

Supplementary Materials

The Magnetosome Protein, Mms6 from *Magnetospirillum magneticum* strain AMB-1, is a Lipid-Activated Ferric Reductase

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Table S1. List of protein sequences used in this work

protein name	Sequence
Mms6	VGGTIWTGKGLGLGLGLGAWGPILGVVGAGAVYAYMKSRDIESAQSDDEEVELRDALA
m1Mms6	VGGTIWTGKGLGLGLGLGAWGPILGVVGAGAVYAYADSEDIESAQSRRKVELEDALA
m2Mms6	VGGTIWTGKGLGLGLGLGAWGPILGVVGAGAVYAYAKDRSIDEAQESDSVELREALA
m3Mms6	VGGTIWTGKGLGLGLGLGAWGPILGVVGAGAVYAYAQSLERAEDEDADISAVEKLSR
C21Mms6	KSRDIESAQSDDEEVELRDALA
GLtoGA	VGGTIWTGKGAGAGAGAGAGAWGPILGVVGAGAVYAYMKSRDIESAQSDDEEVELRDALA
W103A	VGGTIATGKGLGLGLGLGAWGPILGVVGAGAVYAYMKSRDIESAQSDDEEVELRDALA
W119A	VGGTIWTGKGLGLGLGLGAAGPILGVVGAGAVYAYMKSRDIESAQSDDEEVELRDALA
S138A	VGGTIWTGKGLGLGLGLGAWGPILGVVGAGAVYAYMKARDIESAQSDDEEVELRDALA
D140A	VGGTIWTGKGLGLGLGLGAWGPILGVVGAGAVYAYMKSRAIESAQSDDEEVELRDALA
S146A	VGGTIWTGKGLGLGLGLGAWGPILGVVGAGAVYAYMKSRDIESAQDAEEVELRDALA
D147A	VGGTIWTGKGLGLGLGLGAWGPILGVVGAGAVYAYMKSRDIESAQSAEEVELRDALA
E148A	VGGTIWTGKGLGLGLGLGAWGPILGVVGAGAVYAYMKSRDIESAQSDAEVELRDALA
E149A	VGGTIWTGKGLGLGLGLGAWGPILGVVGAGAVYAYMKSRDIESAQSDAEVELRDALA
D154A	VGGTIWTGKGLGLGLGLGAWGPILGVVGAGAVYAYMKSRDIESAQSDDEEVELRAALA
S146E148A	VGGTIWTGKGLGLGLGLGAWGPILGVVGAGAVYAYMKSRDIESAQDAEVELRDALA
Histidine tag:	MGGSHHHHHHGMASMTGGQQMGRDLYDDDDKDPTLGGHM
His-Ubi-Mms6	MGHHHHHHHHHSSGHIDDDAKHMQIFVKTLTGKTITLEVESSDTIDNVKSKI QDKEGIPPDQQRLLIFAGKQLEDGRTLSDYNIQKESTLHLVLRRLRGGHIETLYFQGHMPSTLTPR GGTIWTGKGLGLGLGLGAWGPILGVVGAGAVYAYMKSRDIESAQSDDEEVELRDALA

Legend: All proteins, except for C21Mms6, which was chemically synthesized, were produced as fusion proteins with an N-terminal histidine tag. All except Hi-Ubi-Mms6 included the histidine tag shown in the second to last row. His-Ubi-Mms6 is shown in the last row with its histidine tag.

Table S2. Summary of k_{cat} determinations

Operator	Conditions		10 ⁵ x k _{cat}	Individual preparations (10 ⁵ x k _{cat})							
Individual	prep	date	/sec	1178	1628	1629	1633	1634	1639	14611	14619
2	1178	2/15/2014	2.6	2.6							
1	1628	8/28/2013	1.2		1.2						
2	1633	12/14/2013	1.8				1.8				
2	1634	12/15/2013	2.2					2.2			
2	1629	12/16/2013	2.3			2.3					
2	1629	12/18/2013	2.0			2.0					
2	1633	12/18/2013	2.4				2.4				
2	1634	12/18/2013	3.3					3.3			
2	1629	1/18/2014	1.7			1.7					
2	1633	1/18/2014	0.62				6.2				
2	1639	6/27/2014	0.30						3.0		
3	14611	10/5/2020	1.8							1.8	
3	14611	10/14/2020	1.4							1.4	
3	14619	6/21/2022	16								16
3	14619	6/21/2022	15								15
3	14611	6/24/2022	343							343	
3	14611	6/24/2022	342							342	
3	14611	6/27/2022	5.9							5.9	
3	14611	6/27/2022	11							11	
3	14611	7/1/2022	14							14	
3	14611	7/1/2022	9.4							9.4	
3	14611	7/1/2022	0.60							0.60	
3	14611	7/1/2022	0.44							4.4	

	10 ⁵ x k _{cat} averages		10 ⁵ x k _{cat}							
	All values	minus highest and lowest values	Individual preparations							
Average	30	4.8	2.6	1.2	2.0	1.6	2.7	0.30	69	15
standard deviation	99	5.2			0.27	0.88	0.82		144	0.86
coefficient of variation	326%	109%			14%	56%	30%		209%	6%
Median	2.3	2.2								
Minimum	0.30	0.44								
Maximum	343	16								
N	23	20	1	1	3	3	2	1	10	2

LEGEND: The results of k_{cat} measurements for a range of Mms6 preparations by three different people over a period of 9 years.

