

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Cytochrome c released by HCCS variants

Recombinant GST-HCCS variants with substitutions in A) Domain I, B) Domain II, C) Domain III, and D) Domain IV were co-expressed with cytochrome c in Δ ccm *E. coli* in 1 L cultures. Cells were lysed by sonication and fractionated by ultracentrifugation. UV/vis absorption spectra were recorded for the resulting soluble fractions using 1 mg of total protein, quantified by Nanodrop. The alpha peak region of the recorded spectra is depicted and each colored spectrum represents the indicated mutant in the corresponding color.

Figure S2. N128A and M130A HCCS mutants exhibit distinguishable heme characteristics

Recombinant GST-HCCS: cytochrome c co-complexes were purified from Δ ccm *E. coli* and prepared for UV/vis absorption spectroscopy. Shown are spectra for A) N128A HCCS/ cyt c and B) M130A HCCS cyt c following purification (black line) and chemical reduction with sodium dithionite (red). Arrows indicate the wavelength (nm) of peak absorption maxima. All spectra were performed with equal amounts (100 μ g) of total purified protein. For all proteins, Bradford quantitation was confirmed by Coomassie staining, which also indicated that GST-HCCS proteins were obtained at >90% purity.

Figure S3. ALA treatment enhances the synthetase activity of several HCCS variants

Recombinant GST-HCCS variants were co-expressed with cytochrome c in Δ ccm *E. coli* either in the presence (+) or absence of ALA (-). Cells were lysed with BPER reagent and 100 μ g of

protein extracts were resolved by SDS-PAGE and transferred to nitrocellulose. Released cytochrome c was detected by heme stain. Data shown are representative of 3 replicate experiments.

