Transient complexes of haem proteins
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Appendices

Appendix A

AIR restraints and structural statistics for docking of Cyt b₅ – Cyt c complexes

Table A1. **Active and passive residues used in the definition of the ambiguous distance restraints (AIRs) and flexible segments for docking of Cyt b_5 and Cyt c.**

Ferric Cyt b_5 in complex with ferric Cyt c

Active	A4, H26-Y27, E43-E44, D53, N57, E59-V61, S64, D66, D82, HEM89[†]
Passive	K2-A3, Y6, K28, E37, G42, V45, E48-Q49, E56, G62, T65, R68-L70, P81, R84, S85, K86, T88, HEM89
Flexible segments	S1-T8, I24-Y30, F35-K72, L79-HEM89

Ferric Cyt b_5 in complex with ferrous Cyt c

Active	A4, Y27, E43-E44, 48, E59-V61, S64, D82, HEM89†
Passive	A3, K5-Y6, T8, E10-E11, Q13, N16, H26, K28, P40, G42, V45, R47, Q49, E56-N57, G62, R68, L70, H80-P81, R84
Flexible segments	A1-S18, I24-Y30, E38-G51, A54-S64, D66-K72, E78-K86

Ferric Cyt c

Active	T8, K11-T12, Q16, T69, K72-K73, G77, K79, A81, K86
Passive	K4-K5, R13, L15, K27-V28, Y46-T49, A51, E66, N70, Y74, P76, T78, G83, K87, D90-R91, Y97, HEM104
Flexible segments	G1-H18, P25-P30, E44-A51, M64-K89, HEM104

Ferrous Cyt c

Active	T8, T12-R13, Q16, K72, G77, K79, A81, K86-K87
Passive	K4-K5, K11, L15, V28, S47, T69-N70, K73, P76, G83, E88-K89
Flexible segments	S2-H18, H26-P30, G45-T49, Y67-K89

[†] The solvent-accessible haem atoms CMD (5-CH$_3$) and CAD (6-α-Pr CH$_2$), the 1D resonances of which are affected by the binding of Cyt c, are defined as active.

Table A2. **Structural statistics for Cyt b_5 – Cyt c docking with HADDOCK.** Statistics for the docking of ferric Cyt b_5 – ferric Cyt c (**A**), ferric Cyt b_5 – ferrous Cyt c (**B**), ferric Cyt b_5 – (ferric Cyt $c)_2$ (**C**), and ferric Cyt b_5 – (ferrous Cyt $c)_2$ (**D**). All values are calculated for five best docked structures of each cluster. The clusters are numbered according to the increasing intermolecular non-bonded energy (E_{nb}). [1] Number of structures per cluster; [2] Backbone rmsd (Å) from the lowest-energy structure; [3] Standard deviation; [4] E_{tot} (kcal/mol) – intermolecular total energy ($E_{vdw}+E_{elec}+E_{AIR}$); [5] E_{nb} (kcal/mol) – intermolecular non-bonded energy; [6] E_{vdw} (kcal/mol) – intermolecular van der Waals energy; [7] E_{elec} (kcal/mol) – intermolecular electrostatic energy; [8] E_{AIR} (kcal/mol) – ambiguous intermolecular restraints (AIRs) energy; [9] BSA – buried surface area (Å^2).

A Ferric Cyt b_5 – ferric Cyt c

	N^1	rmsd^2	sd^3	$E_{tot}{}^4$	sd^3	$E_{nb}{}^5$	sd^3	$E_{vdw}{}^6$	sd^3	$E_{elec}{}^7$	sd$_3$	$E_{AIR}{}^8$	sd^3	BSA^9	sd^3
1	2	1.3	1.3	-559	5	-769	8	-62	11	-706	19	209	3	1631	156
2	45	13.7	0.1	-471	36	-678	40	-56	4	-622	42	206	8	1523	21
3	40	13.7	0.1	-422	41	-634	37	-38	5	-597	37	212	6	1276	39
4	6	6.0	0.2	-354	48	-571	40	-34	8	-537	40	217	12	1203	69
5	11	8.8	0.1	-345	27	-559	20	-34	6	-525	22	213	15	1254	52
6	5	11.0	0.2	-334	53	-545	59	-55	5	-490	63	210	10	1575	78
7	25	13.8	0.1	-339	18	-544	21	-47	6	-497	23	204	6	1334	79
8	5	7.4	0.1	-219	34	-463	34	-23	8	-441	32	244	7	930	39
9	10	6.9	0.2	-209	33	-440	31	-33	3	-407	30	230	10	1085	36
10	5	9.9	0.2	-172	34	-412	35	-42	6	-370	38	239	6	1263	35

