Supporting Information

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SI Materials and Methods

Chemicals and Biochemicals. Nitronium tetrafluoroborate (NO_2BF_4), desferrioxamine (DFO), and Mb from equine skeletal muscle were purchased from Sigma-Aldrich. Protein standards for molecular mass determination were from Bruker Daltonics. Hemin (Fe-protoporphyrin IX chloride) and mesoheme (Fe-mesoporphyrin IX chloride) were from Livchem; 3-morpholinosydnonimine hydrochloride (SIN-1) was from Calbiochem. Hydroxylapatite (Bio-Gel HTP) and Chelex resin were from Bio-Rad; DE-52 from Whatman; Sephadex G-75, PD-10, and HisTrap Chelating columns from GE Healthcare; and ampholytes from Serva. All other chemicals and biochemicals, except as otherwise stated, were from Sigma-Aldrich.

Purification of Leghemoglobins, Protein Identification, and Molecular Mass Determination. Nodule soluble extracts were subjected to ammonium sulfate precipitation (35–90%) and leghemoglobins (Lbs) were purified by successive chromatographic steps in hydroxylapatite, Sephadex G-75, and DE-52 columns (1, 2). Anion-exchange chromatography was developed isocratically with 20 mM Tris-HCl (pH 8.0) for elution of Lb*a* and Lba_m, and then with a linear gradient of 0–60 mM NaCl in the same buffer for elution of Lb*c*, Lbc_m Lb*d*, and Lbd_m (1). The protein fractions were treated with ferricyanide and nicotinic acid to ensure complete conversion of Lbs into their ferric Lb-nicotinate complexes. These fractions were further purified on analytical or preparative isoelectric focusing (IEF) minigels containing 7.5% (wt/vol) acrylamide and 5% (vol/vol) ampholytes (pH 4–6) following published protocols (2). Soybean Lb protein bands (red or green) were excised from the IEF gels without staining and nicotinate removed by elution of proteins in 10 mM NH_4HCO_3 (pH 9.2). Proteins were subjected to in-gel digestion with trypsin using a Digest MSPro (Intavis). Peptide and protein identification was performed by peptide mass fingerprinting in a MALDI-TOF instrument (Applied Biosystems) as previously described (3). For molecular mass determination, Lbs were eluted from IEF gel pieces overnight at 4 °C with 10 mM NH_4HCO_3, loaded on PD-10 minicolumns equilibrated with 1 mM NH_4HCO_3, and concentrated on Centricon-10 membranes (Amicon). Molecular masses were determined by MALDI-TOF/MS. Spectra were mass calibrated externally using a standard protein mixture and internally using the signals derived from spiked horse heart cytochrome *c*. For analysis, 1 μL of the protein extracts and 2 μL of matrix (sinapinic acid, 3 mg mL^{-1}) were loaded on the MALDI plate using the sandwich method (4).

Structural Analyses of Hemes and Hemoproteins. ***Mass spectrometry.*** Low-energy MS^n (n = 1–5) analyses of hemes were carried out by nESI-IT/MS^n on a LCQ ion trap mass spectrometer (ThermoFisher). The spray voltage applied was 0.85 kV and the capillary temperature was 110 °C. For MS^n experiments, the isolation window was 3-Da wide and the relative collision energy was 25–50%. High-resolution MS analyses were performed on an ApexQe Fourier transform-ion cyclotron mass spectrometer (Bruker Daltonics), equipped with a 7T actively shielded magnet. Ions were generated using a Combi MALDI-electrospray ionization (ESI) source. For the analysis, the ESI interface was used and a voltage of 4,500 V was applied to the needle, and a counter voltage of 300 V was applied to the capillary. Samples were prepared by adding a spray solution of 70:29.9:0.1 MeOH/H_2O/formic acid to a solution of the sample at a vol/vol ratio of 1–5% to give the best signal-to-noise ratio. Data acquisition and processing were performed using ApexControl (v2.0.0.) and DataAnalysis (v3.4) software, respectively, from Bruker Daltonics.
NMR spectroscopy. ^1H NMR samples were prepared in D_2O containing 50 mM phosphate buffer (pH* 7.2; uncorrected for the deuterium isotope effect) and 2 mM cyanide to ensure complete formation of the ferric Lb-cyano complexes. The entire protein sample available of each Lb was used (~1 mM of Lb*a* and ~0.2 mM of Lbc_m). NMR spectra were collected at 30 °C with the proton chemical shifts referenced to residual water (HDO). 1D and Nuclear Overhauser and Exchange SpectroscopY (NOESY) spectra were obtained on a Bruker DRX-500 NMR spectrometer operating at 499.38-MHz proton Larmor frequency. The Water-Eliminated Fourier Transform (WEFT)-NOESY experiments used 160-ms relaxation delay and 190-ms recovery delay. The mixing time for the WEFT-NOESY experiments was 40 ms. All NOESY spectra were collected with 1,024 or 2,048 datapoints in t_2 and with 256–512 blocks, in t_1 with 512 scans per block for the Lb*a* sample, and 2,048–4,096 scans/block for the Lbc_m sample.
Resonance Raman spectroscopy. Resonance Raman (RR) spectra were recorded using a Jobin-Yvon U1000 spectrometer, equipped with a liquid nitrogen-cooled CCD detector (Spectrum One; Jobin-Yvon). Excitation at 413.1 nm (50 mW) was provided by an Innova Kr^+ laser (Coherent). The signal-to-noise ratios were improved by spectral collections of six cycles of 30-s accumulation time. The reported spectra were the results of averaging five to six single spectra. The spectral analysis was made using the Grams 32 software (Galactic Industries). The frequency precision was 0.5–1 cm^{-1} for the most intense bands and 1.5–2 cm^{-1} for the weakest bands.

