Supporting Information

Targeting and maturation of Erv1/ALR in the mitochondrial intermembrane space

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Supplementary Table 1. ^{15}N transverse relaxation rates R_2 (s^{-1}) and heteronuclear $^{15}N\{^1H\}$-NOEs per residue of fully reduced FAD-free sf-ALR and *E. coli*-purified sf-ALR, collected at 600 MHz in 50 mM phosphate buffer pH 7 at 308K.

	^{15}N R_2		^{15}N $\{^1H\}$-NOEs	
	Fully reduced FAD-free ALR	E. coli-purified sf-ALR	Fully reduced FAD-free ALR	E. coli-purified sf-ALR
D8	7.65	5.74	0.35	0.34
T9	7.50	9.68	-0.07	0.30
K10	9.63	9.86	-0.07	0.28
F11	8.28	11.38	0.23	0.35
R12	9.19	16.70	0.06	0.39
E13	11.14	13.06	0.29	0.19
D14	9.33	19.60	0.05	0.30
C15	17.56	18.56	0.35	0.68
Mean	10.04	13.07	0.15	0.36
A34	23.89	20.01	0.98	0.98
A35	18.08	19.32	0.61	0.67
Y36	23.46	16.73	0.85	0.84
Y37	24.69	17.23	0.86	0.78
L40	23.03	16.83	0.74	0.84
T42	22.65	16.31	0.71	0.83
Q47	27.60	17.96	0.52	0.87
D48	24.35	15.24	0.92	0.87
M49	17.44	18.82	0.54	0.77
A50	15.13	18.42	0.50	0.70
S57	31.19	17.95	0.65	0.99
K58	21.86	16.92	0.60	0.95
F59	20.91	15.48	0.59	0.75
T80	29.37	13.14	0.77	0.97
R81	22.96	19.22	0.65	0.67
T82	34.02	15.86	0.63	0.92
F86	25.29	18.93	0.67	0.83
T87	23.00	16.90	0.47	0.85
Mean	23.83	17.29	0.68	0.84
G102	12.78	15.82	0.28	0.85
K110	13.89	19.03	0.46	0.72
V111	9.86	20.33	-0.06	0.78
D112	9.38	20.87	0.11	0.73
E113	10.87	22.94	-0.04	0.81
R114	12.69	17.23	0.23	0.74
W115	11.90	19.84	0.22	0.65
R116	9.41	19.05	0.19	0.79
D117	8.67	19.37	0.28	0.44
G118	8.24	16.85	0.03	0.68
W119		19.98	0.25	0.69
K120	8.12	15.26	-0.05	0.81
D121		17.45	0.02	0.71
G122	5.51	18.07	-0.48	0.59
S123	5.55	17.87	-0.70	0.75
C124	3.90	19.00	0.37	0.75
D125	2.38	13.51	-1.49	0.55
Mean	8.88	18.38	-0.02	0.709

Supplementary Figure 1. Cysteine redox and aggregation state of fully reduced FAD-free sf-ALR. (**a**) non reducing SDS-PAGE of fully reduced FAD-free sf-ALR before and after modification with 4-acetamido-4-maleimidylstilbene-2,2-disulfonic acid (AMS), a gel shift reagent selective for free thiol groups; (**b**) analytical size-exclusion chromatography comparison of apparent molecular size. Elution profiles of *E. coli* purified sf-ALR (blue dots), FAD-free sf-ALR in the absence (green dots) and the presence (red dots) of 100 mM DTT separated on a Superdex 75 HR 10/30 (Amersham Pharmacia Biotech). In the inset the calibration of gel filtration column obtained from standard profiles corresponding to conalbumin (75 KDa), carbonic anhydrase (29 KDa), ribonuclease (13.7 KDa) and aprotinin (6.5 KDa) and the corresponding apparent MWs of the analyzed ALR states are reported.

Supplementary Figure 2. Maturation process of sf-ALR by sequential addition of Mia40$_{3S-S}$ and FAD. (**a**) Overlay of ^{1}H-^{15}N HSQC spectra of fully reduced FAD-free sf-ALR before (in red) and after addition of 2 equivalents of Mia40$_{3S-S}$ (in black); (**b**) overlay of ^{1}H-^{15}N HSQC spectra of a 1:2:1 fully reduced FAD-free sf-ALR/Mia40$_{3S-S}$/FAD mixture (in red) and of *E. coli*-purified sf-ALR state (in black); (**c**) non reducing SDS-PAGE of Mia40$_{3S-S}$ (1, 1'), fully reduced FAD-free sf-ALR (2, 2') and 1:1 (3, 3') and 1:2 (4, 4') fully reduced FAD-free sf-ALR/Mia40$_{3S-S}$ mixtures in the presence or absence of 150 mM DTT.

