Supplementary material

Re-refinement of the Mm-MIOX structure

Attempts to re-refine the deposited 2HUO file failed initially due to the high anisotropy of the data (~2.8 Å resolution in the c* direction) and the inherent instability of TLS refinement as implemented in REFMAC5 (1). However, after applying an anisotropy correction as described in Strong *et al.* (2) and removing the solvent model it was possible to perform refinement using the PHENIX package (3). After a few rounds of refinement in PHENIX, a new TLS-model was introduced based on TLSMD analysis (4). The N-terminus was rebuilt in the improved density using COOT (5) and the model was extended with the sidechain of Phe²⁸ that was now clearly visible. The main remodelling was however of the position of the backbone NH of Arg²⁹. This entity forms a strong ionic interaction with Asp⁹⁰ (Fig. 2C in the main paper) but in 2HUO this NH-group was pointing outwards toward bulk water. Furthermore, the solvent model was rebuilt and a few sidechains were moved to their closest rotamer. The re-refined model belong to the upper 95th percentile in overall quality in the resolution range 1.75-2.25 Å (12522 structures) according to Molprobity (6) (2HUO belong to the upper 47th percentile).

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Figure S1. Structure-based sequence alignment of 8 eukaryotic myo-Inositol oxygenase

sequences.

HsMIOX	KASFRNYTSGPLL 3	7
MmMIOX	KDSFRNYTSGPLL 3	7
RnMIOX	KGSFRNYTSGPLL 3	7
SsMIOX	KGSFRNYTSGPLL 3	4
PcMIOX	MIPPPSQVVDLEEVSDAIDEVNKLKSARSWNDGSDFDAGKDKTKFRQYEAAC 5.	2
XtMIOX	NDQYRNYKDGPLL 2	6
DrMIOX	KTEFRNFENGDLF 3	0
At1MIOX	MTILIDRHSDQNDAGDEIVEKNQGNGKEEETELVLDAGFEAPHTNSFGRTFRDYDAESER 6	0
	$\alpha 1$ $\alpha 2$ $\beta 1$ $\alpha 3$ $\alpha 4$	
		~
HSMIOX	DR-VFTTYKLMHTHQTVDFVRSKHAQFGGFSYKKMTVMEAVDLLDGLVDESDPDVDFPNS 9	6
MmMIOX	DR-VFTTYKLMHTHQTVDFVSRKRIQYGSFSYKKMTIMEAVGMLDDLVDESDPDVDFPNS 9	6
RNMIOX	DR-VFTTYKLMHTHQTVDFVMRKRIQFGSFSYKKMTVMEAVDMLDDLVDESDPDVDFPNS 9	6
SSMIOX	DR-VFRTYKLMHTWQTVDFVRKKHAQFGGFSYKRMTVLEAVDMLDGLVDESDPDVDFPNS 9	3
PCMIOX	DR-VKAFYKEQHEKQTVEFNIKVRANFKKTVRARMGIWEAMELLNTLVDESDPDTTVSQI 1	11
XtMIOX	DR-VRKTYTLMHTYQTVQFVKEKHVQWGSCTHRKMSVMDALSLLDNLVDESDPDVDFPNS 8	5
DrMIOX	DR-VFNTYKLMHTHQTLDFVKQKHQVWSNCSHFSLSMMDSIDSLDELVDESDPDVDFPNS 8	9
Atimiox	RRGVEEFYRVNHIGOTVDFVRKMREEYEKLNRTEMSIWECCELLNEFIDESDPDLDEPQI 1. * * * * * **::* : : : : : *: ::****** ::	20
	Reserved Reserved	
HsMIOX	FHAFQTAEGIRKAHPDKDWFHLVGLLHDLGKVLALFGEPQWAVVGDTFPVGCRPQAS 1	53
MmMIOX	F <mark>H</mark> AFQTAEGIRKAHPDKDWFHLVGLLH <mark>DL</mark> GKIMALWGEPQWAVV <mark>GD</mark> TFPVGCRPQAS 1	53
RnMIOX	FHAFQTAEGIRKAHPDKDWFHLVGLLH <mark>DL</mark> GKILALWGEPQWAVVGDTFPVGCRPQAS 1	53
SsMIOX	FHAFQTAEGIRKAHPDKDWFHLVGLLH <mark>DL</mark> GKVLVLAGEPQWAVVGDTFPVGCRPQAS 1	50
PcMIOX	EHLLQTAEAIRRDG-KPDWMQVAGLVHDLGKLLHIFGSDGQWDVVGDTFVVGCKFSDK 1	68
XtMIOX	FHAYQTAEGIRRIHPDKDWFQLVGLLH <mark>DV</mark> GKIMALDNEPQWSVV <mark>GD</mark> TFPVGCKFQES 1	42
DrMIOX	FHAFQTAEGIRREHPDKDWFQLVGLIH <mark>DV</mark> GKVMALYSEPQWAVVGDTYPVGCKFQNS 1	46
At1MIOX	EHLLQTAEAIRKDYPDEDWLHLTGLIHDLGKVLLHSSFGELPQWAVVGDTFPVGCAFDES 1	80
	α6 α7	
HsMIOX	VVFCDSTFODNPDLODPRYSTELGMYOPHCGLDRVLMSWGHDEYMYOVMKFNKFSLPPEA 2	13
MmMIOX	VVFCDSTFQDNPDLQDPRYSTELGMYQPHCGLENVLMSWGHDEYLYQMMKFNKFSLPSEA 2	13
RnMIOX	VVFCDSTFODNPDLODPRYSTELGMYOPHCGLENVLMSWGHDEYLYOMMKFNKFSLPSEA 2	13
SsMIOX	VVFCDSTF0DNPDL0DPVYSTELGMY0PHCGLENALMSWGHDEYMY0MMKFNKFSLPGEA 2	10
PcMIOX	NIYP-ETFKGNPDYYDPVYSTEYGVYSPHCGLENVMLSWGHDEYLYHVLK-NQSSLPDEA 2	26
XtMIOX	IVFSDTTFRDNPDTKHPIYSTKYGIYKPNCGLENVLMSWGHDEYLFKVLKFNKSSIPEEG 2	02
DrMIOX	IVFRNSTFEGNPDGKNPAPNTEFGIYEPQCGLDKVLMSWGHDEYLYRVMKFNKCTIPEEG 2	06
At1MIOX	IVHH-KYFKENPDYDNPSYNSKYGIYTEGCGLDNVLMSWGHDDYMYLVAKENOTTLPSAG 2 *. *** .* .:: *:* ***::*****:*:: : * *: ::*	39
HeMTOY	FYMIRFHSFYDWHTCPDYOOLCSOODLAMLDWURFFNKFDLVTKCPDLDDUDKLDDVOC 2	73
MmMIOX	FYMIREHSEVPWHTGGDVROLCSOODLDMLPWVOEFNKEDLVTKCPDLPDVESLBPVVOG 2	73
RoMIOX	FYMUREHSEVDWHTCGDVROLCSOODLDMLDWUOFENKEDLVTKCDDLDEVKSLBDVVOG 2	73
SeMIOX	FYLIREHSEVPWHTGGDVROLCNEODLAMLPWVOEFNKEDLVTKGSDMDDVDELRPVVOG 2	70
POMIOX	LYMIRYHSFYPWHREGAYMHL/TNANDORALEAVRAFNPYDL/YSKSDDPIDPEKVKPYYOS 2	86
X+MIOX	LYMIREHSEVOWHTGGDVOHLCNDKDHRMLNWUKFENKEDLVTKTEDLDDVELLBOVVOG 2	62
DrMIOX	LYMIREHSEVENHSNGDYMHLCNEKDOOMLEWVKEFNKEDLYTKSTELEDVERLKEVVOS 2	66
A+1MTOX	LETTRY HSFYALHKSEAVKHLMNNEDRENMKWLKVENKYDLYSKSKVRVNVEEVKPYYLS 2	99
ACIMICA		
HsMIOX	LIDKYCPGILSW 285	
MmMIOX	LIDKYCPGTLSW 285	
RnMIOX	LIDKYCPGILSW 285	
SsMIOX	LIDKYCPGVLCW 282	
PcMIOX	LIAKFFPDVIEW 298	
XtMIOX	LIDKYCPGVLSW 274	
DrMIOX	LIDKYCPGVLOW 278	
At1MIOX	LTNKYFPSKLKW 311	
	* *: *. : *	