B Ferric Cyt b_5 – ferrous Cyt c

	N^1	rmsd^2	sd^3	$E_{tot}{}^4$	sd^3	$E_{nb}{}^5$	sd^3	$E_{vdw}{}^6$	sd^3	$E_{elec}{}^7$	sd^3	$E_{AIR}{}^8$	sd^3	BSA^9	sd^3
1	4	1.3	0.8	-527	30	-734	37	-395	6	-696	37	195	5	1249	48
2	23	12.2	0.1	-456	37	-662	37	-47	5	-615	37	195	4	1466	51
3	8	13.5	0.2	-400	20	-602	22	-40	1	-562	23	193	6	1174	46
4	19	10.0	0.2	-374	12	-590	9	-46	6	-544	12	205	4	998	36
5	10	6.7	0.3	-377	35	-587	31	-37	5	-550	31	202	2	1217	70
6	9	8.8	0.2	-384	15	-586	12	-37	3	-549	15	190	6	1221	36
7	7	3.1	0.1	-354	17	-565	15	-38	4	-527	16	203	3	1157	32
8	5	11.5	0.2	-340	31	-545	24	-45	4	-499	22	194	4	1263	46
9	18	12.9	0.7	-286	22	-502	22	-49	5	-454	22	208	3	1145	58
10	8	13.6	0.2	-295	29	-502	24	-51	4	-451	26	197	3	1437	73
11	9	12.5	0.2	-277	20	-491	22	-32	1	-458	22	205	6	1002	21
12	10	10.4	0.1	-223	21	-431	23	-35	2	-396	21	198	6	1080	75

(continued)

Table A2 (continued)

C   Ferric Cyt b_5 – (ferric Cyt c)$_2$

	N[1]	rmsd[2]	sd[3]	E_{tot}[4]	sd[3]	E_{nb}[5]	sd[3]	E_{vdw}[6]	sd[3]	E_{elec}[7]	sd[3]	E_{AIR}[8]	sd[3]	BSA[9]	sd[3]
1	8	5.8	4.1	-677	146	-1006	143	-66	14	-940	138	329	11	2530	336
2	14	15.2	0.2	-634	39	-978	39	-84	10	-894	40	344	4	2814	152
3	17	4.5	3.2	-582	118	-917	124	-68	4	-849	124	335	7	2851	276
4	8	11.0	3.5	-576	77	-903	77	-72	10	-830	77	327	3	2640	170
5	6	14.9	1.8	-514	118	-848	110	-75	14	-773	108	333	12	2616	265
6	14	17.8	0.5	-500	25	-841	24	-79	11	-762	21	340	15	2556	272
7	11	18.9	2.0	-435	73	-774	77	-58	13	-716	86	338	7	2285	140
8	4	18.2	2.3	-425	64	-757	59	-80	11	-678	50	332	8	2469	280
9	6	14.4	1.8	-326	101	-666	98	-60	3	-605	99	339	8	2303	36
10	8	14.7	2.7	-293	36	-622	39	-83	13	-539	50	329	10	2506	86

D   Ferric Cyt b_5 – (ferrous Cyt c)$_2$

	N[1]	rmsd[2]	sd[3]	E_{tot}[4]	sd[3]	E_{nb}[5]	sd[3]	E_{vdw}[6]	sd[3]	E_{elec}[7]	sd[3]	E_{AIR}[8]	sd[3]	BSA[9]	sd[3]
1	7	7.4	4.0	-695	45	-1003	46	-80	7	-923	42	308	4	2745	110
2	8	11.3	0.2	-638	38	-943	34	-65	9	-878	40	305	9	2532	127
3	13	9.1	3.0	-617	13	-924	18	-65	7	-859	20	307	12	2318	181
4	6	6.7	2.4	-537	51	-841	47	-79	9	-763	43	304	9	2389	151
5	4	17.9	0.6	-520	56	-828	52	-50	7	-777	46	308	9	2349	250
6	5	17.0	1.8	-527	116	-827	115	-73	5	-754	113	299	7	2459	186
7	5	10.1	3.6	-505	74	-814	76	-56	10	-759	72	309	2	2221	180
8	5	16.8	1.5	-498	60	-810	62	-77	10	-733	59	312	4	2707	134
9	10	17.0	1.6	-490	81	-788	79	-87	12	-701	78	298	2	2861	149
10	4	14.3	1.9	-459	153	-764	154	-59	14	-705	145	305	7	2210	248
11	5	14.6	1.7	-446	79	-762	81	-67	10	-695	83	315	3	2329	126
12	4	18.7	1.6	-454	161	-760	165	-70	10	-690	159	306	5	2399	140
13	6	15.3	1.3	-432	82	-733	83	-84	8	-649	85	301	6	2411	135
14	6	16.3	1.9	-422	41	-723	49	-82	8	-641	50	301	10	2715	132
15	6	17.3	2.0	-421	54	-722	57	-77	11	-645	53	301	7	2558	248
16	8	14.5	0.9	-365	83	-665	79	-68	8	-597	72	299	8	2248	199
17	4	15.4	1.5	-303	148	-601	146	-63	7	-538	141	297	2	2339	274
18	5	16.1	1.9	-245	28	-547	39	-69	4	-478	40	301	11	2225	99

Appendix B

Protein Isolation Protocols

The present protein expression and purification protocols are based on those reported before for Cyt c[41,150], Cyt b_5[58], and CcP[73]. An alternative purification procedure for CcP[74,75] has persistently yielded the protein of inferior quality; therefore, it was scrapped. The minimal medium composition has been adjusted from the published protocol[244].

Cyt c

On the eve of expression, *E. coli* competent cells (JM109 and BL21 for the growth on the rich and minimal media, respectively) are transformed with a relevant pUCcc plasmid (see Appendix D) and incubated on LB/Amp plates overnight at 37° C[i]. The following morning several 3 ml pre-cultures (LB with 0.1 g/L Amp and 1 mM KNO_3 for the rich medium or TB with 0.1 g/L Amp for the minimal medium) are inoculated with single colonies from the overnight plates and are incubated at 37° C and 250 rpm shaking until turbidity is evident (*ca.* 3 – 4 hours)[ii]. At this point, 1 mL of the pre-cultures is used to inoculate the larger cultures.