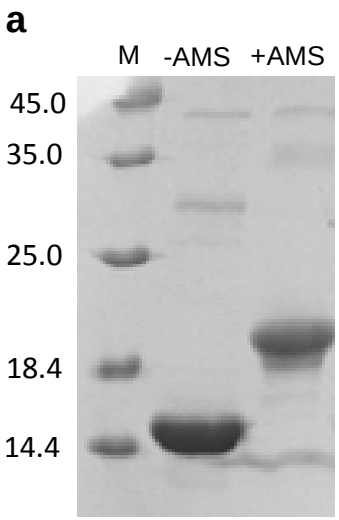
Supplementary Figure 3. Maturation process of sf-ALR by sequential addition of FAD and Mia40$_{3S-S}$. (**a**) Overlay of ^{1}H-^{15}N HSQC spectra of fully reduced FAD-free sf-ALR before (in red) and after addition of 1 equivalent of FAD (in black); (**b**) overlay of ^{1}H-^{15}N HSQC spectra of a 1:2:1 fully reduced FAD-free sf-ALR/Mia40$_{3S-S}$/FAD mixture (in black) and of *E. coli*-purified sf-ALR state (in red); (**c**) residues showing backbone chemical shift variations comparing the ^{1}H-^{15}N HSQC maps of the 1:2:1 fully reduced FAD-free sf-ALR/Mia40$_{3S-S}$/FAD mixture with that of the *E. coli*-purified sf-ALR state are mapped in blue and orange on the ribbon representation of the two

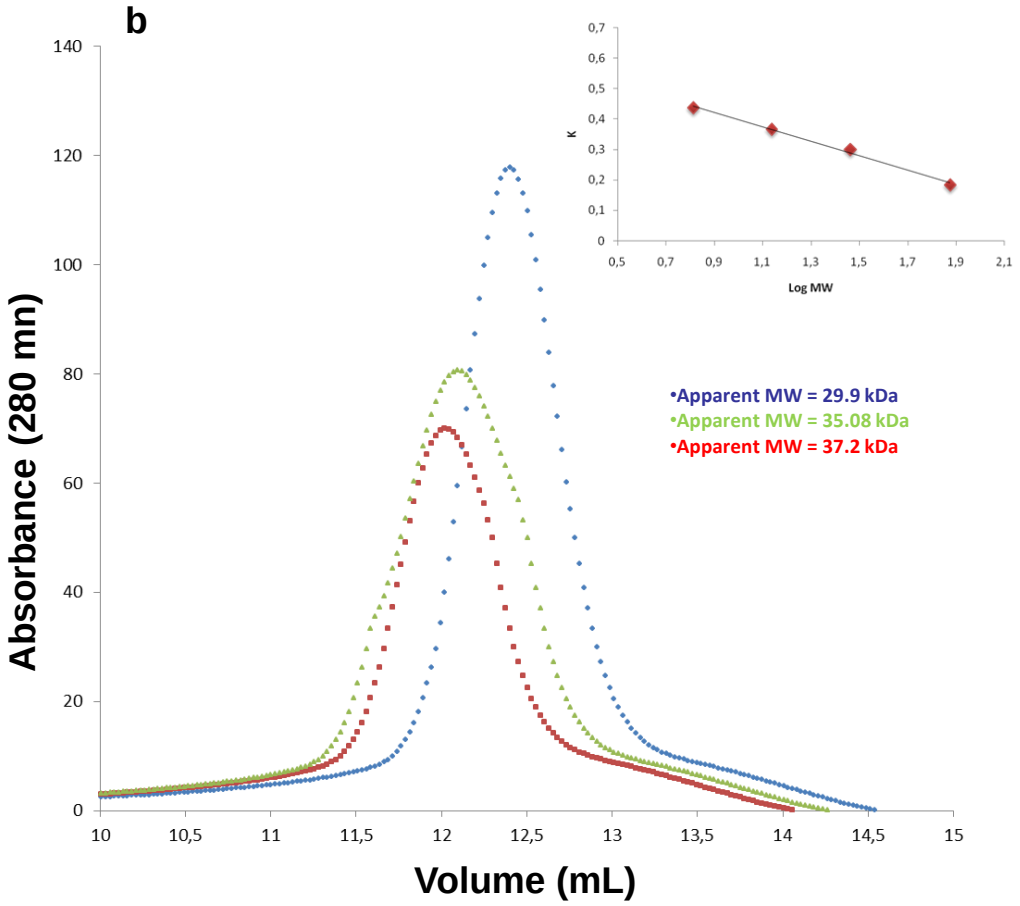
subunits of *E. coli*-purified sf-ALR. FAD is in light blue and cysteine residues are shown as yellow sticks.