Figure S2. Assignment of *myo*-inosose-1 as bound species as opposed to *myo*-inositol. A SIGMAA-weighted (7) 2Fo-Fc map (blue) at 1σ and Fo-Fc (red) at -5σ is rendered from phases from a refinement where *myo*-inositol was present in the model. The final refined model of *myo*-inosose-1 is shown in gray for reference.



Figure S3. Modelling of a putative interaction between Asp⁸⁵ and the diiron cluster that can potentially be formed in the Lys¹²⁷Ser mutant. Hs-MIOX in light brown, energy-minimised model of Lys¹²⁷Ser mutant shown in green.



Figure S4. Experimental spectra of the mixed-valent state of human MIOX in the absence (upper panel, spectrum taken from Fig. 5A) and presence (lower panel, spectrum taken from Fig. 5B) of the substrate *myo*-inositol. Below each experimental spectrum there is a simulated spectrum with the following parameters for g-value and line width: Upper panel, sum of components I and II in a quantitative ratio 0.3/0.7. Component I, $g_{\parallel} = 1.96$, $g_{\perp} = 1.74$, $L_{\parallel} = 25$ G, $L_{\perp} = 160$ G; component II, $g_{\parallel} = 1.94$, $g_{\perp} = 1.72$, $L_{\parallel} = 70$ G, $L_{\perp} = 250$ G. Both components were simulated with Gaussian line shape. Lower panel: simulated spectrum with $g_x = 1.96$, $g_y = 1.82$, $g_z = 1.78$; $L_x = 25$ G, $L_y = 30$ G, $L_z = 60$ G and Gaussian line shape.



Figure S5. The EPR microwave saturation parameter $P_{1/2}$ of MIOX (o) and MIOXinositol (•) as a function of the inverse absolute temperature. Saturation recovery data were collected at 5.5, 7.0 and 10.0 K. The exchange coupling constants, J ($H_{ex} = 2J \cdot S_1 \cdot S_2$), were estimated from the slopes as $\Delta = -3J$ and were determined to be ~20 cm⁻¹ for MIOX and ~10 cm⁻¹ for MIOX-inositol.



Table S1. Re-refinement statistics and quality of Mm-MIOX structure	
Resolution (highest resolution shell) (Å)	42.6-2.0 (2.09-2.0)
R _{cryst} [#] (%)(highest resolution shell)	21.0 (30.4)
R _{free} [#] (%)(highest resolution shell)	25.5 (31.0)
RMS deviation from ideal geometry	
Bond lengths (Å)	0.005
Bond angles (°)	0.76
Average B-factors (Å ²)	56.5
Ramachandran plot (favored, allowed, outliers)(%)*	96.9, 3.1, 0

* According to Molprobity (6)