After being inoculated with the pre-cultures, rich-medium cultures (1.7 L LB per a 2 L flask, 0.1 g/L Amp and 1 mM KNO_3) are grown semi-anaerobically at 37° C, 160 rpm shaking, for 24 hours[iii,iv]. In contrast, minimal-medium cultures (0.33 L M9 minimal medium per a 2 L flask; for the composition of the minimal medium see below) are grown aerobically at 30° C, 250 rpm shaking for 60 – 72 hours, with an optional addition of Amp (0.3 mL of sterile-filtered 0.1 g/mL stock per flask) after first 24 - 36 hours of growth[iv].

The cells are harvested by centrifugation at 6,000 rpm for 15 minutes. The pink pellet is re-suspended in the minimal volume of the lysis buffer (50 mM Tris-HCl, 1 mM

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[i] Use of freshly-transformed cells alleviates plasmid instability, the main problem with Cyt c expression.
[ii] The pre-cultures must not be incubated for longer than 4 – 5 hours as this may lead to a loss of the plasmid from the cells.
[iii] Care must be taken not to exceed the shaking speed as excessive aeration is detrimental to semi-anaerobically grown culture.
[iv] The genes responsible for Cyt c expression and maturation are constitutively expressed; therefore, no induction is required.

EDTA), followed by addition of a few milligrams of lysozyme, DNAse, and RNAse. The mixture is stirred at 4° C until smooth (*ca.* 1 hour) and then passed through a pre-cooled French press at 1,200 lb/psi to break the cell membrane. The red lysate is collected, combined with 326 g/L of $(NH_4)_2SO_4$[i], and stirred at 4° C for 30 minutes to precipitate out the debris. The solution is cleared by centrifugation at 8,000 rpm for 30 minutes, leaving intensely pink Cyt *c* in the supernatant. Several dialysis bags (3,000 M_r cut-off) are filled with the protein solution, leaving three-fourths of the bag empty to allow for expansion. The cell extract is dialysed against 2 L of 46 mM NaP_i pH 6.8 for 3 hours, followed by a fresh change of the buffer and an overnight dialysis.

The resulting dialysate is cleared by centrifugation at 6,000 rpm for 15 minutes and loaded to the CM column equilibrated in 46 mM NaP_i pH 6.8. Once loaded, the column is washed with the equilibration buffer (*ca.* 200 mL) followed by 46 mM NaP_i 75 mM NaCl pH 6.8 (*ca.* 200 mL), and finally the protein is eluted with 46 mM NaP_i pH 6.8 containing 0.3 – 0.4 M NaCl. The fractions corresponding to the second peak on the chromatogram contain Cyt *c*. These are pooled, oxidized with $K_3[Fe(CN)_6]$, and prepared for the final purification procedure.

During the last purification step, an FPLC with either SP-Sepharose or Hi-Load G-75 Superose (Amersham Pharmacia, Uppsala, Sweden) column is used. For the purification on an SP column, the protein is exchanged into 20 mM NaP_i pH 7.0 in an Amicon fitted with a YM3 membrane (Millipore, Billerica, MA), concentrated to *ca.* 20 mL, and loaded onto the SP column equilibrated in 20 mM NaP_i pH 7.0. The pure Cyt *c*, eluted with a linear salt gradient of 20 mM NaP_i pH 7.0 and 20 % per hour of 1 M NaCl at 4 mL/min flow rate, appears as a single peak on a chromatogram recorded at 280 nm. For the purification on a G-75 gel-filtration column, the protein is exchanged into 20 mM NaP_i 0.1 M NaCl pH 6.0, concentrated down to *ca.* 2 – 2.5 mL[ii], loaded onto the column equilibrated in the same buffer, and eluted at 1.5 – 2 mL/min flow rate. Usually, a single peak corresponding to the pure protein is recorded at 280 nm.

Pure Cyt *c*, with a UV-vis peak ratio of $A_{410} / A_{280} \geq 4.0$ for the oxidized protein, is concentrated to 1 – 2 mM and stored in 20 mM NaP_i 0.1 M NaCl pH 6.0 at -20° C. Typical

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[i] For T12A Cyt *c* variant, 275 g/L of $(NH_4)_2SO_4$ was used.
[ii] For a large-scale protein preparation, it is more practical to reduce the volume to *ca.* 5 – 7.5 mL and run the column several times, each time loading not more than 2 – 2.5 mL of the sample to ensure a good separation.

final yields for different variants of Cyt *c* grown on rich and minimal media are given in Table B1.

Table B1. **Yields of Cyt *c* variants after the purification (mg/L of cell culture**).

Medium used	Variant of Cyt *c*			
	wt	T12A	R13A	R13K
Rich	12 - 17	15	10	10
Minimal	7 - 8	3.5	4	6.5

Cyt b_5

Competent *E. coli* JM109 cells are transformed with pUC-cytb_5 plasmid[58], and the protein is expressed using the procedure described above for Cyt *c*. The final cultures (1 L LB per a 2 L flask, 0.1 g/L ampicillin, 1 mM KNO_3, and 0.1 mM δ-aminolevulinic acid for the rich medium or 1 L M9 minimal medium per a 2 L flask, for the composition of the minimal medium see below) are incubated at 37° C, 250 rpm shaking for 24 hours.