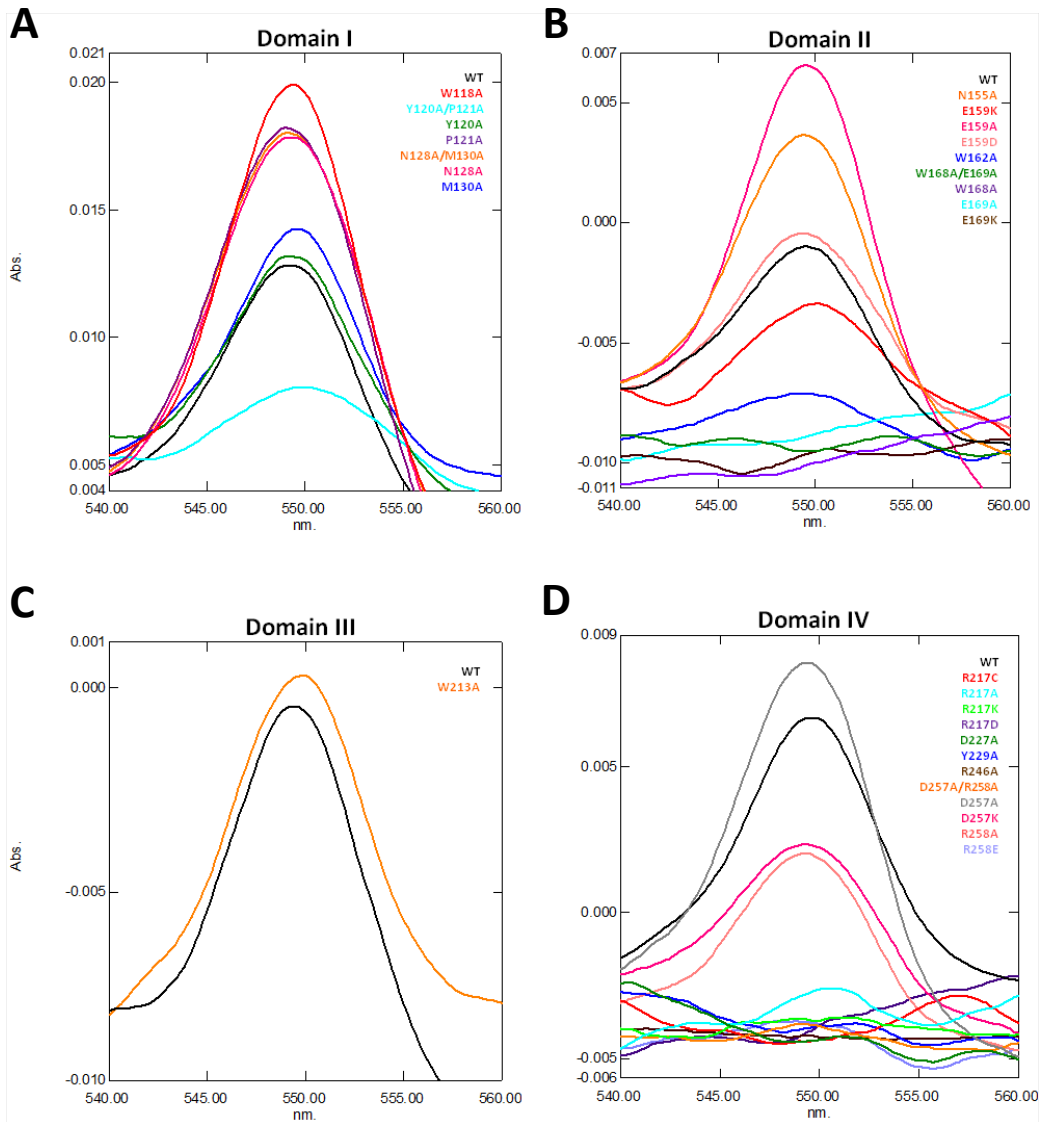


Figure S1

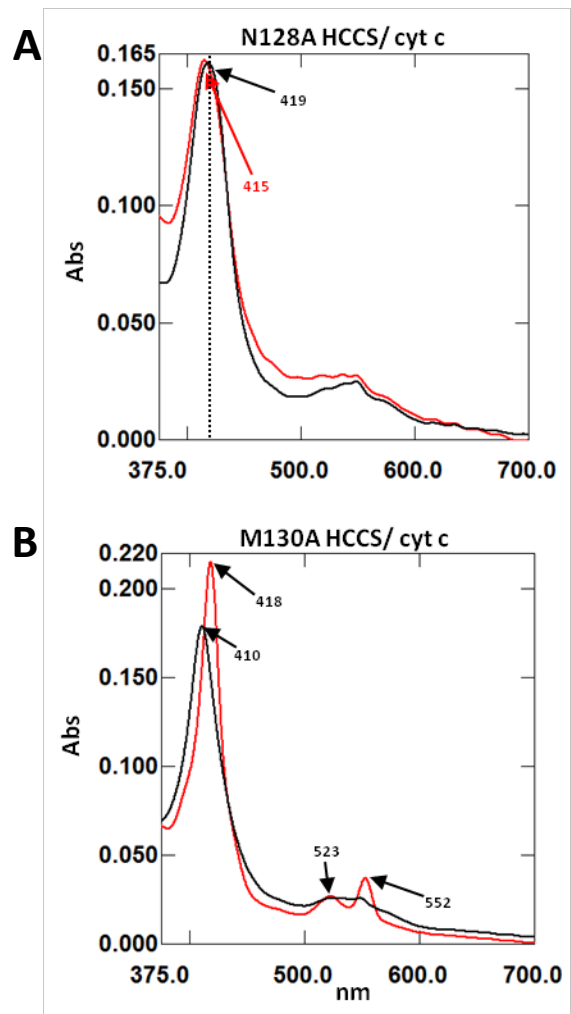


Figure S2

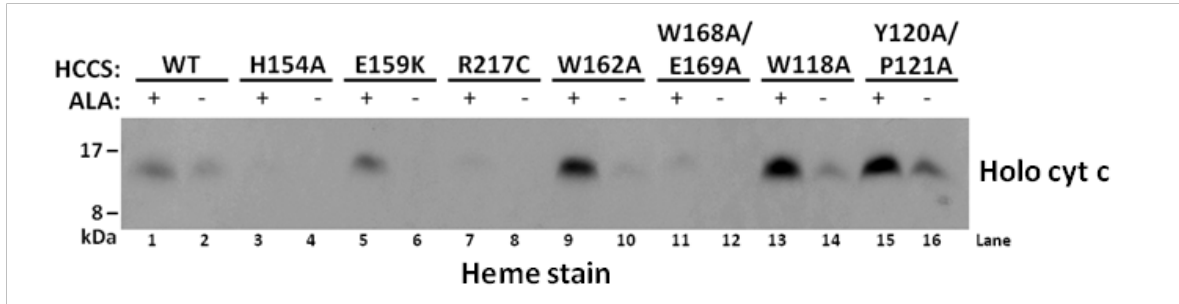


Figure S3

Table S1. Oligonucleotide primers and plasmids

	Oligo ID	Sequence (5'-3')	Constructed plasmid
1	W118A-F	AGC AGA TTC AGA GAA AAA GGC GGT TTA CCC TTC TGA GCA G	HCCS_W118A
	W118A-R	CTG CTC AGA AGG GTA AAC CGC CTT TTT CTC TGA ATC TGC T	
2	Y120A/P121A-F	GAT TCA GAG AAA AAG TGG GTT GCC GCT TCT GAG CAG ATG TTC TGG A	HCCS_Y120A/ P121A
	Y120A/P121A-R	TCC AGA ACA TCT GCT CAG AAG CGG CAA CCC ACT TTT TCT CTG AAT C	
3	Y120A-F	CAG ATT CAG AGA AAA AGT GGG TTG CCC CTT CTG AGC AGA TGT TC	HCCS_Y120A
	Y120A-R	GAA CAT CTG CTC AGA AGG GGC AAC CCA CTT TTT CTC TGA ATC TG	
4	P121A-F	GAG AAA AAG TGG GTT TAC GCT TCT GAG CAG ATG TTC T	HCCS_P121A
	P121A-R	AGA ACA TCT GCT CAG AAG CGT AAA CCC ACT TTT TCT C	
5	N128A/M130A-F	CCC TTC TGA GCA GAT GTT CTG GGC TGC AGC GTT AAA GAA AGG GTG GAA GTG G	HCCS_N128A/ M130A
	N128A/M130A-R	CCA CTT CCA CCC TTT CTT TAA CGC TGC AGC CCA GAA CAT CTG CTC AGA AGG G	
6	N128A-F	CCT TCT GAG CAG ATG TTC TGG GCT GCA ATG TTA AAG AAA GGG TG	HCCS_N128A
	N128A-R	CAC CCT TTC TTT AAC ATT GCA GCC CAG AAC ATC TGC TCA GAA GG	
7	M130A-F	GAG CAG ATG TTC TGG AAT GCA GCG TTA AAG AAA GGG TGG AAG TG	HCCS_M130A
	M130A-R	CAC TTC CAC CCT TTC TTT AAC GCT GCA TTC CAG AAC ATC TGC TC	
8	N155A-F	GGA TAT GTA TAA TAT CAT TAG AAT TCA CGC TCA GAA TAA CGA GCA GGC TTG GAA G	HCCS_N155A
	N155A-R	CTT CCA AGC CTG CTC GTT ATT CTG AGC GTG AAT TCT AAT GAT ATT ATA CAT ATC C	