Table S3. Homologs identified by TM-Align

PDB	Homologs identified by TM-Align	Microbial source
3V2H	D-beta-hydroxybutyrate dehydrogenase	<i>Sinorhizobium meliloti</i>
3GVI	Lactate/malate dehydrogenase in complex with ADP	<i>Brucella melitensis</i>
3FG2	Ferredoxin Reductase for the CYP199A2 System	<i>Rhodopseudomonas palustris</i>
7DNM	AgCarB2-C2 complex	<i>Arthrobacter globiformis</i>
5LC5	Mammalian respiratory complex I, class2	<i>Bos taurus</i>
6AGS	L310R/Q401C mutant of malic enzyme	<i>Escherichia coli</i>
2VCF	Cytosolic ascorbate peroxidase with isoniazid (INH)	<i>Glycine max</i>
6T15	III2-IV(5B)1 respiratory supercomplex	<i>Saccharomyces cerevisiae</i>
1D4C	Flavocytochrome c fumarate reductase, uncomplexed	<i>Shewanella putrefaciens</i> strain mr-2
3EF6	Toluene 2,3-dioxygenase reductase	<i>Pseudomonas putida</i>
1YCG	Nitric oxide reductase	<i>Moorella thermoacetica</i> FprA
3KD9	Pyridine nucleotide disulfide oxidoreductase	<i>Pyrococcus horikoshii</i>

LEGEND: Enzymes identified by TM-Align as structural homologs of Mms6. The top three structures were used by I-TASSER as the basis of structural prediction.

Table S4. Summary of reported kinetic parameters

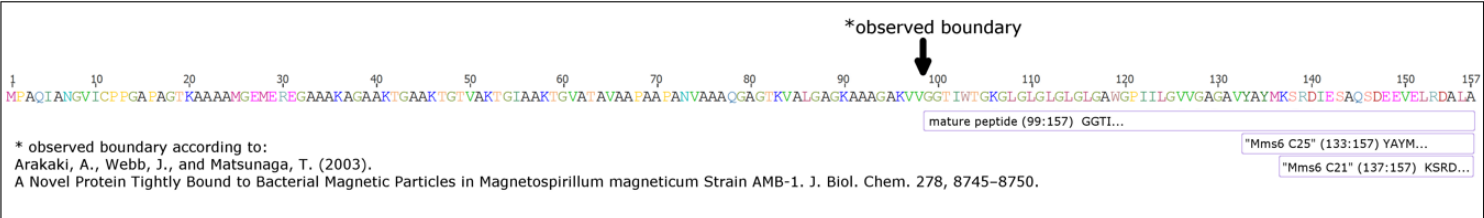
Bacterium	MW	pH optimum	electron donor	cofactor	electron acceptor (K_d)		Specific activity (nmole/min/mg)	Citations
			NADH or NADPH	FMN vs. FAD				
Mms6, <i>M. magnetotacticum</i>			NADH preferred	FAD		Fe-Citrate with FAD, NADH (76)	Fe-citrate (5.7)	this work
Mms6, <i>M. magnetotacticum</i> in bicelles							Fe-citrate (20)	this work
<i>Archaeoglobus fulgidus</i>	18	7	both	both	Fe-EDTA with NADH (61), with NADPH (80); flavin not specified	Fe-citrate (20.6)		56
<i>Azotobacter vinelandii</i>	44.6 and 69	7.5	preferred (16)	FMN preferred	Fe-Citrate with NADH, FMN (10)	Fe-citrate (21)	Fe-citrate (197)	57
<i>Bacillus subtilis</i>	N/R		NADPH	FMN	Fe-HBA with NADPH, FMN (55)	Fe-citrate (197)	Fe-DCIP (3.8)	58
<i>Bacillus subtilis</i>	13		NADPH (78) with oxygen as acceptor	FMN (12.5) with oxygen as acceptor	Fe-DCIP (385)	Fe-DCIP (3.8)		41
<i>E. coli</i>	26		both equal	both but FMN preferred				53
<i>E. coli</i>	26		NADH (9), NADPH (30)	FMN (1.5), FAD (1.9)				53
<i>L. pneumophila</i>	38		NADH (11), NADPH (9)				Fe-citrate (7)	59
<i>M. magnetotacticum</i>	36	7	NADH (1.3) NADPH (119)	FMN (0.035)	Fe-Citrate with FMN, NADH (15)	Fe-citrate (3111)		60
<i>R. sphaeroides</i>	32	7.1	NADH (18) not NADPH	FMN (3.2), FAD (14.1)	Fe-Citrate with FMN, NADH (8.3)			61
<i>R. sphaeroides</i>	41		NADH (20) not NADPH		Fe-Citrate with FMN, NADH (4.2)	Fe-citrate (1300), Fe-EDTA (13100)		61
<i>Acidithiobacillus ferrooxidans</i>	28	6.5	NADPH (76) not NADH	FMN	Fe-EDTA with NADPH, FMN (30)	Fe-citrate (1300), ferric-EDTA (13000)		62
<i>Paracoccus denitrificans</i>	19		NADH (5.5) not NADPH	FMN (20)	Fe-NLA with FMN, NADH (17)	Fe-EDTA (0.005); Fe-NLA (7.6)		63
<i>Paracoccus denitrificans</i>	18		NADH (2.6)	FAD		Fe-NLA (3.1), Fe-EDTA (0.16), Fe-citrate (0.08)	Fe-NLA (4.8)	63
<i>Pseudomonas putida</i>	29.5		NADPH (6.3)	both	Fe-EDTA with NADH, FAD (19), with NADH, FMN (8.7), with NADPH, FMN or FAD (~7); Fe-citrate with NADH, FAD (12), with FAD, NADPH (6.4)			64
<i>Pseudomonas putida</i>	29.4		NADH (2.4)	both	Fe-EDTA with NADH, FAD (13), with NADH, FMN (6.5), with NADPH, FMN or FAD (~15); Fe-citrate with NADH, FAD (6.2), with FAD, NADPH (12)			64
<i>Magnetospirillum gryphiswaldense</i>	16	6.5	NADH not NADPH	FMN (0.24)	Fe-citrate with FAD, NADPH (45)			65
<i>G. sulfurreducens</i>	78, 87	5.5	NADPH (25) not NADH	none added, a flavin is associated with the enzyme	Fe-NLA with NADPH (1000)	Fe-NLA with NADPH (65000)	Fe-NLA (65000)	66