Supplementary Figure 4. Non reducing SDS-PAGE analysis of FAD and Mia40$_{3S-S}$/Mia40$_{2S-S}$ mixture. (**a**) (1, 2) Fully reduced FAD-free sf-ALR before and after modification with 4-acetamido-4-maleimidylstilbene-2,2-disulfonic acid (AMS); (3, 4) a 1:1 fully reduced FAD-free sf-ALR/FAD mixture before and after AMS modification; (5, 6) a 1:1:0.5 fully reduced FAD-free sf-ALR/FAD/Mia40$_{3S-S}$ mixture before and after AMS modification; (7, 8) a 1:1:1 fully reduced FAD-free sf-ALR/FAD/Mia40$_{3S-S}$ mixture before and after AMS modification; (9, 10) a 1:1:1.5 fully reduced FAD-free sf-ALR/FAD/Mia40$_{3S-S}$ mixture before and after AMS modification. (**b**) (1, 2) Mia40$_{2S-S}$ before and after AMS modification, (3, 4) a 1:1:2 fully reduced FAD-free sf-ALR/FAD/Mia40$_{2S-S}$ mixture before and after AMS modification; (5, 6) a 1:1:1 fully reduced FAD-free sf-ALR/FAD/Mia40$_{2S-S}$ mixture before and after AMS modification; (7, 8) a 1:2:2 fully reduced FAD-free sf-ALR/FAD/Mia40$_{2S-S}$ mixture before and after AMS modification.

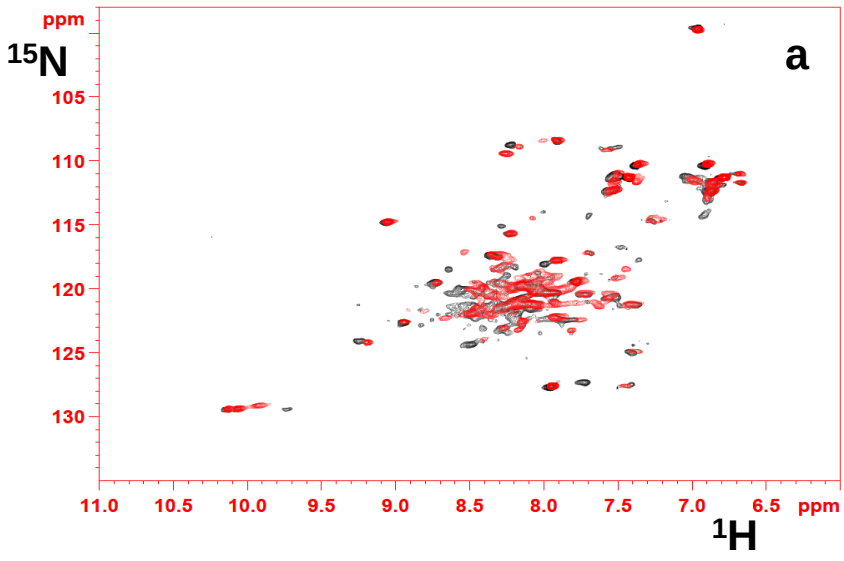
Supplementary Figure 5. Catalytic activity of the reconstituted sf-ALR enzyme. ALR-mediated reduction of cytochrome *c* was measured over time by measuring absorbance increase at 550 nm in the following reconstitution procedures: (i) fully reduced FAD-free sf-ALR was mixed with oxidized cytochrome *c* (black line); (ii) fully reduced FAD-free sf-ALR was mixed with Mia40$_{3S-S}$ and, after 1 h, with FAD and oxidized cytochrome *c* (blue line); (iii) fully reduced FAD-free sf-ALR was mixed with FAD and, after 1 h, with Mia40$_{3S-S}$ and oxidized cytochrome *c* (magenta line); (iv) fully reduced FAD-free sf-ALR was mixed with FAD and oxidized cytochrome *c* (green line); (v) fully reduced FAD-free sf-ALR was mixed with Mia40$_{3S-S}$ and, after 1 h, with oxidized cytochrome *c* (olive line). In the inset, a selected region of the absorbance spectrum of oxidized cytochrome *c* (red line) is compared with that collected on the mixture (ii) (blue line) to show that