First part of the purification procedure, including the French press, is similar to that described above for Cyt *c*. After the lysis with the French press, the cell debris are removed by centrifugation at 8,000 rpm for 30 minutes, and a pink supernatant is collected. This is followed by addition of KCl and PEG 4,000 to the final concentrations of 0.4 M and 6 % (w/w), respectively, after which the solution is stirred for 15 minutes at 4° C and then centrifuged at 8,000 rpm for 30 minutes. The supernatant is transferred to a dialysis bag (3,000 M_r cut-off) and dialysed against 2 L of 20 mM NaP_i pH 7.2 for *ca.* 2 hours, followed by a fresh change of the buffer and an overnight dialysis.

The resulting dialysate is cleared by centrifugation at 8,000 rpm for 30 minutes and loaded onto a DEAE column equilibrated in 50 mM NaP_i pH 7.2. Once loaded, the column is washed with *ca.* 150 mL of the equilibration buffer, and the protein is eluted with a linear gradient of 50 – 150 mM NaP_i pH 7.2, 500 mL each. The red-coloured fractions containing Cyt b_5 are pooled, oxidised with $K_3[Fe(CN)_6]$, exchanged into 50 mM NaP_i pH 7.2, and concentrated in an Amicon fitted with a YM3 membrane (Millipore) to *ca.* 20 – 30 mL.

Then the concentrated protein solution is loaded onto a Mono-Q FPLC column equilibrated with 50 mM NaP_i pH 7.2, and Cyt b_5 is eluted with a linear salt gradient of 50 mM NaP_i pH 7.2 and 4 % per minute of 0.3 M NaCl at 4 mL/min flow rate. Usually, a single peak corresponding to the pure protein is recorded at 280 nm. Pure Cyt b_5, with a UV-vis peak ratio of $A_{412.5} / A_{280} \geq 4.0$ for the oxidised protein, is exchanged into 20 mM NaP_i pH 6.0, concentrated to $1 - 2$ mM and stored at -20 °C. The final yield of the protein is *ca.* 12 and 3 mg per litre of culture for the growth on rich and minimal media, respectively.

CcP

Competent *E. coli* BL21 cells are transformed with a relevant pT7CcP plasmid (see Appendix D) and incubated on LB/Amp plates overnight at 37° C. The following morning several 3 ml pre-cultures (SB with 0.1 g/L Amp) are inoculated with single colonies from the overnight plates and are incubated at 37° C and 250 rpm shaking until turbidity is evident (*ca.* $3 - 4$ hours). At this point, 0.5 mL of each pre-culture is used to seed several larger cultures (50 mL SB/Amp per a 250 mL flask), which are incubated for a further 3 hours and are used to inoculate the final cultures (750 mL SB/Amp per a 2 L flask) with the aliquots of 0.75 mL. The cultures are incubated for 10 - 11 hours[i] at 37° C and 250 rpm shaking, followed by addition of 1.6 mL IPTG (0.125 g/L stock, sterile-filtered) per flask and a further 3 hours of growth.

After the total incubation time of $13 - 14$ hours, the cells are harvested by centrifugation at 8,000 rpm for 10 minutes. The pellet is re-suspended in the minimal volume of the lysis buffer (0.1 M KP_i 1 mM EDTA pH 6.0 and 1 mM PMSF[ii]), followed by addition of a few milligrams of lysozyme and stirring at 4° C until a smooth consistency (*ca.* 1 hour). The smooth cell paste is flash-frozen in the liquid nitrogen and stored at -80° C for the later use.

The cell paste is thawed, few milligrams of DNAse and RNAse and $10 - 20$ mL 1 M $MgCl_2$ are added to it, and the mixture is stirred for 1 hour at 4° C until smooth, followed by centrifugation at 8,500 rpm for 30 minutes. The brownish supernatant is placed into two

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[i] Usually done overnight. Care must be taken not to overgrow the cultures as this reduces the level of protein expression upon induction.
[ii] PMSF is insoluble in water; first, it is dissolved in $1 - 2$ ml of ethanol and then added drop-wise to the buffer.

dialysis bags (6,000 M_r cut-off) leaving room for expansion and dialysed overnight at 4° C against 4 L of 50 mM KP_i 1 mM EDTA pH 6.3.

The dialysate is cleared by centrifugation at 8,500 rpm for 1 hour, and then loaded onto the DEAE column equilibrated in 50 mM KP_i pH 6.0. Once loaded, the column is washed with *ca.* 200 mL of the equilibration buffer, followed by the elution of the protein with a linear gradient of 50 – 500 mM KP_i pH 6.0, 300 mL each. The chromatogram contains several peaks, the biggest of which corresponds to apo-CcP. The fractions of the apo-protein are analysed by SDS-PAGE, and those containing the protein band are pooled. In case of the CcP cysteine mutants, the pooled fractions are incubated with 10 mM DTT at RT for *ca.* 2 – 3 hours in order to reduce the intermolecular disulfide bonds. The protein is exchanged into 0.1 M KP_i 1 mM EDTA pH 6.0 (also containing 1 mM DTT for the cysteine mutants) and concentrated in an Amicon fitted with a YM10 membrane (Millipore) to *ca.* 20 mL[i].