Production of Recombinant Soybean Lb*a*. To overproduce the protein, nodule RNA was reverse-transcribed and gene-specific primers (5′-CACCATGGTTGCTTTCACTGAG-3′ and 5′-CTGCAATAGATACTAATTATGCCTTC-3′; the sequence underlined was required for directional cloning) were used to isolate a full-length cDNA clone in the Champion pET200/D-TOPO vector (Invitrogen). The construct, encoding an N-terminal His tag, was introduced into *Escherichia coli* BL21 (DE3) cells, which were grown in LB medium at 37 °C until an OD_{600} of 1.0 was reached. The recombinant protein was expressed by incubation of cells at 37 °C for 2 h with 0.1 mM isopropylthio-β-D-galactoside and purified on a Ni^{2+} affinity column. The protein was fully oxidized with an excess of ferricyanide and extensively dialyzed against 20 mM phosphate buffer pH 7.0. The spectrum confirmed that Lb*a* was in the aquo-ferric form.

1. Jun H-K, Sarath G, Wagner FW (1994) Detection and purification of modified leghemoglobins from soybean root nodules. *Plant Sci* 100:31–40.
2. Jun H-K, et al. (1994) Characteristics of modified leghemoglobins isolated from soybean (*Glycine max* Merr.) root nodules. *Plant Physiol* 104:1231–1236.
3. Casanovas A, Carrascal M, Abián J, López-Tejero MD, Llobera M (2009) Discovery of lipoprotein lipase pI isoforms and contributions to their characterization. *J Proteomics* 72:1031–1039.
4. Kemptner J, et al. (2009) Evaluation of matrix-assisted laser desorption/ionization (MALDI) preparation techniques for surface characterization of intact *Fusarium* spores by MALDI linear time-of-flight mass spectrometry. *Rapid Commun Mass Spectrom* 23: 877–884.

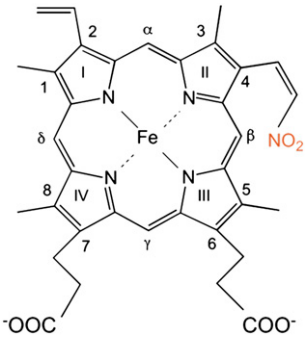

Fig. S1. Heme structure bearing a 4-nitrovinyl group. The isomer with the NO_2 group in the Cβ atom and in *cis*-configuration is shown. Fisher's numbering of protoporphyrin IX was followed.