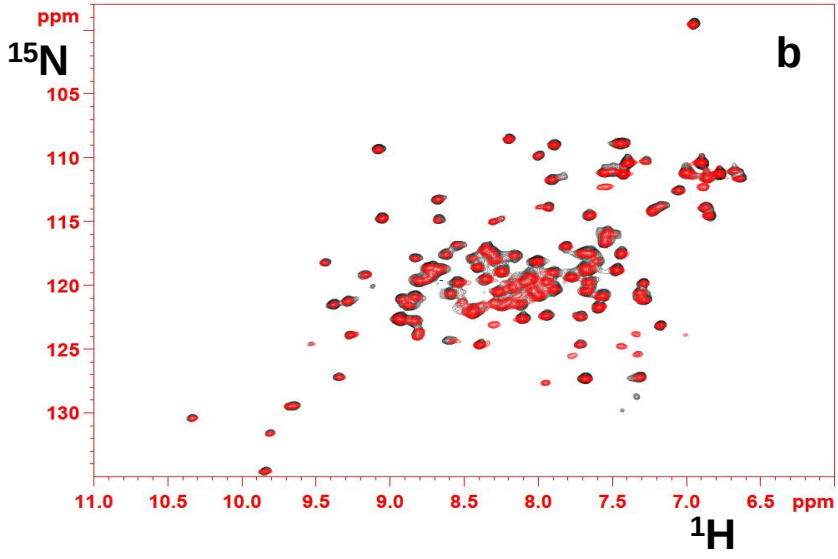
the observed increase of absorbance at 550 nm allows to monitor the electron transfer from ALR to cytochrome *c*.