The concentrated protein is loaded onto a regular, prep-grade G-75 column, equilibrated in 0.1 M KP_i 1 mM EDTA pH 6.0 (also containing 1 mM DTT for the cysteine mutants), and eluted with the equilibration buffer at 1 – 2 mL/min flow rate[ii]. Again, two peaks are observed at 280 nm, and the second one contains apo-CcP. The fractions are collected and again analysed by SDS-PAGE. Those containing reasonably pure protein are pooled and concentrated down to *ca.* 30 mL. At this step the amount of the apo-protein is estimated from the UV absorbance peak at 280 nm (ε = 55 $mM^{-1}cm^{-1}$, M_r = 33,484).

The protein solution is poured into a dialysis bag (6,000 M_r cut-off), leaving three-fourths of the bag empty, and dialysed against 4 L of 0.1 M KP_i 5 mM sodium ascorbate pH 7.5 at 4° C for *ca.* 8 hours. At this point 1.1 molar equivalents of hemin is added to 1 mL of 0.1 M NaOH in an Eppendorf tube and vortexed vigorously[iii]. The solution is transferred to a 50 mL Corning tube containing *ca.* 30 mL of the dialysis buffer and briefly vortexed again. The pH of the hemin solution should be between 7.0 and 7.5; if not, it is adjusted by adding more buffer. The solution is vortexed once more and then poured into the dialysis bag containing the apo-CcP. After incubating the mixture for 1 hour at 4° C, the

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[i] Handle with care to avoid frothing. If the solution turns cloudy, clear it by centrifugation at 10,000 rpm for 10 – 15 minutes.
[ii] Run the column several times, each time loading not more than 5 – 7 mL of the sample to ensure a good separation.
[iii] Wrap aluminium foil around the vessels containing hemin solutions to prevent photo-degradation.

dialysis bag, now containing brown holo-protein, is transferred into a beaker containing 4 L of 0.1 mM KP_i 5 mM sodium ascorbate pH 6.2, and the protein is dialysed overnight at 4° C.

After the overnight dialysis, the reconstituted holo-CcP is cleared by centrifugation at 8,000 rpm for 10 – 15 minutes, diluted three-fold with 10 mM sodium citrate 5 mM sodium ascorbate pH 6.0, and loaded onto the DEAE column equilibrated in the dilution buffer. Once loaded, the column is washed with *ca.* 200 mL of the equilibration buffer, and the protein is eluted with a linear gradient of 50 – 500 mM sodium citrate 5 mM sodium ascorbate pH 6.0, 300 mL each. The brown CcP band moves down the column leaving a black residue of the excess hemin at the top of the column[i]. The fractions of holo-CcP are pooled, exchanged into 0.2 M sodium citrate pH 5.0, concentrated down to *ca.* 35 mL, transferred into a dialysis bag (6,000 M_r cut-off) leaving half of the bag empty, and dialysed against 4 – 5 L of water (changed several times) for two days at 4° C.

Dialysis against water promotes crystallisation of CcP, which is insoluble at zero ionic strength[245]. After two days of dialysis, the crystals are collected by centrifugation at 8,000 rpm for 10 minutes, rinsed with cold water, re-dissolved in 20 mL of ice-cold 0.2 M sodium citrate pH 5.0, and cleared by centrifugation at 8,000 rpm for 10 minutes. At this point the yield of the protein is estimated from the UV-vis absorbance at 408 nm (ε = 98mM^{-1}cm^{-1}, M_r = 34,100). The protein solution is placed into a dialysis bag and dialysed against water (changed several times) for another two days. After the second crystallisation, the crystals of CcP are collected by centrifugation at 8,000 rpm for 10 minutes, re-suspended in 10 – 20 mL of ice-cold water, aliquoted in Eppendorf tubes, and flash-frozen in liquid nitrogen. The crystalline protein is stored in water suspension at -80° C. Typical final yields for different variants of CcP grown on rich medium are given in Table B2.

Table B2. **Yields of CcP variants after the final purification (mg/L of cell culture**).

Variant of CcP						
wt	N38C	T137C	N200C	S225C	S263C	T288C
31 - 38	11 - 20	23	13 - 19	8 - 10	47	26

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[i] As the hemin binds irreversibly to the DEAE, discard the top layer of the hemin-bound material before regenerating the column.

Composition of M9 minimal medium for the expression of haem proteins

M9 Minimal Medium (per 1 L): 960 mL M9 salts, 10 mL trace elements, 10 mL vitamin mixture, 10 mL 1 M MgCl$_2$[i], 6 mL thiamine (5 g/L stock, sterile-filtered), 1 mL ampicillin (0.1 g/mL stock, sterile-filtered), 5 g D-glucose, 0.017 g δ-aminolevulinic acid.

Stock solution of M9 salts (g/L)[i]: KH$_2$PO$_4$ (13.0), K$_2$HPO$_4$·3H$_2$O (10.0), Na$_2$HPO$_4$ (9.0), Na$_2$SO$_4$ (2.4), ^{15}NH$_4$Cl (1.0).