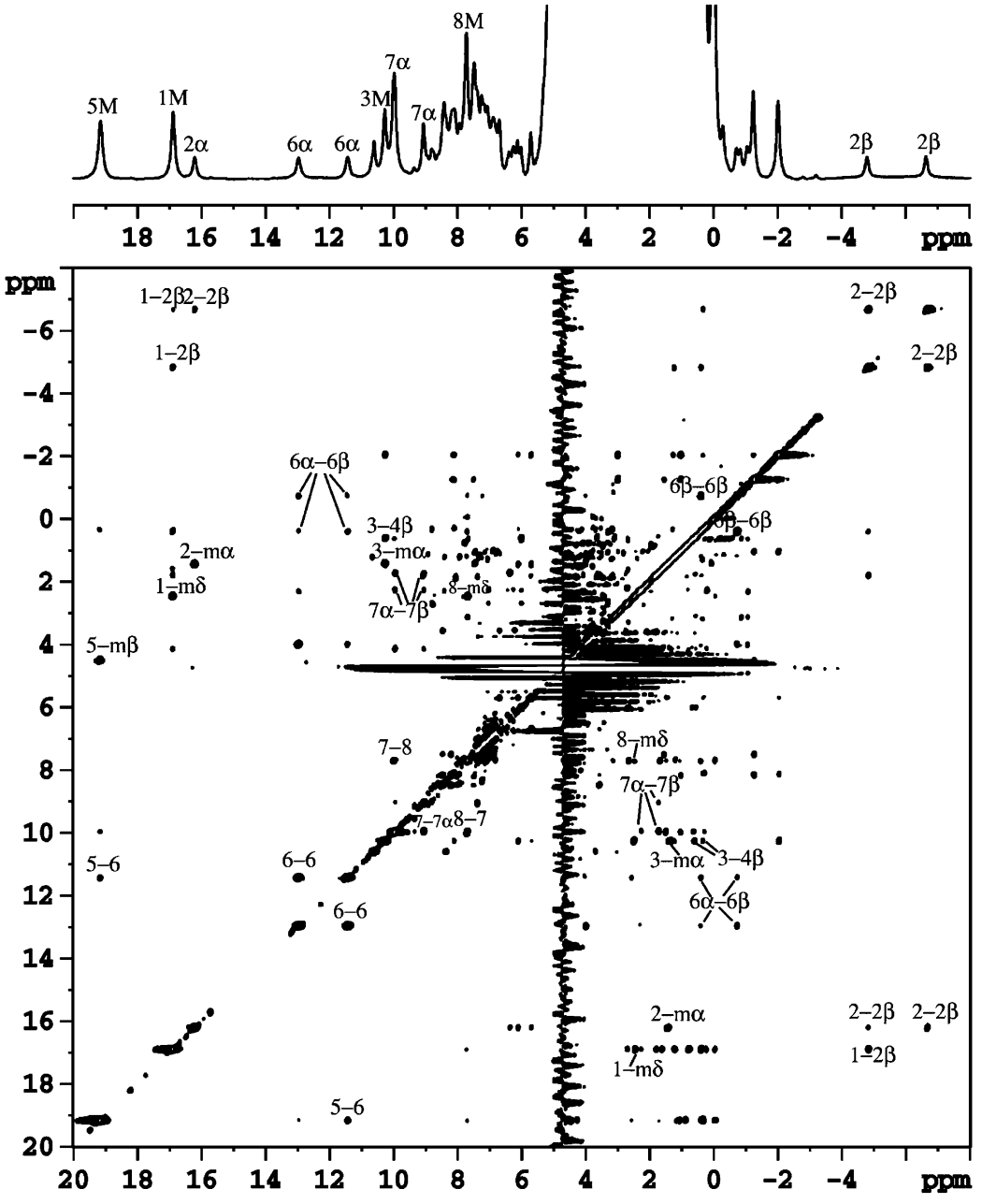

Fig. S2. 1D ^{1}H and WEFT-NOESY spectrum of the ferric Lba-cyanide complex (~1 mM), recorded at 500 MHz, pH* 7.2, 30 °C.