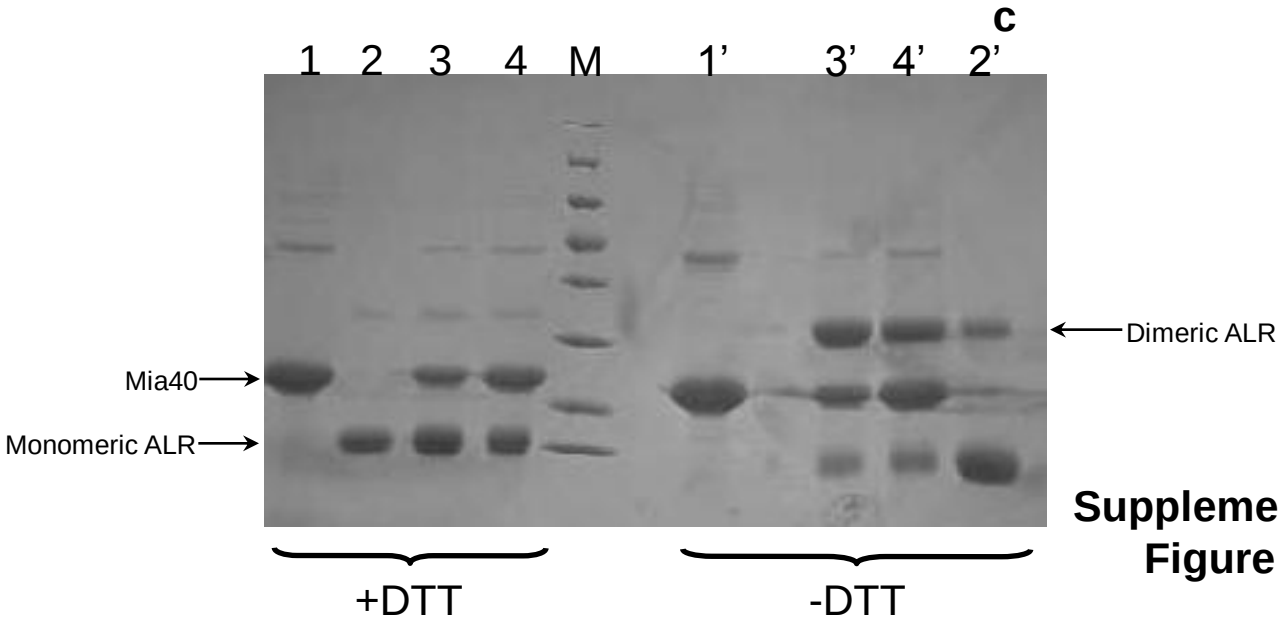




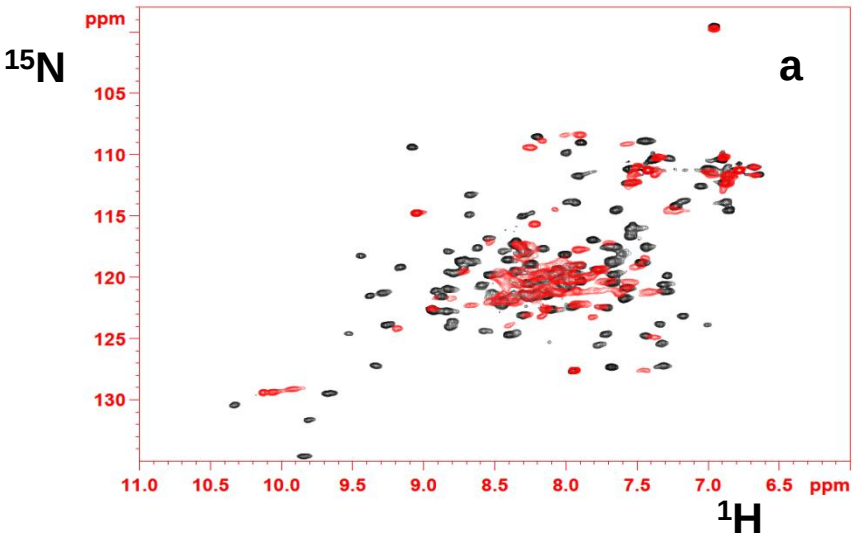
Supplementary Figure 1

