Supporting Information

Structural and biochemical investigations of the [4Fe-4S] cluster-containing fumarate hydratase from *Leishmania major*

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Table S1. Primers used for site-directed mutagenesis of LmFH-2

Name	Forward primer	Reverse primer
LmFH-2-D135A	5' CCG TCG TGC CAG GCT ACC GGC ACG GCC 3'	5' GGC CGT GCC GGT AGC CTG GCA CGA CGG 3'
LmFH-2-R173A	5' TAC CAC AAC CTG GCT TAC AGC CAG ACG 3'	5' CGT CTG GCT GTA AGC CAG GTT GTG GTA 3'
LmFH-2-H334A	5' CGG TTG CCC CGC GCC GGT GCC TCC TGC 3'	5' GCA GGA GGC ACC GGC GCG GGG CAA CCG 3'
LmFH-2-R421A	5' CTC ATC GTT GCC GCT GAT ATT GCC CAC 3'	5' GTG GGC AAT ATC AGC GGC AAC GAT GAG 3'
LmFH-2-T467A	5' CC TTT GGC CCG GCG ACG GCC GGC CG 3'	5' CG GCC GGC CGT CGC CGG GCC AAA GG 3'
LmFH-2-R471A	5' ACG ACG GCC GGC GCC ATG GAC TCC TAC 3'	5' GTA GGA GTC CAT GGC GCC GGC CGT CGT 3'

Oligonucleotide sequences were based on the *Leishmania major* genome sequence (GeneDB accession code LmjF.29.1960).
Underlined letters represent the nucleotides changed for the desired mutation.

Table S2. Tacsimate composition and its variations[a-e] to obtain structures with particular ligands bound

Co-crystallized ligand	100% Tacsimate pH 5	100% Tacsimate[a] pH 5 with *S*-malate	100% Tacsimate[b] pH 5 with fumarate	100% Tacsimate[c] pH 5 with malonate	100% Tacsimate[d] pH 5 with succinate	100% Tacsimate[e] pH 5 without ligands
Ammonium citrate tribasic	0.25 M	0.25 M	0.25 M	0.25 M	0.25 M	0.25 M
Sodium acetate trihydrate	0.40 M	0.40 M	0.40 M	0.40 M	0.40 M	0.40 M
Sodium formate	0.50 M	0.50 M	0.50 M	0.50 M	0.50 M	0.50 M
Ammonium tartrate dibasic	0.16 M	0.16 M	0.16 M	0.16 M	0.16 M	0.16 M
RS-Malic acid	0.30 M	-	-	-	-	-
Malonic acid	1.83 M	-	-	1.83 M	-	-
Succinic acid	0.12 M	-	-	-	0.12 M	-
S-malate	-	0.30 M	-	-	-	-
Fumarate	-	-	0.30 M	-	-	-