Statistical parameters	MW (KDa)	pH optimum	electron donor (K_d)	Cofactor (K_d)	Fe(III)-EDTA (K_M)	Fe(III)-citrate (K_M)	Specific activity (nmole/min/mg)
Average	38	7	27	8.3	27	13	1775
Standard deviation	21	1	35	8.3	26	11	4103
Coefficient of variation	56%	10%	1.3%	1.0%	1.0%	0.84%	231%
Median	31	7	16	7.9	15	12	14
Number of values	18	7	15	6.0	9.0	12	10
Minimum value	13	5.5	1.3	0.035	6.5	4.2	5×10^{-03}
Maximum value	87	7.5	119	20	80	45	13,100

LEGEND: Summary of kinetic parameters for other reductases reported in the literature (upper table). The numbers in parentheses are the reported K_d or K_M or specific activity values. The lower table summarizes the average, media, maximum, and minimum values reported in the upper table. Citations refer to the citation list in the published manuscript.

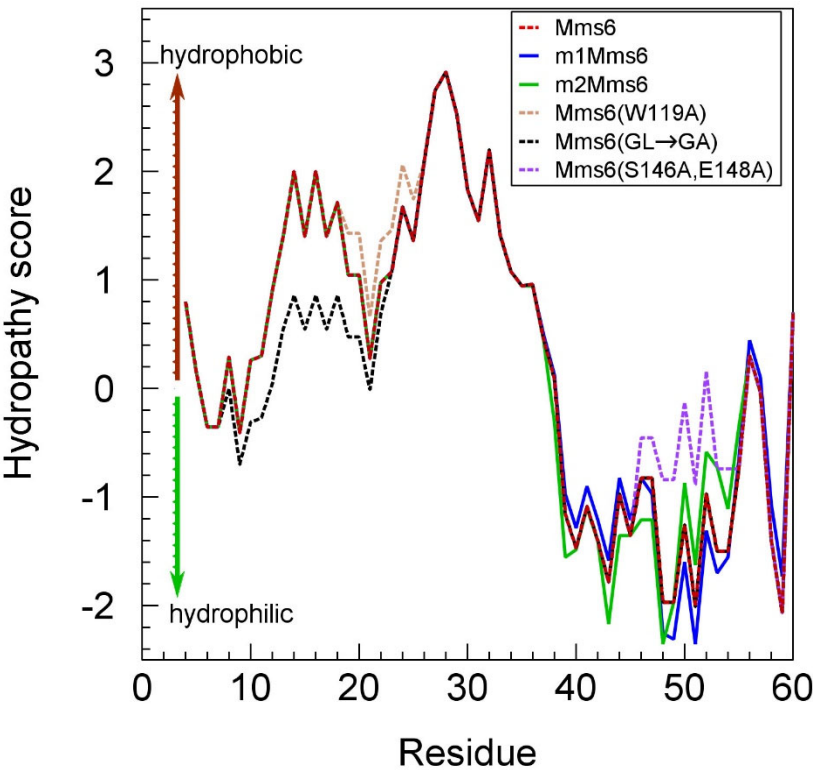
FIGURES

Figure S1. Residue numbering for Mms6 and mutants



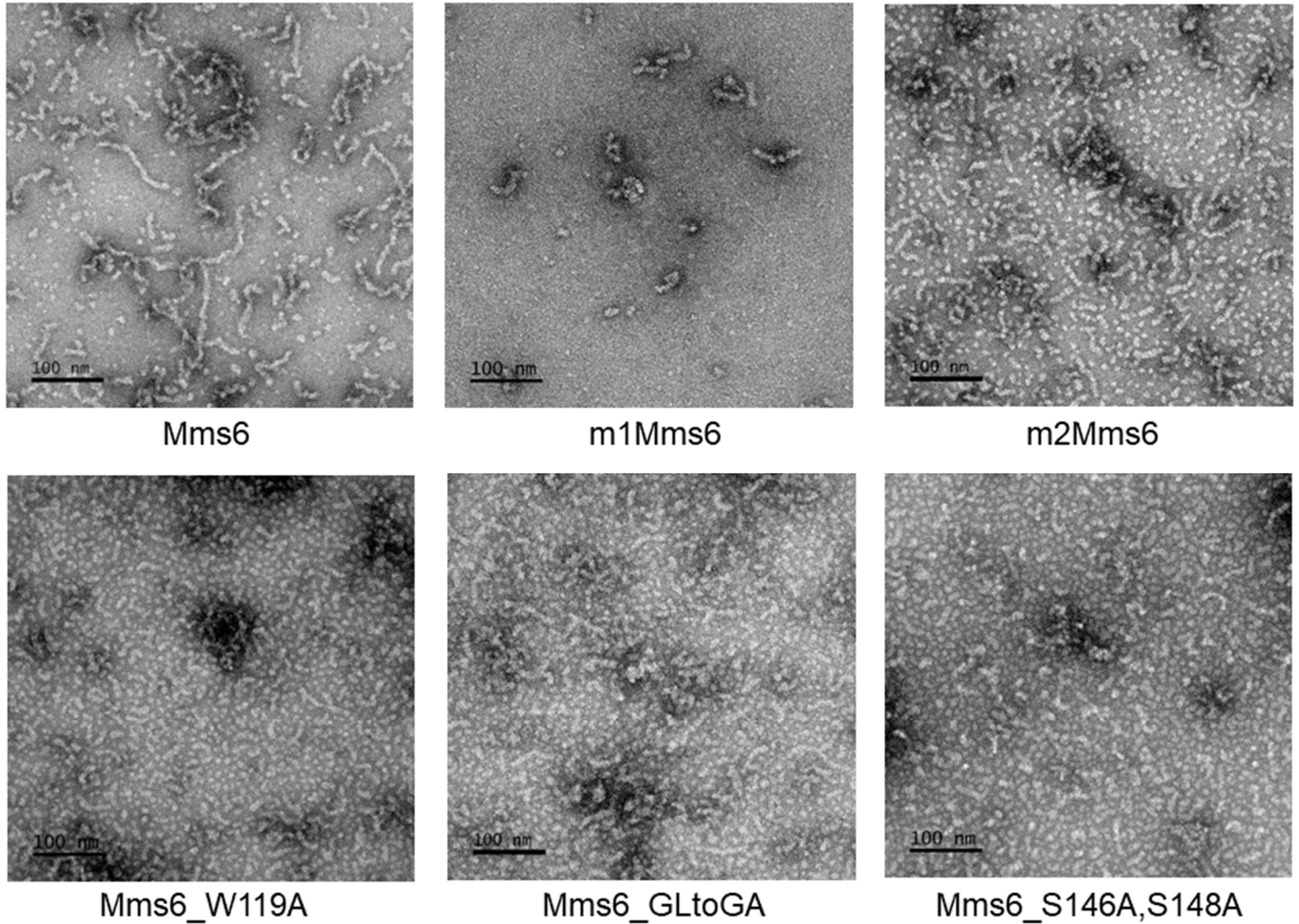
LEGEND: The numbering scheme for Mms6 residues relative to the translation of the complete gene sequence.

Figure S2. Hydropathy plots of Mms6 and its sequence variants used in this work.



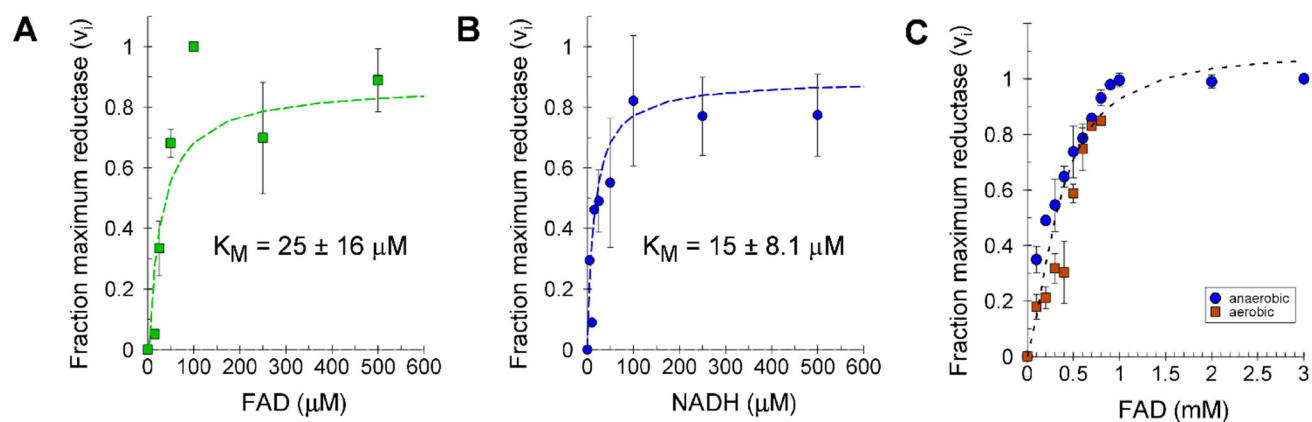
LEGEND: Hydropathy plots were obtained using the Web tool: ProtScale (on the ExPASy Server) <http://expasy.org/tools/protscale.html>. Parameter settings were: window size = 7, Relative weight of the window edges = 100%, Choices in modeling were: Weight variation model = linear, and normalized scale = no.

Figure S3. The effects of C-terminal sequence scrambles on the ability of Mms6 to form micelles.