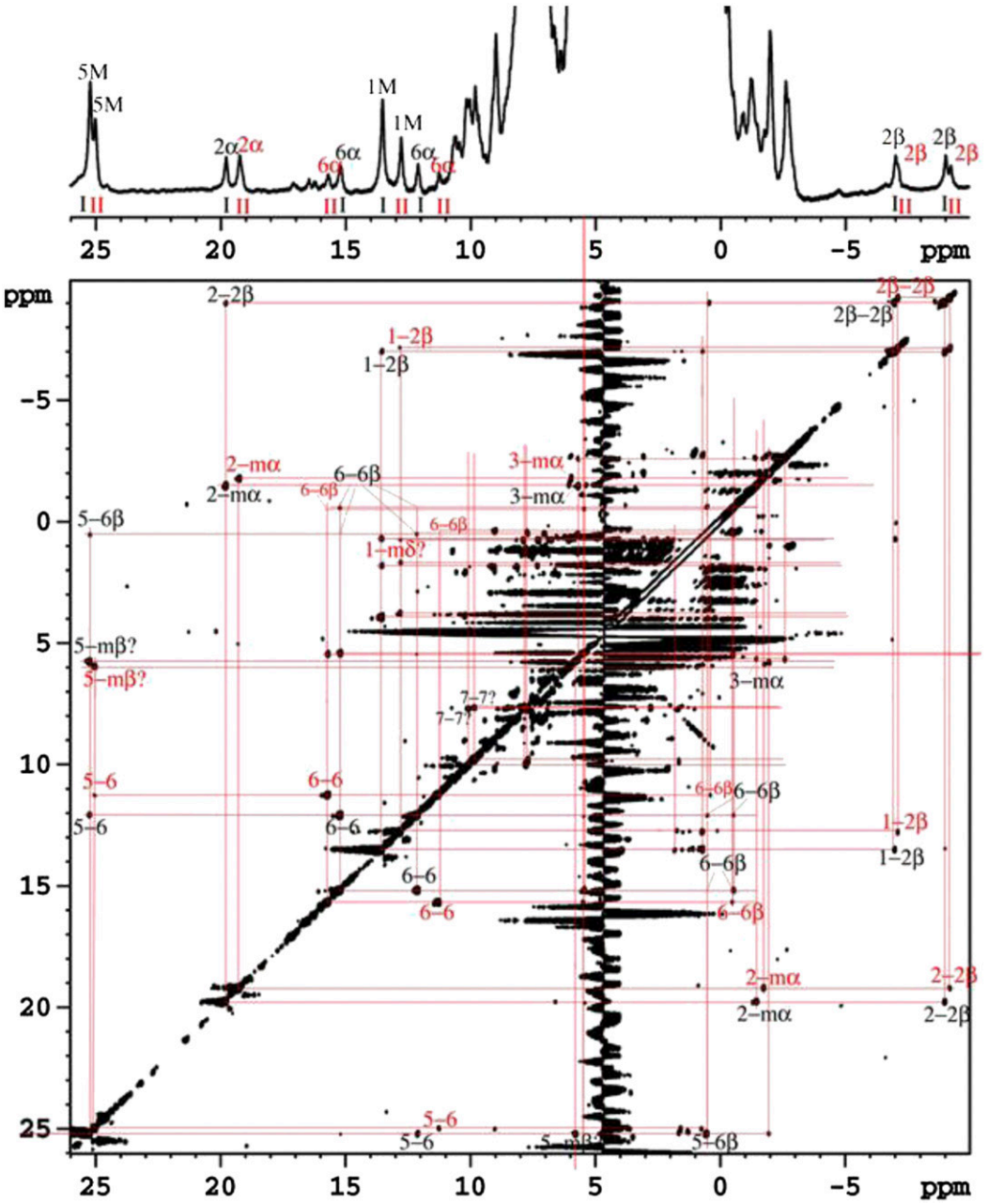

Fig. S3. 1D ^{1}H and WEFT-NOESY spectrum of the ferric Lbc$_{m}$-cyanide complex (~0.2 mM), recorded at 500 MHz, pH* 7.2, 30 °C. Two sets of proton resonances are marked in black and red and correspond to the isoproteins Lbc$_{m1}$ and Lbc$_{m2}$+c$_{m3}$, although not necessarily in this order.