Table S3. Data collection and refinement statistics of LmFH-2

Data name	**LmFH-2-holo** (PDB ID 6UNZ)	**LmFH-2-mal** (PDB ID 6UO0)	**LmFH-2-malo** (PDB ID 6UOI)	**LmFH-2-suc** (PDB ID 6UOJ)
Co-crystallization	-	*S*-malate	Malonate	Succinate
Ligand in the structure	-	*S*-malate	Malonate	Succinate
Data collection				
Space group	$P2_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions				
a, b, c (Å)	159.09, 66.07, 238.08	65.61, 84.68, 239.66	65.58, 85.10, 240.23	65.61, 84.84, 240.54
α, β, γ (°)	90, 90.02, 90	90, 90,90	90, 90,90	90, 90,90
Wavelength (Å)	0.9795	0.9795	0.9795	0.9795
Resolution (Å)	50-3.2 (3.26-3.2)*	50-1.85 (1.88-1.85)*	50-1.95 (1.98-1.95)*	50-2.35 (2.39-2.35)*
No. of unique reflections	75,134	110,293	98,420	56,220
R_{sym}	0.219 (0.855)*	0.139 (0.906)*	0.136 (0.747)*	0.137 (0.531)*
I/σ(I)	5.0 (1.0)*	7.5 (1.9)*	12.2 (1.6)*	6.75 (1.34)
CC1/2[$]	(0.334)*	(0.597)*	(0.699)*	(0.721)*
Completeness (%)	90.4 (78.1)*	96.0 (86.1)*	99.8 (99.0)*	98.7 (98.4)*
Redundancy	3.0 (2.6)*	5.3 (4.4)*	6.0 (3.8)*	3.6 (3.0)*
Refinement				
No. of reflections used	75,122 (6,448)*	109,580 (9,785)*	98,288 (9,640)*	56,154 (5,496)*
R_{work} / R_{free}**	0.2453/0.2888	0.1560/0.1888	0.1471/0.1760	0.1480/0.2019
No. of atoms				
Protein	31,167	8,435	8,555	8,299
No. of molecules				
[4Fe-4S] cluster	8	2	-	2
[3Fe-4S] cluster	-	-	2	-
Fe	-	-	2	-
S-malate	-	4	-	-
Malonate	-	-	6	-
Succinate	-	-	-	2
Glycerol	2	4	1	2
Water	3	902	882	603
Average B-factors (Å^2)				
Protein	63	27	27	32
[4Fe-4S] cluster	72	20	-	30
[3Fe-4S] cluster	-	-	20	-
Fe	-	-	27	-
S-malate	-	28	-	-
Malonate	-	-	28	-
Succinate	-	-	-	30
Glycerol	50	48	36	66
Water	42	38	38	38
R.m.s deviations				
Bond lengths (Å)	0.007	0.006	0.005	0.006
Bond angles (°)	1.40	1.10	1.09	1.10
Ramachandran analysis[#] (%)				
Favored	96.6	97.7	97.4	97.4
Allowed	3.1	2.3	2.6	2.6
Outliers	0.3	0	0	0

*Highest resolution shell is shown in parenthesis.

**R_{free} was calculated with 5% of the data.

[$]The overall CC1/2 was not available from the version of HKL2000 that was used.

[#]Distribution of dihedral angles in the Ramachandran diagram was calculated with the *MolProbity* program[1].