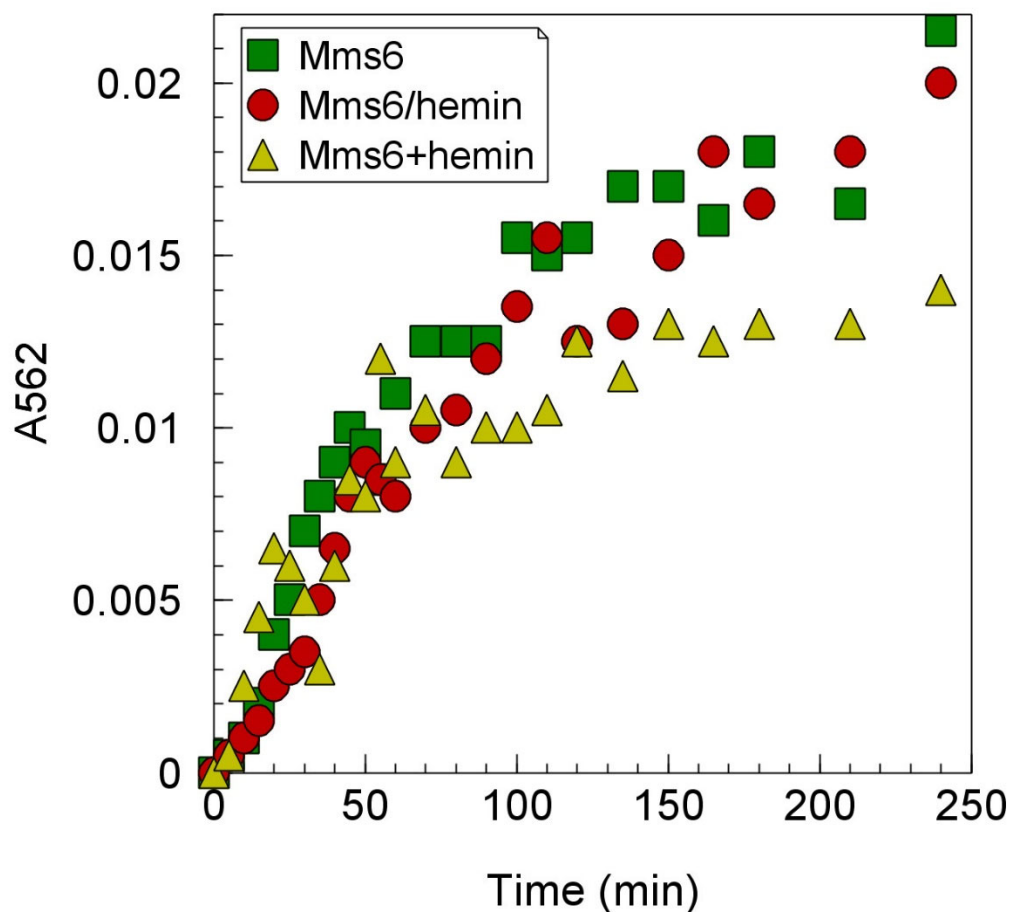
LEGEND: Morphologies of Mms6 mutants observed in TEM images. All images are shown at the same resolution (100 nm scale bar). Note that the m1Mms6 protein preparation was at a lower concentration than others but includes the same characteristic in all images of micelles frequently in strings. The concentration of protein in the m1Mms6 preparation was much lower than for the other preparations, which resulted in a sparser distribution of particles.

Figure S4. K_M estimates for FAD and NADH and a comparison between aerobic and anaerobic conditions.



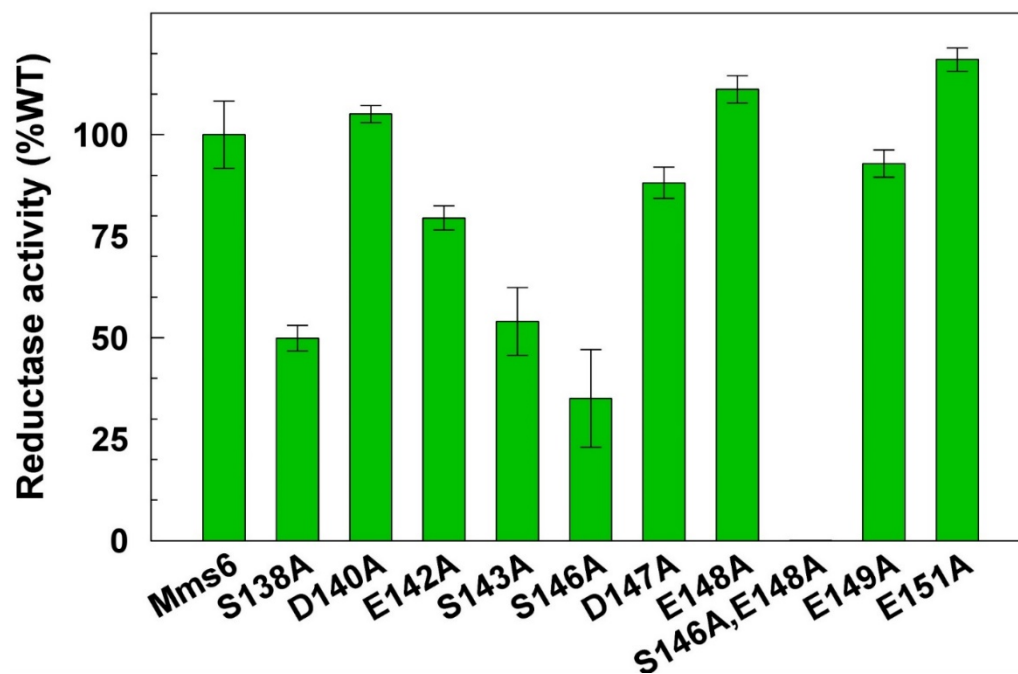
LEGEND: Reductase activity (initial velocity) was determined under aerobic conditions over a range of (A) FAD concentrations and (B) NADH concentrations. Calculated K_M values are shown. These are considered rough estimates as the signal was low, the errors are large and very few points have contributed to the fit. This is reflected by the goodness of fit values (A; $r^2 = 0.81$, standard error of the estimate = 0.17; B: $r^2 = 0.84$, standard error of the estimate = 0.15). (C) In a separate set of experiments, the reductase activity at various concentrations of FAD was compared under aerobic and anaerobic conditions

Figure S5. The effect of hemin on Mms6 reductase activity.