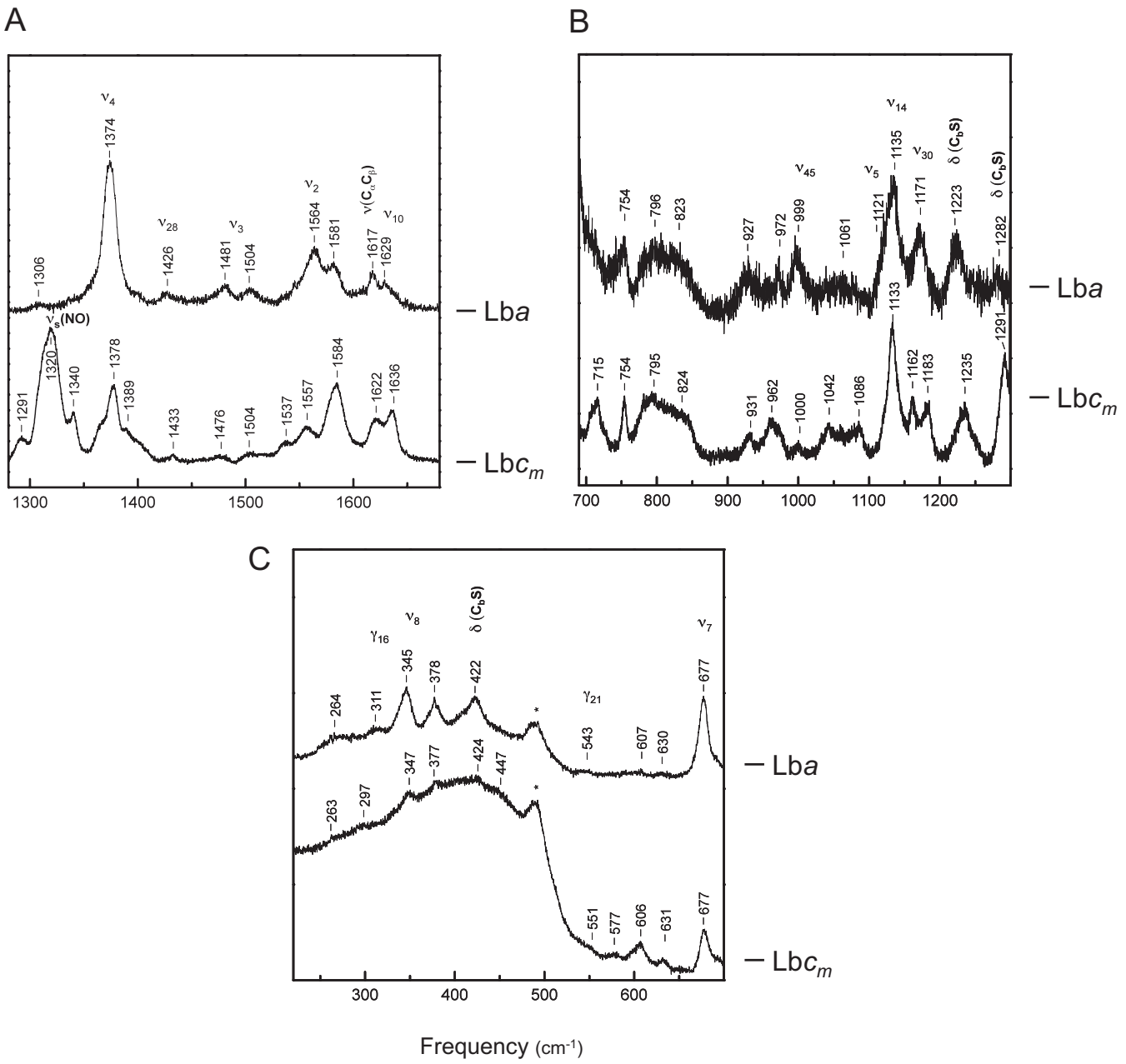

Fig. S4. (*A*) High- (1,280–1,700 cm^{-1}), (*B*) mid- (700–1,300 cm^{-1}), and (*C*) low- (230–700 cm^{-1}) frequency regions of RR spectra of ferric Lb*a* and Lbc$_m$, excited at 413.1 nm. (*A*) The major modification in the high-frequency spectra of Lbc$_m$ is the appearance of a strong band at 1,320 cm^{-1}. This band corresponds to a symmetric stretching of NO_2 [ν_s(NO)] bound to ferriprotoheme. Modes of the porphyrin skeleton (ν_2, ν_3, ν_4, ν_{10}, ν_{12}, ν_{28}) were shifted, indicating an effect on the tetrapyrrole geometry (1, 2). The stretching mode of the $-C_\alpha{=}C_\beta$ vinyl bonds seen at 1,617 cm^{-1} for Lb*a* was apparently upshifted for Lbc$_m$, forming a broad band at 1622 cm^{-1} (2). (*B*) Bands detected in the 900–1,300 cm^{-1} region were assigned to stretching or deformation modes of the peripheral C_b(pyrrole)-substituent bonds (C_b-S) [ν_5, ν_{14}, ν_{30}, ν_{45}, $\delta(C_b - S)$] (2). The spectra of Lb*a* and Lbc$_m$ show that all these modes are modified in terms of intensity and/or frequency. In particular, the intensity of the C_b(pyrrole) – C_α(vinyl) stretching mode at ~1,000 cm^{-1} is strongly decreased upon NO_2 binding (2). Moreover, new bands were found at 962, 1,042, and 1,086 cm^{-1} in the Lbc$_m$ spectrum. Therefore, this frequency range strongly indicates a peripheral modification of heme upon nitration. (*C*) Bands observed at 400–450 cm^{-1} represent major contributions of C_b(pyrrole) – C_α= C_β(vinyl) and C_b(pyrrole)-Me deformations (2). Lb*a* showed a major band at 422 cm^{-1} and a shoulder at 409 cm^{-1}, whereas Lbc$_m$ exhibits a weak band at 424 cm^{-1} and a new line at 447 cm^{-1}. The porphyrin ν_8 mode is shifted from 345 cm^{-1} for Lb*a* to 347 cm^{-1} for Lbc$_m$. This upshift is diagnostic of an increased deformation of the porphyrin core in Lbc$_m$ (1). Shifts of the pyrrole deformation γ_{16} and γ_{21} modes (311/543 cm^{-1} for Lb*a*, and 297/551 cm^{-1} for Lbc$_m$, respectively) confirm this conformational change. It is interesting to note that the frequency of the propionate mode was not significantly different for Lb*a* and Lbc$_m$ (377–378 cm^{-1}). In *C*, the bands marked with an asterisk result from the Raman cell itself.

