, D. E., and Friesner, R. A. (2004) A hierarchical approach to all-atom protein loop prediction. *Proteins* 55, 351–367.

(5) Jacobson, M. P., Friesner, R. A., Xiang, Z., and Honig, B. (2002) On the role of the crystal environment in determining protein side-chain conformations. *J Mol Biol* 320, 597–608.

(6) Schrödinger, LLC. (2013) Prime, version 3.3. New York, NY.

(7) Rickert, K., and Klinman, J. P. (1999) Nature of hydrogen transfer in soybean lipoxygenase 1: separation of primary and secondary isotope effects. *Biochemistry* 38, 12218–12228.

(8) 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.

(9) Schrödinger, LLC. The PyMOL Molecular Graphics System, Version 1.6.0.0.