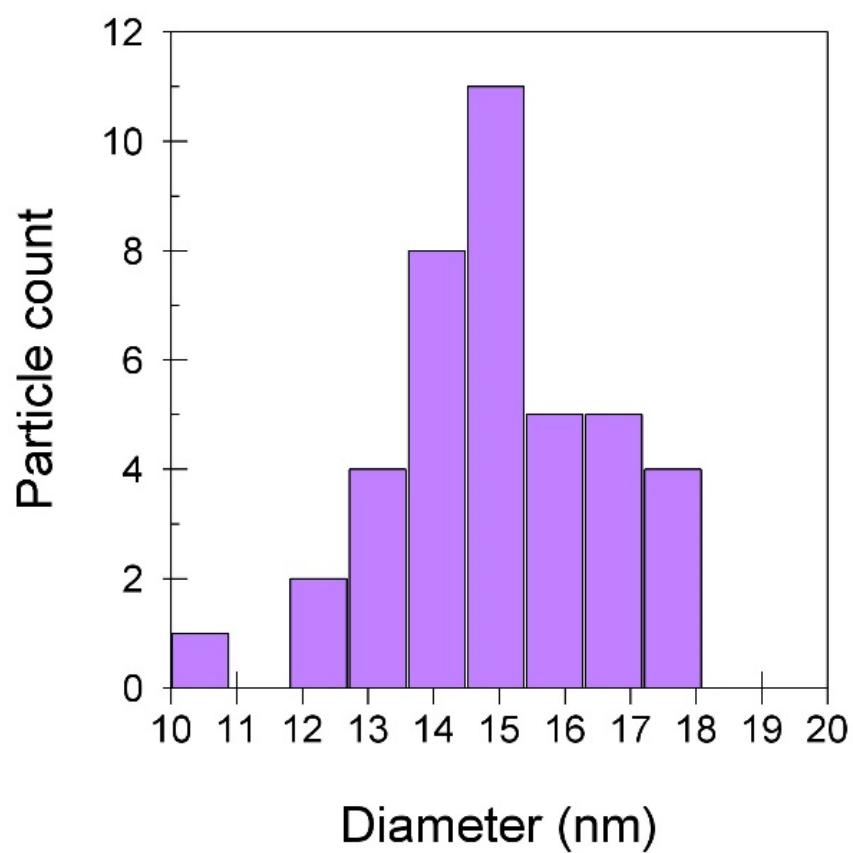
LEGEND: Freshly prepared Mms6 (45 μ M) in Buffer A (20 mM Tris, 100 mM KCl, 0.05% (v/v) NP-40, 10 mM 2-mercapto-ethanol, 0.2 mM PMSF, pH 7.9) with 7M urea, 200 mM imidazole was dialyzed at 4 °C against Buffer A with consecutive changes to buffers containing incrementally lower concentrations of urea (4, 2, 1, 0.5, 0 M). After the urea was removed, the sample was divided into two and dialyzed with or without 0.4 mM hemin over a period of 2 hours followed by dialysis against Buffer A for 14 h with one buffer change. The protein dialyzed against hemin is referred to as Mms6/hemin and the Mms6 dialyzed against Buffer A without hemin was named as Mms6. For the sample Mms6+hemin, 20 μ M hemin was added directly before the assay was initiated. Twenty μ M Mms6 was assayed for reductase activity in the presence and absence of 20 μ M hemin in 20 mM Tris, 100 mM KCl, pH 7.5 with 100 μ M each of NADH and FAD and 0.8 mM ferrozine. Mms6/hemin was assayed under the same conditions but in the absence of additional added hemin.

Figure S6. Ferric reductase activities of Mms6 and mutants with Ala-substitutions in the C-terminal domain.