Table S4. Data collection and refinement statistics of LmFH-2 variants

Data name	**LmFH-2-T467A-tacs** (PDB ID 6UP9)	**LmFH-2-T467A-mal** (PDB ID 6UPM)	**LmFH-2-H334A-mal** (PDB ID 6UPO)	**LmFH-2-R421A-tacs** (PDB ID 6UQ8)	**LmFH-2-R421A-mal** (PDB ID 6UQ9)	**LmFH-2-R471A-tacs** (PDB ID 6UQB)	**LmFH-2-R471A-mal** (PDB ID 6UQL)	**LmFH-2-R173A-mal** (PDB ID 6UQM)	**LmFH-2-R173A-fum** (PDB ID 6UQN)
Co-crystallization	*RS*-Malate, malonate, and succinate	*S*-Malate	*S*-Malate	*RS*-Malate, malonate, and succinate	*S*-Malate	*RS*-Malate, malonate, and succinate	*S*-Malate	*S*-Malate	Fumarate
Ligands in the structure	Malonate	*S*-Malate	*S*-Malate	*S*-Malate and malonate	*S*-Malate	*S*-Malate and malonate	*S*-Malate	*S*-Malate	Fumarate and *S*-malate
Data collection									
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions									
a, *b*, *c* (Å)	65.65, 84.94, 240.21	65.62, 84.72, 239.67	65.93, 84.59, 240.36	65.73, 84.75, 240.12	65.76, 84.80, 240.22	65.46, 84.94, 240.27	65.71, 84.80, 240.22	65.62, 85.07, 240.42	65.15, 85.41, 240.72
α, β, γ (°)	90, 90,90	90, 90,90	90, 90,90	90, 90,90	90, 90,90	90, 90,90	90, 90,90	90, 90,90	90, 90,90
Wavelength (Å)	0.9795	0.9795	0.9792	0.9791	0.9791	0.9791	0.9791	0.9792	0.9792
Resolution (Å)	50-1.95 (1.98-1.95)*	50-2.03 (2.07-2.03)*	50-3.11 (3.16-3.11)*	50-1.34 (1.36-1.34)*	50-2.30 (2.34-2.30)*	50-1.95 (1.98-1.95)*	50-2.1 (2.14-2.1)*	50-2.0 (2.03-2.0)*	50-3.3 (3.36-3.3)*
No. of unique reflections	97,272	87,005	22,910	283,622	59,015	98,175	78,203	90,969	20,385
R_{sym}	0.107 (0.987)*	0.228 (0.984)*	0.262 (1.109)*	0.077 (1.202)*	0.348 (0.923)*	0.119 (0.849)*	0.162 (0.860)*	0.166 (0.723)*	0.302 (1.130)*
I/σ(I)	9.3 (1.1)*	7.7 (1.2)*	6.2 (1.0)*	22.2 (1.0)*	7.9 (1.9)*	16.0 (2.2)*	9.0 (1.6)*	7.2 (1.2)*	4.5 (1.0)*
CC1/2[$]	(0.453)*	(0.445)*	(0.323)*	(0.605)*	(0.776)*	(0.815)*	(0.742)*	(0.649)*	(0.335)*
Completeness (%)	98.5 (98.3)*	99.6 (97.7)*	92.0 (81.5)*	94.3 (88.4)*	97.8 (93.5)*	99.9 (99.9)*	98.6 (97.7)*	99.5 (98.1)*	96.7 (99.0)*
Redundancy	3.5 (3.1)*	5.5 (3.0)*	5.5 (3.4)*	9.5 (6.9)*	10.4 (7.3)*	9.1 (8.1)*	6.5 (5.9)*	5.9 (3.5)*	3.6 (3.2)*
Refinement									
No. of reflections used	97,209 (9,291)*	86,927 (8,449)*	22,858 (1,950)*	283,518 (25,497)*	58,941 (5,420)*	98,085 (9,515)*	78,118 (7,603)*	90,865 (8,826)*	20,341 (2,018)*
R_{work} / R_{free}**	0.1574/0.1936	0.1563/0.1930	0.2001/0.2541	0.1341/0.1435	0.1420/0.1892	0.1434/0.1742	0.1462/0.1834	0.1475/ 0.1879	0.2054/0.2630
No. of atoms									
Protein	8,249	8,373	8,059	8,727	8,249	8,341	8,274	8,463	8,101
No. of molecules									
[4Fe-4S] cluster	2	2	2	2	2	2	2	2	2
S-malate	-	2	2	2	2	2	2	2	1
Fumarate	-	-	-	-	-	-	-	-	1
Malonate	6	-	-	4	-	4	-	-	-
Glycerol	1	2	-	5	1	2	2	6	1
Water	783	867	12	871	643	919	733	833	17
Average B-factors (Å^2)									
Protein	27	24	48	18	25	28	27	25	56
[4Fe-4S] cluster	20	17	45	12	17	20	19	17	52
S-malate	-	20	46	13	18	21	21	19	57
Fumarate	-	-	-	-	-	-	-	-	60
Malonate	30	-	-	21	-	30	-	-	-
Glycerol	37	46	-	30	41	42	46	47	59
Water	37	35	39	27	32	38	36	35	27
R.m.s deviations									
Bond lengths (Å)	0.006	0.005	0.004	0.008	0.005	0.005	0.006	0.008	0.004
Bond angles (°)	1.11	1.09	0.97	1.32	1.09	1.09	1.10	1.18	0.97
Ramachandran analysis[#] (%)									
Favored	97.4	97.5	95.6	97.6	97.2	97.5	97.4	97.6	96.0
Allowed	2.6	2.5	4.4	2.2	2.8	2.5	2.6	2.4	4.0
Outliers	0	0	0	0.2	0	0	0	0	0

*Highest resolution shell is shown in parenthesis.
**R_{free} was calculated with 5% of the data.
[$]The overall CC1/2 was not available from the version of HKL2000 that was used.
[#]Distribution of dihedral angles in the Ramachandran diagram was calculated with the *MolProbity* program[1].