Stock solution of trace elements (g/L)[ii]: FeSO$_4$·7H$_2$O (6.0), CaCl$_2$·2H$_2$O (6.0), MnCl$_2$·4H$_2$O (1.2), CoCl$_2$·6H$_2$O (0.8), ZnSO$_4$·7H$_2$O (0.7), CuCl$_2$·2H$_2$O (0.3), H$_3$BO$_3$ (0.02), EDTA (5.0).

Stock solution of vitamin mixture[ii]: 0.1 g/L of biotin, choline chloride, folic acid, niacinamide, D-pantothenate, and pyridoxal; 0.01 g/L riboflavin.

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[i] Autoclaved and stored at RT.
[ii] Sterile-filtered and stored at 4º C in a vial wrapped in aluminium foil to prevent photo-degradation.

Appendix C

PRE-based distance restraints for structure calculations of Cyt c – CcP complexes

Table C1. **Distance restraints used in structure calculations of the Cyt *c* – CcP complexes.** The distances (Å) from the indicated CcP-MTSL to the backbone amides of Cyt *c* (n denotes a residue number). Restraints with a single upper or lower bound are in bold or italic, respectively. The restraints that are violated in the lowest-energy structure are shaded. All values correspond to the 100 % bound form of the 100% (wt and T12A Cyt *c*) or 25 % (R13A Cyt *c*) single-orientation complex. For more information consult Chapters III – VI.

Cyt *c*	wt				T12A				R13A		
n	N38C	N200C	T288C	S263C	N38C	N200C	T288C	S263C	N38C	N200C	T288C
1	18.0	*22.5*	*22.3*	*22.0*	19.2				13.8	*16.7*	15.3
2	16.1	*21.8*	17.1		19.7	*22.0*	*22.0*	*22.0*	12.3	*16.7*	13.2
4	18.3	*22.5*	*23.1*	*22.0*	**18.5**			*22.0*	13.4	*16.7*	*16.7*
5	14.9	*22.5*	18.0	*22.0*				*22.0*	11.4	15.2	12.1
6	15.6	*22.6*	18.9		**14.5**	*22.0*	*22.0*		12.1	*16.7*	13.4
7	**13.7**	19.2	18.0	*22.0*	**14.8**	*22.0*	19.7	*22.0*	15.2	14.0	12.6
8	**13.3**	14.4	18.3		**14.8**	17.8		*22.0*	**10.1**	11.0	12.7
9	**14.0**	15.8	19.7	*22.0*		**15.5**	*22.0*	*22.0*	**10.5**	11.7	12.5
11	**13.7**	**14.1**	19.8	*22.0*		17.5	17.4	*22.0*	**10.4**	**10.7**	11.0
12	**15.1**	**14.7**	16.2	*22.0*	**15.5**	17.7	*22.0*	*22.0*	**10.3**	**10.5**	11.1
13	**13.6**	**14.6**	15.1	*22.0*			20.3		**10.0**	**10.3**	12.8
14	**14.8**	16.0	16.6	*22.0*	**15.1**	17.2	18.7	*22.0*			
15	**14.4**	**15.5**	18.0	*22.0*	**14.4**	18.0	21.0		**10.2**	11.8	13.0
16			16.1	*22.0*					**10.2**	**10.7**	11.5
17	**15.3**	**15.9**	18.7	*22.0*			*22.0*		**10.7**	13.4	12.2
18	**15.6**	17.0	17.6	*22.0*			*22.0*		**11.4**	15.6	15.2
19	17.4		17.9	*22.0*			19.3	*22.0*	11.8	*16.7*	14.7
20	15.1		*22.1*	*22.0*	**16.0**	*22.0*	*22.0*	*22.0*	**10.4**	*16.7*	14.5
21	17.0	*21.8*	*21.8*						**11.4**	*16.7*	16.0
22	17.4	*22.4*	*22.9*	*22.0*	19.0	*22.0*	*22.0*	*22.0*	11.0	*16.7*	15.8
23	*22.4*	*22.2*	*23.0*	*22.0*	*22.0*	*22.0*	20.7	*22.0*	14.8	*16.7*	15.8
24	19.3	*22.5*	*22.1*	*22.0*	20.1	*22.0*		*22.0*	13.5	*16.7*	*16.7*
27	19.1	**14.0**	19.6	*22.0*	19.3	**14.7**	20.7	*22.0*	14.4	15.4	14.1
28	17.4		16.6	*22.0*			*22.0*		12.4	11.7	11.7
29	19.9	**15.6**	17.4			**15.5**		*22.0*	12.8	12.6	13.4
31		18.4	*22.4*	*22.0*	*22.0*	**15.8**			14.5	*16.7*	16.1

(continued)

Table C1 (continued)