9	E159K-F	ATT AGA ATT CAC AAT CAG AAT AAC AAG CAG GCT TGG AAG GAG	HCCS_E159K
	E159K-R	CTC CTT CCA AGC CTG CTT GTT ATT CTG ATT GTG AAT TCT AAT	
10	E159A-F	TCA CAA TCA GAA TAA CGC GCA GGC TTG GAA GGA GA	HCCS_E159A
	E159A-R	TCT CCT TCC AAG CCT GCG CGT TAT TCT GAT TGT GA	
11	E159D-F	AAT TCA CAA TCA GAA TAA CGA TCA GGC TTG GAA GGA GAT TTT G	HCCS_E159D
	E159D-R	CAA AAT CTC CTT CCA AGC CTG ATC GTT ATT CTG ATT GTG AAT T	
12	W162A-F	TCA GAA TAA CGA GCA GGC TGC GAA GGA GAT TTT GAA GTG G	HCCS_W162A
	W162A-R	CCA CTT CAA AAT CTC CTT CGC AGC CTG CTC GTT ATT CTG A	
13	W168A/E169A-F	GCT TGG AAG GAG ATT TTG AAG GCG GCA GCC CTT CAT GCT GCA	HCCS_W168A/ E169A
	W168A/E169A-R	TGC AGC ATG AAG GGC TGC CGC CTT CAA AAT CTC CTT CCA AGC	
14	W168A-F	TGG AAG GAG ATT TTG AAG GCG GAA GCC CTT CAT GCT GC	HCCS_W168A
	W168A-R	GCA GCA TGA AGG GCT TCC GCC TTC AAA ATC TCC TTC CA	
15	E169A-F	GGA GAT TTT GAA GTG GGC AGC CCT TCA TGC TGC AG	HCCS_E169A
	E169A-R	CTG CAG CAT GAA GGG CTG CCC ACT TCA AAA TCT CC	
16	E169K-F	AGG AGA TTT TGA AGT GGA AAG CCC TTC ATG CTG CA	HCCS_E169K
	E169K-R	TGC AGC ATG AAG GGC TTT CCA CTT CAA AAT CTC CT	
17	W213A-F	GCC TTT TGA TAG GCA CGA TGC GAT CAT AAA CCG TTG CGG G	HCCS_W213A
	W213A-R	CCC GCA ACG GTT TAT GAT CGC ATC GTG CCT ATC AAA AGG C	
18	R217C-F	AGG CAC GAT TGG ATC ATA AAC TGT TGC GGG ACA GAA	HCCS_R217C
	R217C-R	TTC TGT CCC GCA ACA GTT TAT GAT CCA ATC ATC GTG CCT	
19	R217A-F	AGG CAC GAT TGG ATC ATA AAC GCT TGC GGG ACA GAA G	HCCS_R217A