Table S5. The number of residues visualized in each LmFH-2 structure

Enzyme	Chain A	Chain B	Chain C	Chain D	Chain E	Chain F	Chain G	Chain H
LmFH-2-holo	21-375 / 385-568	27-377 / 386-568	28-375 / 385-568	28-375 / 388-568	29-375 / 385-568	28-375 / 386-568	28-375 / 385-568	28-375 / 386-568
LmFH-2-mal	28-375 / 385-568	28-375 / 385-568	-	-	-	-	-	-
LmFH-2-malo	26-376 / 385-568	28-375 / 385-568	-	-	-	-	-	-
LmFH-2-suc	26-376 / 385-568	28-375 / 385-568	-	-	-	-	-	-
LmFH-2-T467A-tacs	28-375 / 385-568	28-375 / 385-568	-	-	-	-	-	-
LmFH-2-T467A-mal	28-375 / 385-568	28-375 / 385-568	-	-	-	-	-	-
LmFH-2-H334A-mal	28-376 / 385-568	28-376 / 385-568	-	-	-	-	-	-
LmFH-2-R421A-tacs	28-375 / 385-568	28-374 / 386-568	-	-	-	-	-	-
LmFH-2-R421A-mal	28-376 / 385-568	28-375 / 386-568	-	-	-	-	-	-
LmFH-2-R471A-tacs	28-377 / 384-568	28-375 / 385-568	-	-	-	-	-	-
LmFH-2-R471A-mal	28-375 / 385-568	28-377 / 385-568	-	-	-	-	-	-
LmFH-2-R173A-mal	28-377 / 385-568	28-375 / 386-568	-	-	-	-	-	-
LmFH-2-R173A-fum	26-375 / 385-568	28-375 / 385-568	-	-	-	-	-	-