Cyt *c*	wt				T12A				R13A		
n	N38C	N200C	T288C	S263C	N38C	N200C	T288C	S263C	N38C	N200C	T288C
32					20.6	*22.0*		22.0	13.4	*16.7*	
34		*22.5*	*22.5*	*22.0*					12.5	*16.7*	
35	20.4	*23.9*	*22.7*	*22.0*	20.3	*22.0*	*22.0*	22.0	14.1	*16.7*	*16.7*
36	18.9	*22.8*	*23.0*	*22.0*	22.0	*22.0*	20.3		15.2	*16.7*	*16.7*
37	20.6	*22.9*	*22.4*	*22.0*	22.0	*22.0*	20.9	22.0		*16.7*	*16.7*
38		*22.6*	*23.1*		19.4			22.0	16.6	*16.7*	*16.7*
39		*22.3*			20.1	*22.0*	*22.1*			*16.7*	*16.7*
40	18.7	*22.1*	*22.1*	22.0		*22.0*		22.0	14.8	*16.7*	16.1
41		19.7	21.2	*22.0*	22.0			*22.0*	*16.7*	*16.7*	
42	19.8	19.0	*22.1*	22.0		18.3	22.0	22.0			
43	20.2		*23.3*	*22.0*	21.1	*22.0*	20.8	22.0	16.4	*16.7*	*16.7*
44		*22.6*	*23.1*	*22.0*	19.0	*22.0*		*22.0*	14.9	*16.7*	*16.7*
45		*22.5*	*22.6*	*22.0*	21.4	18.1		*22.0*	14.6	*16.7*	
46	20.0	17.2	22.8	22.0	22.0	17.3	*22.0*	22.0	16.1	*16.7*	*16.7*
47	15.9	**14.0**	20.5	22.0	18.9	**14.4**	19.4	22.0	11.1	**10.6**	13.7
48		**14.0**	20.8	22.0	22.0	**15.0**	22.0	22.0	15.4	16.5	
49	18.0	**15.1**	17.1	22.0	22.0	**15.3**	16.9	22.0	14.1	12.4	15.1
51	19.9	**13.9**	17.2	22.0	21.4	**14.4**	17.4	22.0	12.8	12.0	13.2
52	19.5	**14.4**	17.0		22.0			22.0	15.7	*16.7*	14.4
53	*22.5*		17.8	22.0							
54		17.9	19.4	22.0	21.6	18.7	19.5	22.0	15.5	*16.7*	13.6
55	20.7	19.2	19.3	22.0	21.4	19.0	18.2	22.0	15.6	*16.7*	13.5
57	19.4	*23.1*	22.7		18.8	21.2	18.8	22.0		*16.7*	*16.7*
58		*22.6*	20.5			22.0	22.0	22.0	16.2	*16.7*	
59	21.0	22.2	22.3	22.0	22.5	22.0		22.0	15.8	*16.7*	*16.7*
60	*22.2*	22.5	22.7	22.0	20.5	*22.0*	20.3	22.0	15.0	*16.7*	*16.7*
61	19.6	19.5	21.5	22.0	**15.3**		*22.0*	22.0	13.3	*16.7*	*16.7*
62		22.5	22.3	22.0	22.0	*22.0*	22.0	22.0	15.3	*16.7*	*16.7*
63									15.9	*16.7*	
65	19.5			22.0	20.8		18.7	*22.0*			
67	16.9	*21.7*	15.8	22.0		*22.0*	16.2	22.0	14.7	*16.7*	15.1
68	18.5	20.5	15.6	22.0	22.0	22.0	**14.1**		14.1	*16.7*	14.4
69	18.7	*23.0*	15.1			22.0	**14.4**	22.0	13.8	*16.7*	13.8
70	19.2		**13.3**	22.0	22.0	19.4	**14.0**	22.0	13.8	*16.7*	12.5
72	19.1	**14.4**	13.4	20.0	22.0	**14.3**	**15.0**			15.7	
73		16.2	**13.2**		20.6	17.0	**14.2**	22.0	13.4	14.0	**10.1**

(continued)

Cyt c	wt				T12A				R13A		
n	N38C	N200C	T288C	S263C	N38C	N200C	T288C	S263C	N38C	N200C	T288C
75	18.8		**13.4**	*22.0*	17.6	15.9	**13.5**	*22.0*		*16.7*	
77	17.8	**14.2**	14.3	19.0	*22.0*	**14.7**	**14.1**	*22.0*	11.6	**10.7**	**10.4**
78	18.4	**13.8**	**13.2**	*22.0*	21.4	**14.4**	**13.6**	*22.0*	13.7	11.8	11.9
82	**15.6**		**15.2**	*22.0*			**14.6**		12.7	**11.2**	12.9
85	17.8		**13.8**	*22.0*					**10.3**	12.3	12.0
86					18.8	*22.0*	14.6	*22.0*	**10.6**	**11.0**	**10.5**
87	16.5	17.2	14.2	*22.0*	17.4	*22.0*	15.8	*22.0*			
89	14.8	*22.4*	15.4	*22.0*	17.6		18.1	*22.0*	**10.1**	12.8	10.9
90	17.3	20.2	16.8	*22.0*	20.0	*22.0*	*22.0*	16.5	11.9	15.9	12.6
91	17.4	*22.7*	17.6	*22.0*		*22.0*	*18.8*	*22.0*	13.4	*16.7*	14.4
92	20.0	*21.8*	*22.5*	*22.0*	*22.0*			*22.0*	13.2	*16.7*	15.8
93	17.7	*23.0*	*23.1*	*22.0*	19.4	*22.0*	*22.0*	*22.0*	13.6	*16.7*	*15.0*
96	16.7	*22.1*	22.8		19.2	*22.0*	*22.0*		14.0	*16.7*	16.6
97	17.1	*22.7*	*18.7*	*22.0*	17.5	*22.0*		*22.0*	13.0	*16.7*	16.0
98	17.8	*21.8*	19.2	*22.0*	17.4	*22.0*	19.6		12.4	*16.7*	

Table C2. Three classes of distance restraints used in structure calculations. Number of restraints from different MTSL positions is given for each class. Numbers in brackets refer to restraints violated in the lowest-energy structure. For more information consult Chapters III – VI.