1. Le Moigne C, et al. (2009) Redox effects on the coordination geometry and heme conformation of bis(*N*-methylimidazole) complexes of superstructured Fe-porphyrins. A spectroscopic study. *Inorg Chem* 48:10084–10092.
2. Desbois A, Henry Y, Lutz M (1984) Influence of peripheral substituents on the resonance Raman spectra of ferroporphyrin-2-methylimidazole complexes. *Biochim Biophys Acta* 785: 148–160.

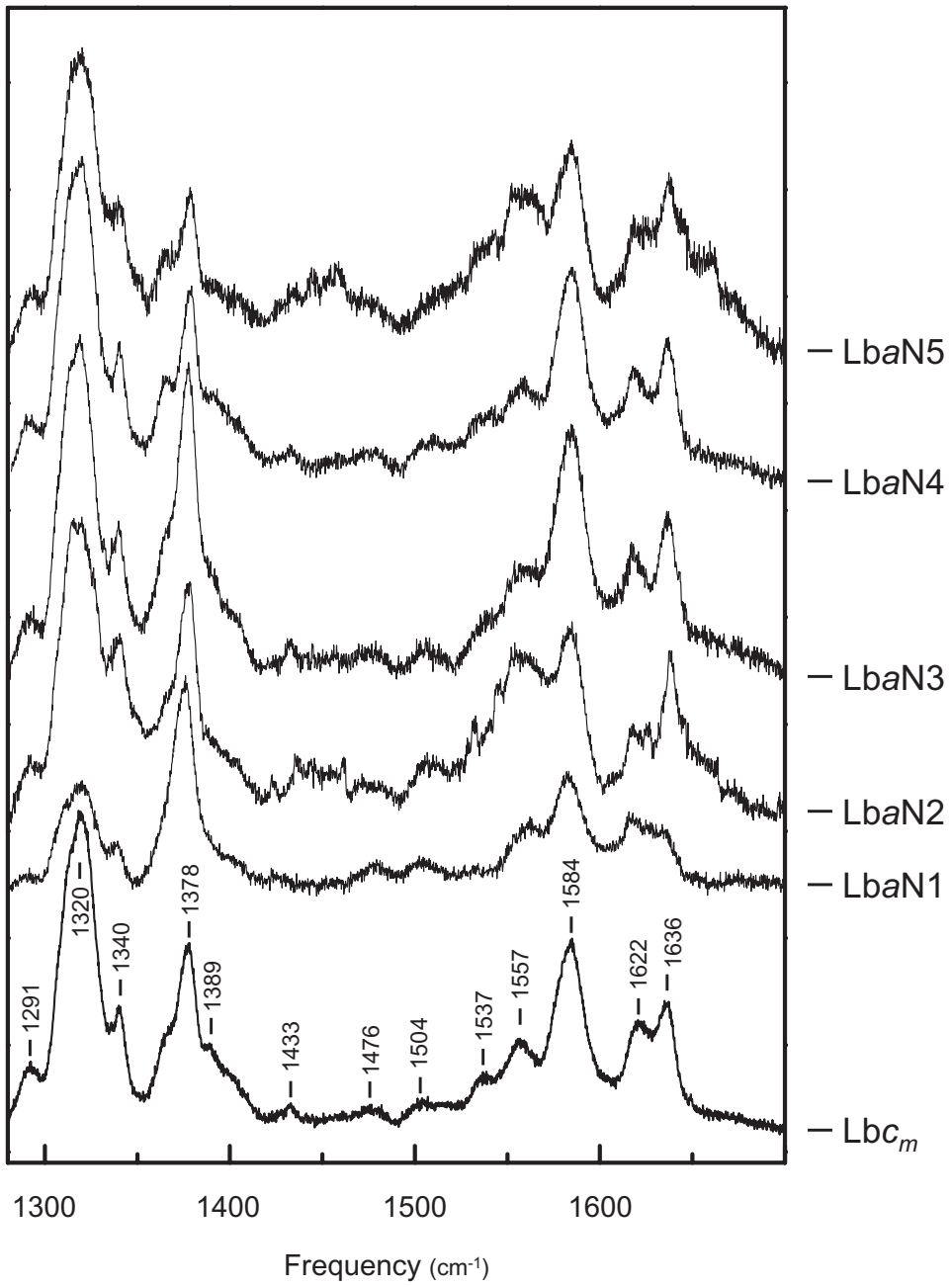

Fig. S5. High-frequency regions (1,280–1,700 cm^{-1}) of RR spectra of ferric Lbc$_m$ and Lba*N* derivatives, excited at 413.1 nm.