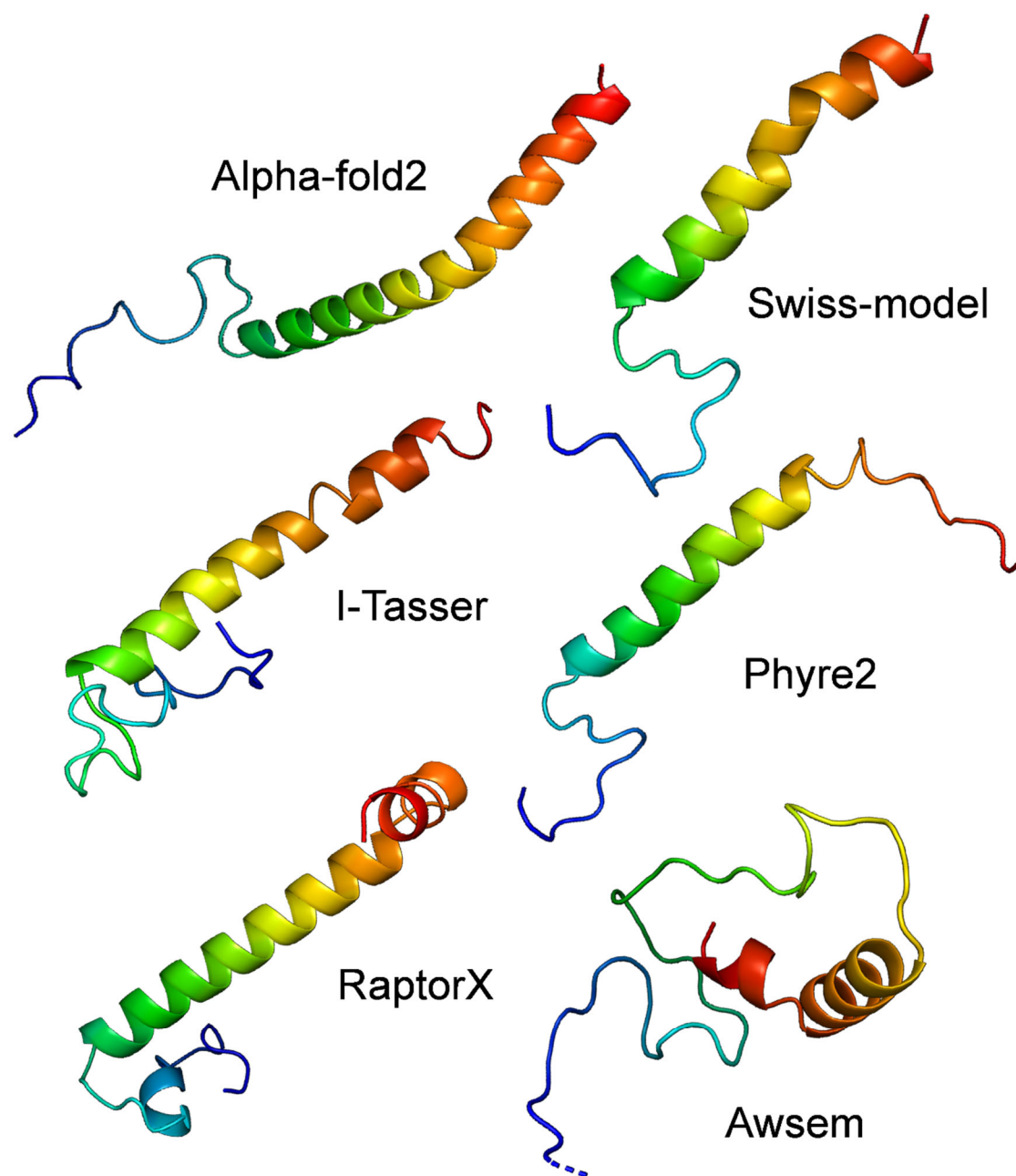
LEGEND: The ferric reductase activity of each recombinant protein was determined as described in Materials and Methods with 20 μ M protein and 100 μ M ferric-citrate.

Figure S7. Mms6 micelle diameters



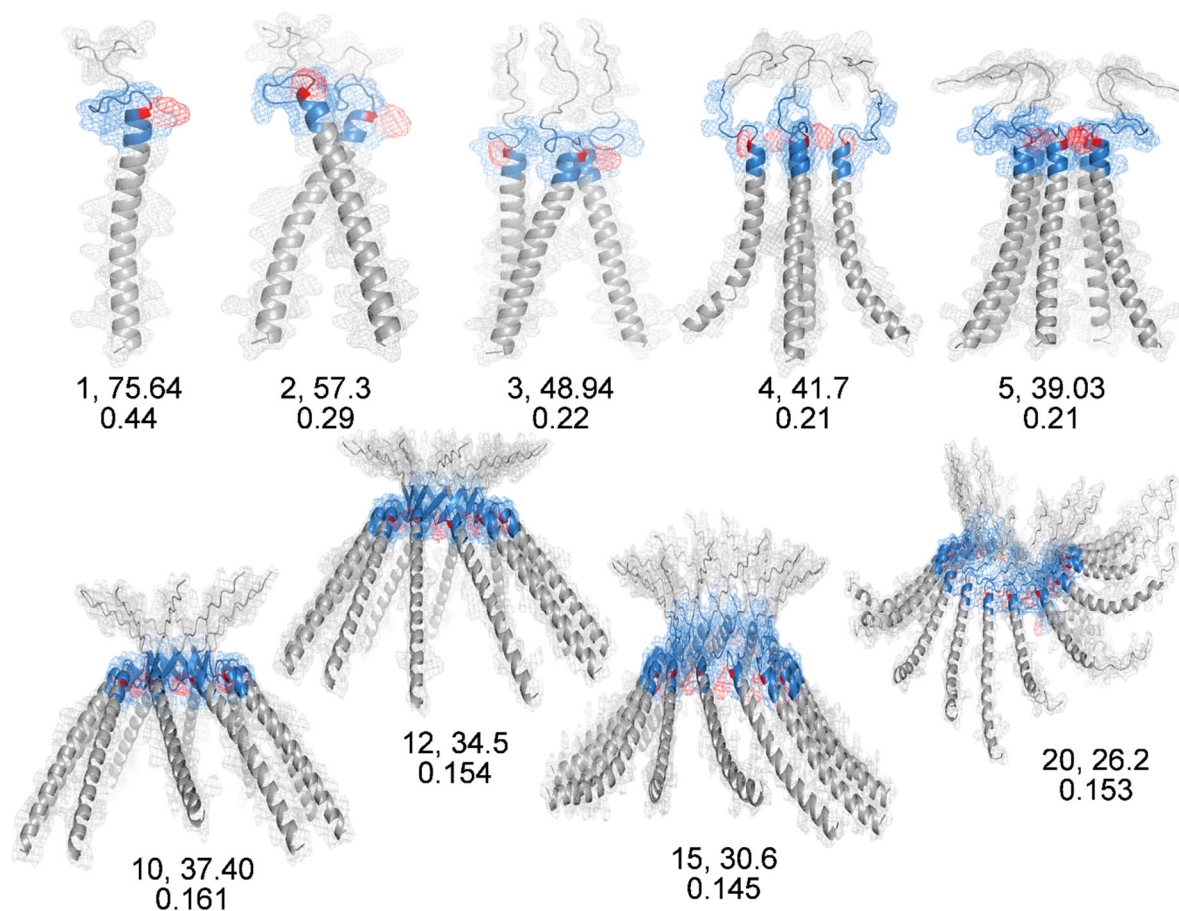
LEGEND: Forty spherical particles were measured for their diameter and grouped by size with one group containing all particles within a 1 nm range. Particle counts are shown per group with the bar centered on the average diameter in the group.