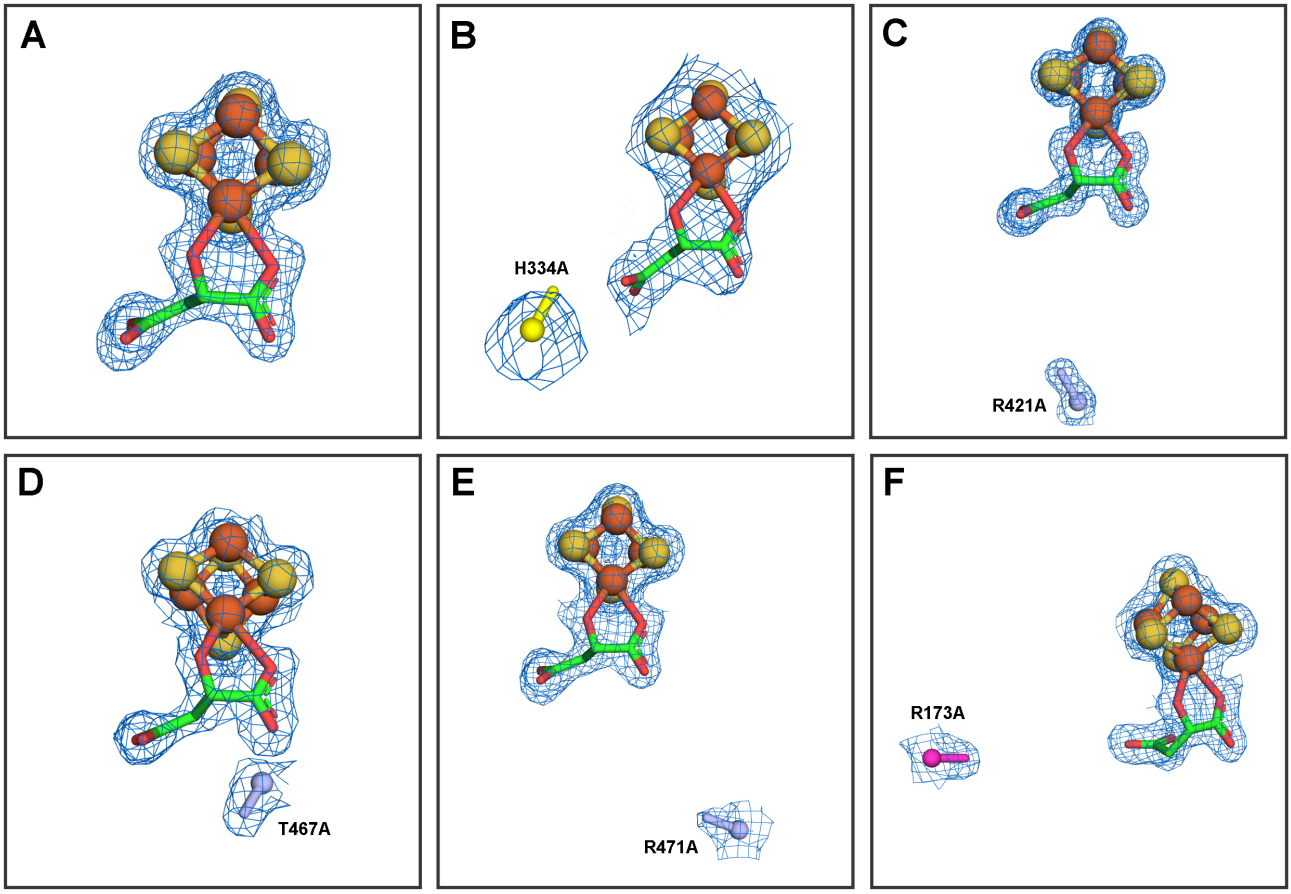

Figure S1. Electron density maps of LmFH-2 and variants with *S*-malate. **(A)** Crystal structure of LmFH-2 in a complex with *S*-malate at 1.85 Å resolution. **(B)** Crystal structure of LmFH-2-H334A in a complex with *S*-malate at 3.11 Å resolution. **(C)** Crystal structure of LmFH-2-R421A in a complex with *S*-malate at 1.34 Å resolution. **(D)** Crystal structure of LmFH-2-T467A in a complex with *S*-malate at 2.03 Å resolution. **(E)** Crystal structure of LmFH-2-R471A in a complex with *S*-malate at 1.95 Å resolution. **(F)** Crystal structure of LmFH-2-R173A in a complex with *S*-malate at 2.0 Å resolution. $2F_o - F_c$ electron density maps contoured at 1.5 RMSD are shown in blue mesh for *S*-malate (green), [4Fe-4S] cluster (orange (Fe) and yellow (S) spheres), and the substitutions H334A (yellow), R421A (light blue), T467A (light blue), R471A (light blue), and R173A (magenta).