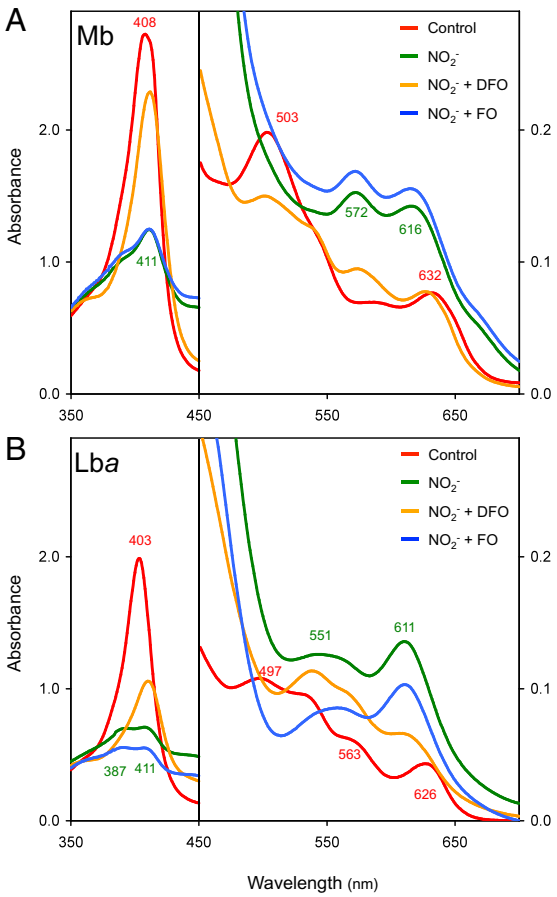

Fig. S6. Inhibitory effect of DFO on nitration of (*A*) Mb and (*B*) Lb*a* after 48 h. Ferrioxamine (FO) had only a slight effect. The reaction mixtures contained 50 mM Na-phosphate buffer (pH 7.0), 200 μM Mb or 150 μM Lb*a* and, where indicated, 200 mM $NaNO_2$, 1 mM DFO, or 1 mM FO. Cyanide (10 mM) formed ferric cyano complexes, which completely suppressed nitration. In contrast, this reaction was not inhibited by addition of superoxide dismutase, catalase, diethylenetriamine pentaacetic acid (DTPA), or Chelex resin (see text for details). The reaction was not inhibited either with 1–5 mM *p*-hydroxyphenylacetic acid, a more hydrophilic analog of Tyr, which is a good scavenger of the NO_2• radical (1). Protein concentrations were standardized using extinction coefficients of 3.7 $mM^{-1}{\cdot}cm^{-1}$ at 630 nm for horse-heart Mb (2) and of 141 $mM^{-1}{\cdot}cm^{-1}$ at 405 nm for soybean Lb*a* (3). Each experiment was repeated twice with identical results.

1. Bartesaghi S, et al. (2007) Protein tyrosine nitration in hydrophilic and hydrophobic environments. *Amino Acids* 32:501–515.
2. Bowen WJ (1949) The absorption spectra and extinction coefficients of myoglobin. *J Biol Chem* 179:235–245.
3. Appleby CA, Bergersen FJ (1980) *Methods for Evaluating Biological Nitrogen Fixation*, ed Bergersen FJ (John Wiley, Chichester), pp 315–335.

Table S1. Spectral characteristics of Lb*a* and Lbc_m of soybean nodules and of the Lb*a* nitrated derivatives generated in vitro

	Lb^{2+}		Lb^{2+} NO			Lb^{2+} nicotinate			Lb^{2+} pyridine					Lb^{3+}					
Isoprotein	γ	α	γ	β	α	γ	β	α	γ	sh	β	α	sh	γ	sh	CT	sh	sh	CT
Lb*a*	427	555	414	545	570	419	525	554	417	474	524	555		404		496	531	563	625
Lbc_m	422	557	413	545	571	417	525	555	415	473	522	553	580	389	436		wk	wk	615
LbaN1	425	557	414	545	570	418	524	554	415	473	522	553	580	402	434		wk	wk	615
LbaN2	424	557	414	546	570	418	525	554	415	473	522	553	580	403	433		wk	wk	615
LbaN3	424	557	414	543	570	418	526	554	415	472	522	553	580	394	434		wk	wk	615
LbaN4	423	557	414	546	570	418	525	554	415	472	522	553	580	391	436		wk	wk	615
LbaN5	423	557	413	542	570	418	524	554	414	472	522	553	580	392	433		wk	wk	615