Figure S8. The structure of Mms6 as predicted by a range of modeling algorithms



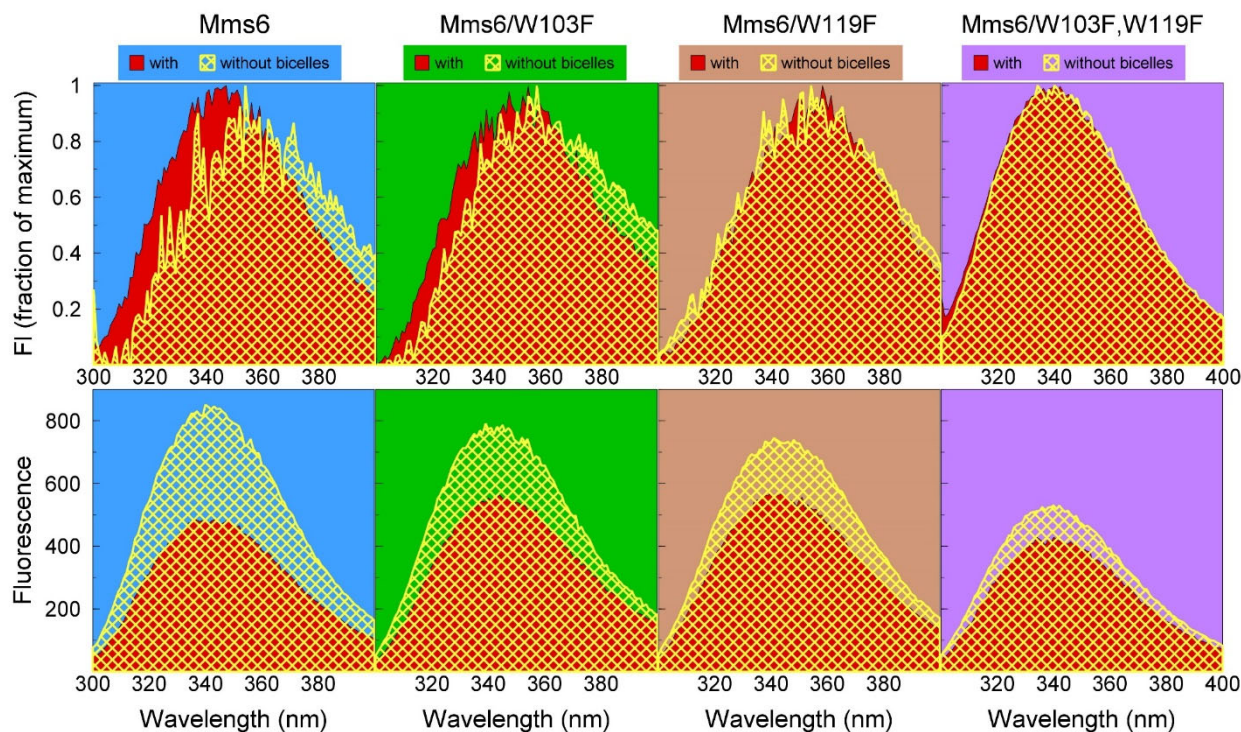
LEGEND: All modeling algorithms, except for Awsem, predicted a flexible C-terminal alpha helical domain and a largely unstructured N-terminal domain. All modeling algorithms accessed on 12 June, 2021.

Figure S9. Predicted quaternary structure of Mms6



LEGEND: The quaternary structure of Mms6 was modeled up to eicosamers by AlphaFold2 (accessed on 15 August, 2022). The first row of numbers under each predicted structure identifies the number of Mms6 monomers in the structure and the pLDDT score (predicted local distance difference test, a per-residue confidence metric). A pLDDT of less than 50 predicts likelihood of disorder. The number in the second row is the pTM (predicted template modeling) score, which reflects the level of confidence in the overall predicted structure.

Figure S10. Identification of the Trp that changes in its hydrophobic environment when Mms6 associates with bicelles.



LEGEND: Mms6 and the mutant proteins Mms6/W103F, Mms6/W119F and Mms6W103F,W109F at 20 μ M were each incorporated into 25 mM q=1 DMPC/DHPC bicelles in a buffer of 20 mM Tris, 100 mM KCl, pH 7.5. The preparations were scanned by a Varian, Cary Eclipse (medium scan rate, PMT detector set at 680V) over the wavelength range 300 to 400 nm in quartz cuvettes with an excitation wavelength of 290 nm. The slit width for both excitation and emission was 5 nm. These results show that the fluorescence shift is lost with W119F but not with W103F.