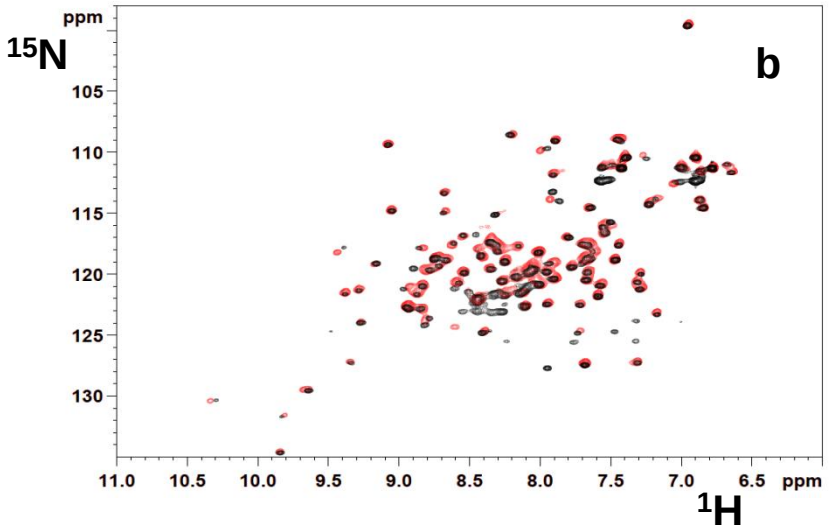


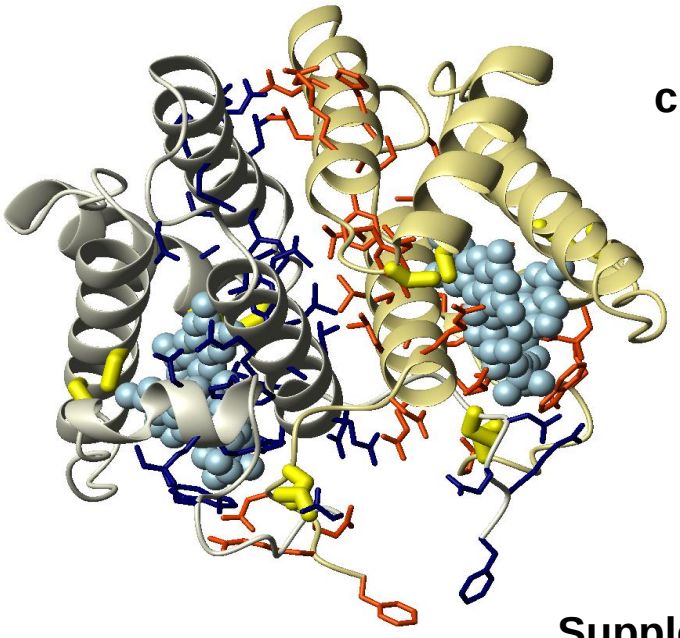




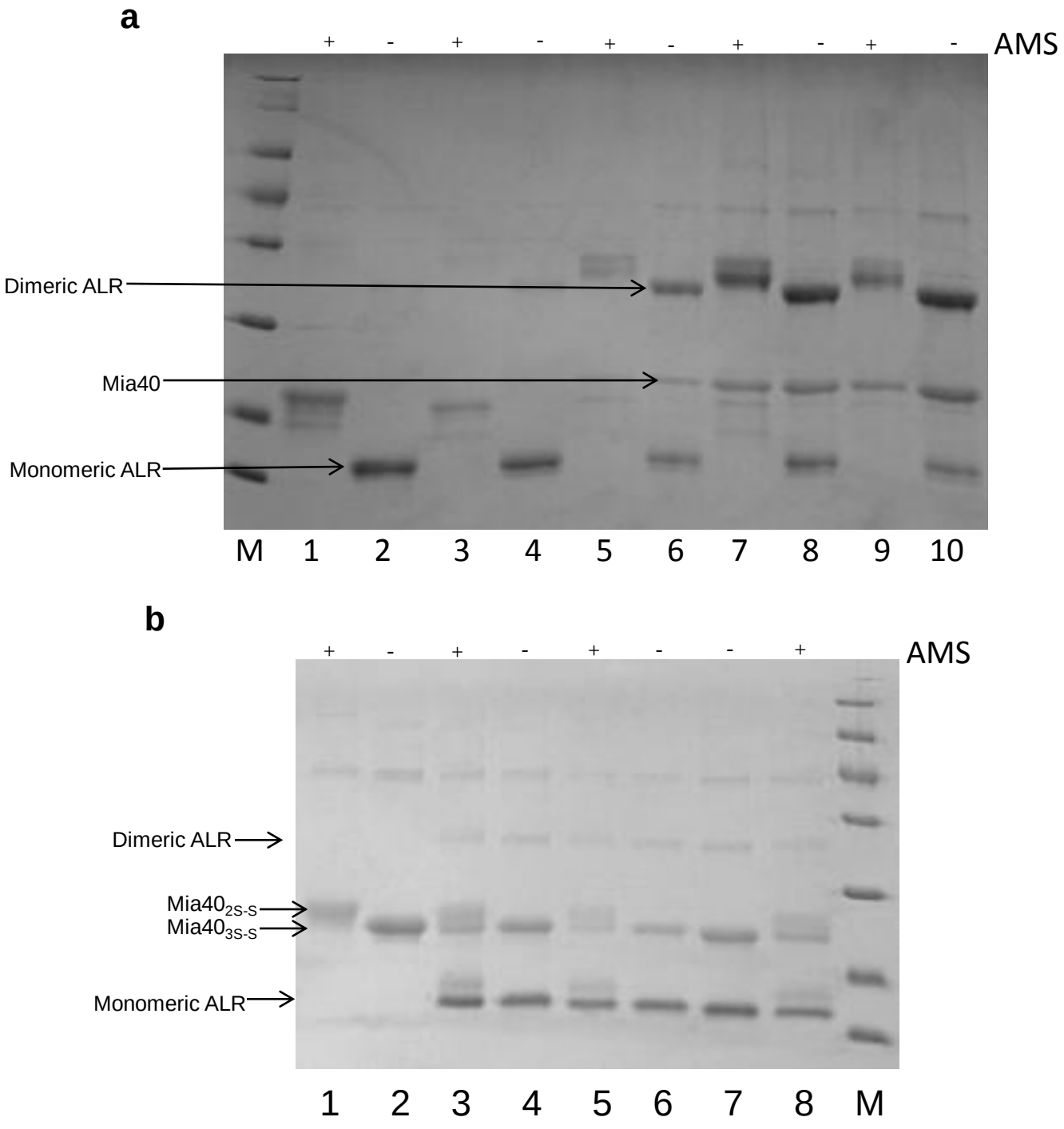