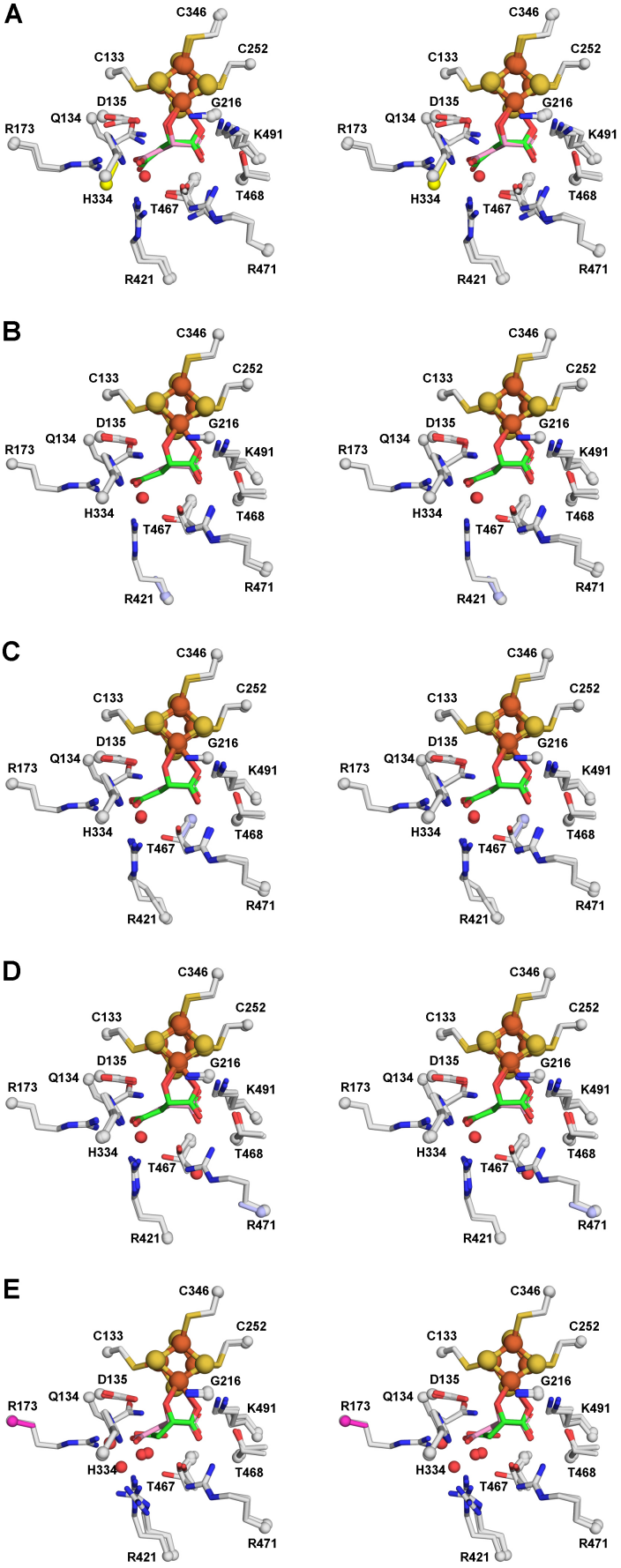

Figure S2. Stereoviews of LmFH-2 and variants in a complex with *S*-malate. **(A)** Superposition of LmFH-2 and LmFH-2-H334A in a complex with *S*-malate. **(B)** Superposition of LmFH-2 and LmFH-2-R421A in a complex with *S*-malate. **(C)** Superposition of LmFH-2 and LmFH-2-T467A in a complex with *S*-malate. **(D)** Superposition of LmFH-2 and LmFH-2-R471A in a complex with *S*-malate. **(E)** Superposition of LmFH-2 and LmFH-2-R173A in a complex with *S*-malate. The substrate *S*-malate is shown in pink for wild type and green for variants. The [4Fe-4S] cluster is shown in orange (Fe) and yellow (S) spheres. The water molecules are shown in red spheres. The substituted residue H334A is shown in yellow. The substitutions R421A, T467A, and R471 are shown in light blue. The substituted residue R173A is shown in magenta.