The major spectral features in the Soret (γ-band) and visible (α- and β-bands) regions of the deoxyferrous and ferric Lb forms, as well as of some representative ferrous Lb complexes, are shown. The spectra of Lbc_m were obtained separately for the Lbc_{1m} and Lbc_{2m}+c_{3m} isoproteins, but they are identical. CT, charge-transfer absorption band; sh, shoulder; wk, weak absorption band.

Table S2. High-resolution MS analysis of hemes from soybean Lbs

Protein	Mol mass (found)	Mol mass (calc)	Mol formula	Fragment loss
Lb*a*, Lb*c*, Lb*d*	**616.1769**	**616.1768**	C_{34} H_{32} Fe N_4 O_4	—
	557.1630	557.1634	C_{32} H_{29} Fe N_4 O_3	CH_2COOH
	498.1498	498.1501	C_{30} H_{26} Fe N_4	2 CH_2COOH
Lba_m, Lbc_m, Lbd_m	**661.1618**	**661.1618**	C_{34} H_{31} Fe N_5 O_6	—
	644.1586	644.1591	C_{34} H_{30} Fe N_5 O_5	OH
	629.1338	629.1356	C_{33} H_{27} Fe N_5 O_5	OH CH_3
	627.1559	627.1563	C_{34} H_{29} Fe N_5 O_4	2 OH
	615.1684	615.1689	C_{34} H_{31} Fe N_4 O_4	NO_2
	602.1481	602.1485	C_{32} H_{28} Fe N_5 O_4	CH_2COOH
	600.1438	600.1455	C_{33} H_{28} Fe N_4 O_4	NO_2 CH_3
	584.1375	584.1380	C_{32} H_{26} Fe N_5 O_3	CH_2COOH H_2O
	571.1297	571.1301	C_{31} H_{25} Fe N_5 O_3	CH_2CH_2COOH OH
	556.1553	556.1556	C_{32} H_{28} Fe N_4 O_2	NO_2 CH_2COOH
	542.1396	542.1400	C_{31} H_{26} Fe N_4 O_2	NO_2 CH_2CH_2COOH
	525.1235	525.1220	C_{30} H_{23} Fe N_5 O	2 CH_2COOH H_2O
	512.1157	512.1142	C_{29} H_{22} Fe N_5 O	CH_2COOH CH_2CH_2COOH OH
	496.1336	496.1345	C_{30} H_{24} Fe N_4	NO_2 CH_2COOH $COOH$ CH_3
	483.1259	483.1267	C_{29} H_{23} Fe N_4	NO_2 CH_2COOH CH_2CH_2COOH
	468.1025	468.1032	C_{28} H_{20} Fe N_4	NO_2 CH_2COOH CH_2CH_2COOH CH_3

Molecular ions of hemes are highlighted in boldface. calc, calculated monoisotopic mass.

Table S3. Assignments of proton resonances of ferric Lb*a*-cyanide and ferric Lbc_m-cyanide complexes

		Ferric Lbc_m-cyanide	
Proton	Ferric Lb*a*-cyanide	I	II
1-Me	16.9	13.5	12.8
3-Me	10.3	6.2*	6.0*
5-Me	19.2	25.2	25.0
8-Me	7.7	8.0*	7.9*
2-Vinyl-α	16.2	19.8	19.2
2-Vinyl-β	−4.8, −6.7	−7.0, −9.0	−7.2, −9.2
4-Vinyl-α	NA	NA	NA
4-Vinyl-β	NA	NA	NA
6-Prop-α	13.0, 11.4	15.2, 12.1	15.7, 11.2
6-Prop-β	0.4, −0.7	0.5, -0.5	0.7, −0.5
7-Prop-α	10.0, 9.1	10.2, 7.9	9.9, 7.8
7-Prop-β	2.3, 1.7	NA	NA
meso-α	1.4	−1.3	−1.7
meso-β	4.5	5.8*	6.0*
meso-γ	NA	NA	NA
meso-δ	2.4	1.7	1.5
Me order	5 > 1 > 3 > 8	5 > 1 > 8 > 3	5 > 1 > 8 > 3
av Me	13.5	13.2	12.9

Chemical shifts are given in parts per million. NA, not assigned; Prop, propionate.
*Tentative assignment.