Supplementary Figure 2



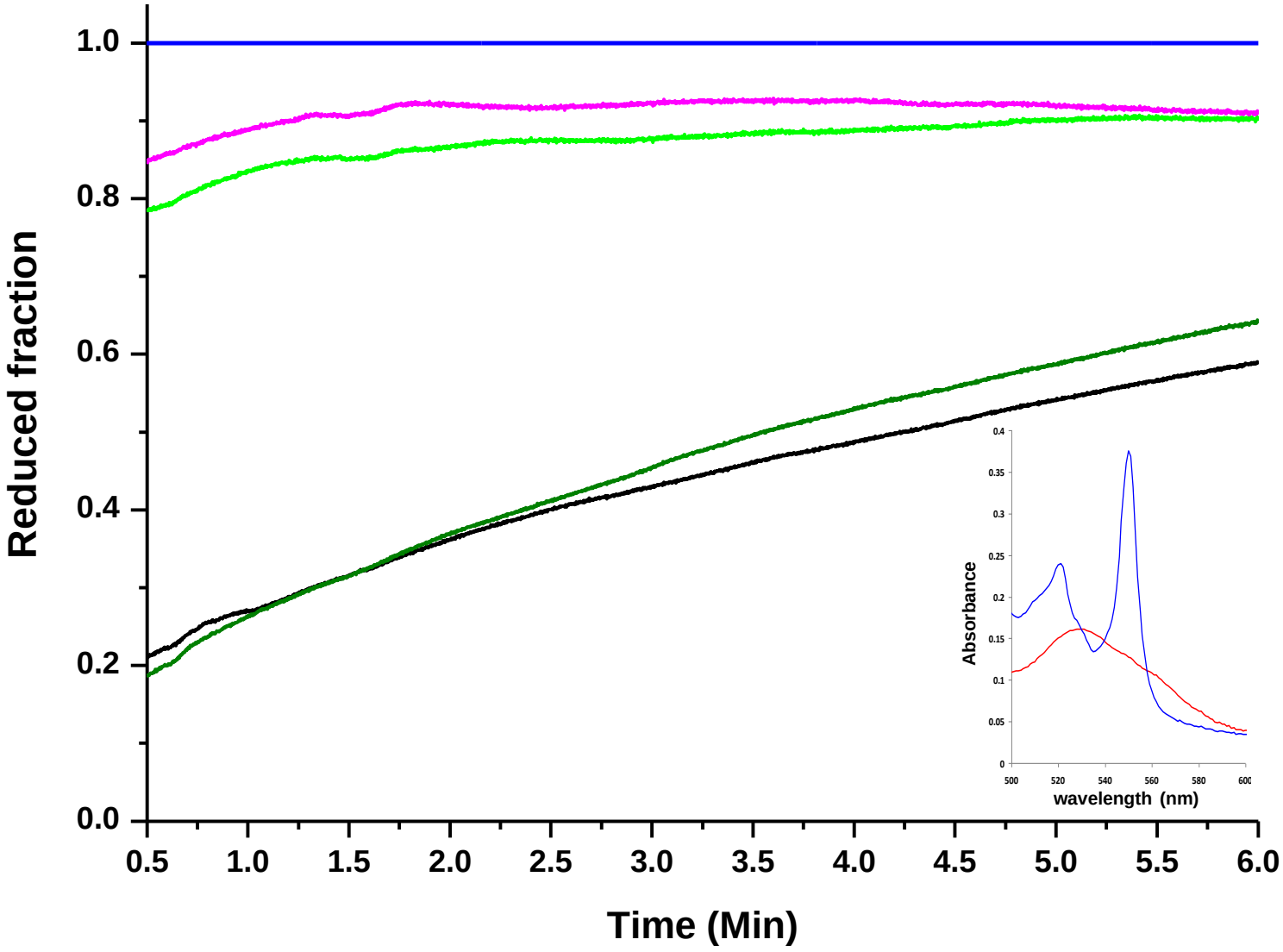




Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5