	R217A-R	CTT CTG TCC CGC AAG CGT TTA TGA TCC AAT CGT GCC T	
20	R217K-F	CTT TTG ATA GGC ACG ATT GGA TCA TAA ACA AGT GCG GGA CAG AAG TTA GA	HCCS_R217K
	R217K-R	TCT AAC TTC TGT CCC GCA CTT GTT TAT GAT CCA ATC GTG CCT ATC AAA AG	
21	R217D-F	ATA GGC ACG ATT GGA TCA TAA ACG ATT GCG GGA CAG AAG T	HCCS_R217D
	R217D-R	ACT TCT GTC CCG CAA TCG TTT ATG ATC CAA TCG TGC CTA T	
22	D227A-F	GAC AGA AGT TAG ATA TGT GAT TGC TTA TTA TGA TGG TGG TGA AGT CA	HCCS_D227A
	D227A-R	TGA CTT CAC CAC CAT CAT AAT AAG CAA TCA CAT ATC TAA CTT CTG TC	
23	Y229A-F	CGG GAC AGA AGT TAG ATA TGT GAT TGA TTA TGC TGA TGG TGG TGA AGT C	HCCS_Y229A
	Y229A-R	GAC TTC ACC ACC ATC AGC ATA ATC AAT CAC ATA TCT AAC TTC TGT CCC G	
24	R246A-F	CAC CAT CCT GGA CGT CGC TCC TGC CTT AGA TTC A	HCCS_R246A
	R246A-R	TGA ATC TAA GGC AGG AGC GAC GTC CAG GAT GGT G	
25	D257A/R258A-F	TCA CTT TCG GCA GTA TGG GCC GCA ATG AAA GTC GCT TGG TGG	HCCS_D257A/ R258A
	D257A/R258A-R	CCA CCA AGC GAC TTT CAT TGC GGC CCA TAC TGC CGA AAG TGA	
26	D257A-F	ACT TTC GGC AGT ATG GGC CAG AAT GAA AGT CGC TT	HCCS_D257A
	D257A-R	AAG CGA CTT TCA TTC TGG CCC ATA CTG CCG AAA GT	
27	D257K-F	TTC ACT TTC GGC AGT ATG GAA GAG AAT GAA AGT CGC TTG GT	HCCS_D257K
	D257K-R	ACC AAG CGA CTT TCA TTC TCT TCC ATA CTG CCG AAA GTG AA	
28	R258A-F	CTT TCG GCA GTA TGG GAC GCA ATG AAA GTC GCT TGG TG	HCCS_R258A
	R258A-R	CAC CAA GCG ACT TTC ATT GCG TCC CAT ACT GCC GAA AG	
29	R258E-F	CAC TTT CGG CAG TAT GGG ACG AGA TGA AAG TCG CTT GGT GGC G	HCCS_R258E
	R258E-R	CGC CAC CAA GCG ACT TTC ATC TCG TCC CAT ACT GCC GAA AGT G	