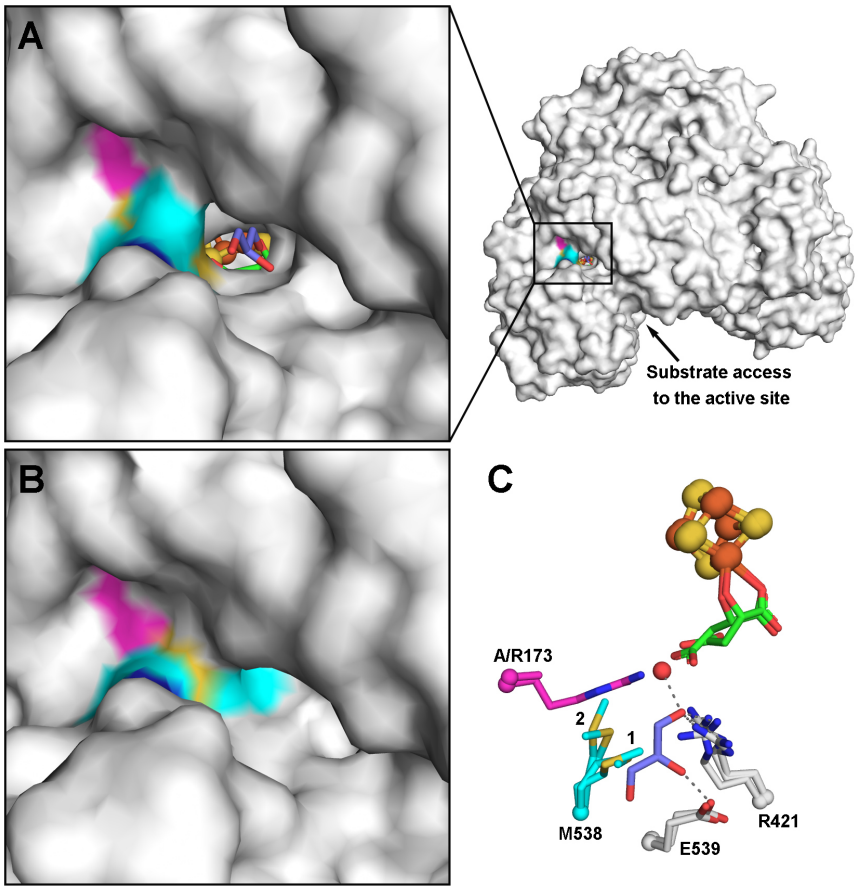

Figure S3. An LmFH-2-R173A structure reveals a solvent-exposed pocket that is not observed in wild type LmFH-2. **(A)** The right panel shows the surface of the *S*-malate-bound LmFH-2-R173A structure indicating the location of the solvent-exposed pocket (black rectangle). This pocket is created by movement of M538 residue (position 2 in panel C) due to the substitution of R173 to alanine. The left panel shows a closer view of this pocket with access to the active site, where a molecule of glycerol (slate blue) was found. **(B)** The surface of the *S*-malate-bound LmFH-2 structure showing the absence of the pocket when the M538 residue is in its typical location (position 1 in panel C). **(C)** Superposition of *S*-malate-bound LmFH-2 and LmFH-2-R173A structures. The hydrogen bonds between glycerol (slate blue) and the residues (white) in the solvent-exposed pocket are shown as gray dashed lines. The double conformation of M538 residue from LmFH-2 (position 1) and LmFH-2-R173A (position 2) are shown in cyan. The R173 residue and the R173A substitution are shown in magenta. The *S*-malate is shown in green. The [4Fe-4S] cluster is shown in orange (Fe) and yellow (S) spheres.