Table S2. Quantitation of released cytochrome c from two methods

Putative Domain	HCCS variant	BPER^a	UV/vis^b
	WT	1	1
Domain I	W118A	1.7±0.25	1.6±0.26
	Y120A/P121A	1.1±0.13	0.5±0.09
	Y120A	0.7±0.28	1.2±0.03
	P121A	0.9±0.91	1.9±0.22
	N128A/M130A	1.3±0.38	1.9±0.29
	N128A	1.5±0.15	1.9±0.27
	M130A	1.2±0.41	1.3±0.17
Domain II	N155A	2.0±0.33	1.9±0.12
	E159K	0.1±0.01	0.7±0.05
	E159A	1.9±0.76	1.8±0.11
	E159D	1.3±0.75	0.9±0.08
	W162A	0.3±0.10	0.3±0.04
	W168A/E169A	<0.1	nd
	W168A	<0.1	nd
	E169A	<0.1	nd
Domain III	E169K	<0.1	nd
	W213A	1.4±0.20	1.1±0.06
Domain IV	R217C	<0.1	nd
	R217K	<0.1	nd
	R217D	<0.1	nd
	R217A	<0.1	nd
	D227A	<0.1	nd
	Y229A	<0.1	nd
	R246A	<0.1	nd
	D257A/R258A	<0.1	nd
	D257A	1.3±0.48	0.9±0.31
	D257K	0.2±0.19	0.4±0.16
	R258A	<0.1	0.6±0.13
	R258E	<0.1	nd

Each value is relative to the amount of cytochrome c released by WT HCCS, which has been set to 1. Data shown represent the average of at least two separate experiments ± SEM.

^aBPERS were performed as described in Materials and Methods. Calculated values were based on the densitometric analysis of chemiluminescent signal from heme stains.

^bCalculated values were based on the reduced alpha peak height in the UV/vis absorption spectrum of 1 mg total protein from the soluble fractions of 1 L cultures co-expressing each respective variant with cytochrome c.

nd = none detected

Table S3. Quantitation of purified protein

Putative Domain	HCCS variant	Protein Levels	
		Expressed alone	Co-expressed with cytochrome c acceptor
	WT	1	1
Domain I	W118A	0.55	0.91
	Y120A/P121A	0.50	0.53
	Y120A	0.82	0.40
	P121A	0.95	0.28
	N128A/M130A	0.95	0.65
	N128A	0.76	0.61
	M130A	0.75	0.61
Domain II	N155A	0.48	0.54
	E159K	0.51	0.39
	E159A	0.65	0.55
	E159D	0.76	0.23
	W162A	0.20	0.15
	W168A/E169A	0.14	0.22
	W168A	0.29	<0.10
	E169A	0.30	<0.10
	E169K	0.20	<0.10
Domain III	W213A	0.45	0.59
Domain IV	R217C	0.14	0.21
	R217K	0.18	0.12
	R217D	0.23	<0.10
	R217A	0.23	<0.10
	D227A	0.52	0.15
	Y229A	0.80	0.11
	R246A	0.23	<0.10
	D257A/R258A	0.33	0.13
	D257A	0.59	0.33
	D257K	0.20	0.15
	R258A	0.33	0.45
	R258E	0.13	0.11

Each value is based on the Bradford measurement of total milligrams of GST-HCCS protein purified from 1 L of culture relative to the amount of total protein purified from 1 L of WT HCCS, which has been set to 1, under each respective condition. Bradford quantitation was confirmed by Coomassie staining, which also indicated that GST-HCCS proteins were obtained at >90% purity. Data shown represent the average yield from at least two separate experiments.