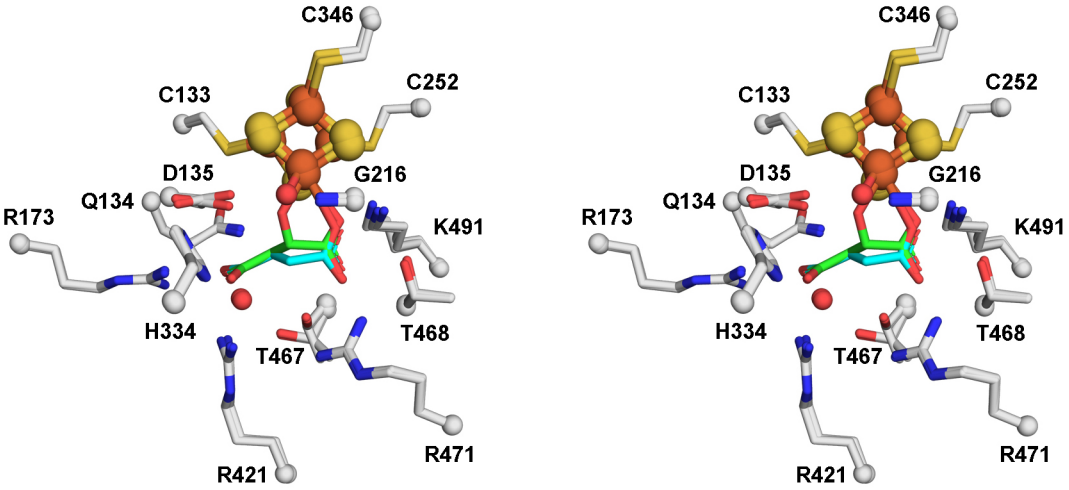

Figure S4. Stereoview of the superposition of LmFH-2 active sites in a complex with *S*-malate and succinate. The substrate *S*-malate is shown in green. Succinate is shown in cyan. The [4Fe-4S] cluster is shown in orange (Fe) and yellow (S) spheres. The water molecules are shown in red spheres.

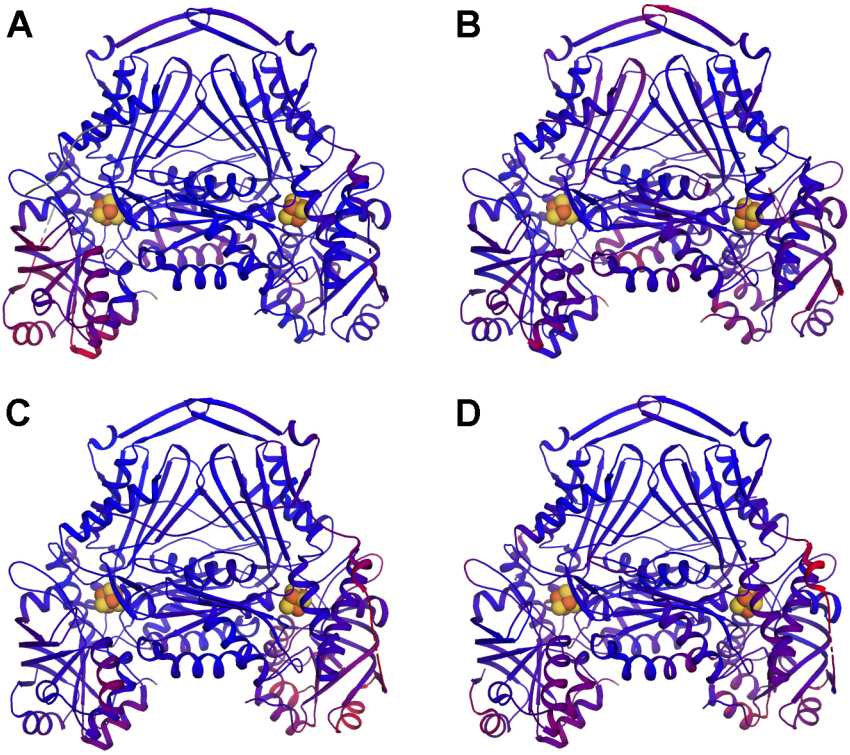

Figure S5. LmFH-2-holo dimers colored by RMSD. **(A)** LmFH-2-holo chains A and B. **(B)** LmFH-2-holo chains C and D. **(C)** LmFH-2-holo chains E and F. **(D)** LmFH-2-holo chains G and H. Each dimer of LmFH-2-holo structure was aligned with LmFH-2 structure in a complex with *S*-malate and spectrum colored by per-residue Cα RMSD values using the ColorByRMSD PyMol script. Blue was used for minimum pairwise RMSD and red for maximum pairwise RMSD. The [4Fe-4S] cluster is shown in orange (Fe) and yellow (S) spheres.

Reference

(1) Chen, V. B., Arendall, W. B., III, Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S., and Richardson, D. C. (2010) MolProbity: all-atom structure validation for macromolecular crystallography, *Acta Crystallographica Section D-Biological Crystallography 66*, 12–21.