MTSL position	Number of restraints			
	Upper bound	Lower bound	Both bounds	Total
wt Cyt *c* – CcP				
N38C	11 (0)	3 (0)	47 (25)	61 (25)
N200C	15 (4)	32 (1)	16 (7)	63 (12)
T288C	7 (0)	27 (2)	42 (17)	76 (19)
S263C	0 (0)	66 (0)	2 (0)	68 (0)
Total	33 (4)	128 (3)	107 (49)	**268 (56)**
T12A Cyt *c* – CcP				
N38C	8 (1)	14 (0)	33 (19)	55 (20)
N200C	11 (1)	30 (1)	14 (4)	55 (6)
T288C	10 (0)	19 (1)	25 (11)	54 (12)
S263C	0 (0)	60 (0)	1 (1)	61 (1)
Total	29 (2)	123 (2)	73 (35)	**225 (39)**
R13A Cyt *c* – CcP				
N38C	15 (4)	1 (1)	51 (42)	67 (47)
N200C	8 (5)	44 (1)	19 (11)	71 (17)
T288C	3 (0)	15 (0)	44 (32)	62 (32)
Total	26 (9)	60 (2)	114 (85)	**200 (96)**

Appendix D

Mutagenesis of Cyt c and CcP

Site-directed mutagenesis of Cyt *c*

To generate pUCcc plasmid that was used throughout this study, part of the original pBPCYC1(T-5A)/3 plasmid[41] containing *CYC1* (Swiss-Prot entry P00044) and *CYC3* (Swiss-Prot entry P06182) genes – coding for yeast *iso*-1-Cyt *c* and yeast Cyt *c* haem lyase, respectively – has been inserted into the polycloning linker site of pUC18 plasmid with *Xma*I and *Hin*dIII restriction enzymes. Note that the protein referred to as wt Cyt *c* is in fact the T-5A/C102T variant.

To introduce single point mutations (T12A, R13A, and R13K; Chapters V-VI) into Cyt *c*, site-directed mutagenesis was carried out using the whole plasmid synthesis (WHOPS) polymerase chain reaction (PCR) protocol (Quik Change™, Stratagene, La Jolla, CA) with the primers given in Table D1 and pUCcc plasmid as a template. The DNA constructs for T12A and R13A Cyt *c* have been prepared by Dr. J. A. R. Worrall. All engineered plasmids have been verified by DNA sequencing.

Table D1. **Primers used in WHOPS PCR site-directed mutagenesis of Cyt *c*.** Introduced codon-changing (bold underlined) and silent (bold) mutations are indicated. For each mutant, the primers' lengths and the restriction site created by the silent mutation are indicated in parentheses.

Cyt *c*

T12A (33 bp; *Pst*I)

fw	5'-CTA CAC TTT TCA AG**G** CTA GAT GTC T**G**C A**G**T GCC-3'
rev	5'-GGC A**C**T G**C**A GAC ATC TAG **C**CT TGA AAA GTG TAG-3'

R13A (36 bp; *Pst*I)

fw	5'-GCT ACA CTT TTC AAG ACT **GC**A TGT CT**G** CA**G** TGC CAC-3'
rev	5'-GTG GCA **C**TG **C**AG ACA T**GC** AGT CTT GAA AAG TGT AGC-3'

R13K (36 bp; *Pst*I)

fw	5'-GCT ACA CTT TTC AAG ACT **AA**A TGT CT**G** CA**G** TGC CAC-3'
rev	5'-GTG GCA **C**TG **C**AG ACA T**TT** AGT CTT GAA AAG TGT AGC-3'

Site-directed mutagenesis of CcP

In order to remove the native cysteine of CcP, C128, the original CCP(MKT) plasmid[73] was used as a template for site-directed mutagenesis carried out by WHOPS PCR (see above) with C128A primers (Table D2). The resulting pT7CcP-C128A plasmid has been used as a template in subsequent PCR rounds to introduce desired single-cysteine CcP mutations: N38C, T137C, N200C, S263C, and T288C (Chapters III-VI) and S225C (not reported in this thesis). While T137C, S263C, and T288C have been introduced by WHOPS PCR with the primers given in Table D2, N38C, N200C, and S225C have been constructed differently as described below.

To generate N38C/C128A CcP, pT7CcP-C128A plasmid has been amplified with FWD and N38C_rev primers (Scheme D1 and Table D2), cut with *Xba*I and *Apa*I restriction enzymes and inserted into the starting plasmid digested with the same endonucleases. S225C/C128A CcP has been generated in an analogous way using S225C_rev primer (Scheme D1 and Table D2) to introduce the mutation and *Xba*I and *Bsr*BI restriction enzymes to insert the amplified fragment into the original plasmid.

N200C/C128A CcP has been engineered from pT7CcP-C128A plasmid using two PCR steps. First, an *Eco*RI restriction site has been introduced by PCR with the REV and N200C_fw primers (Scheme D1 and Table D2), and the PCR product digested with *Eco*RI and *Hin*dIII. Second, both *Eco*RI restriction site and N200C mutation were generated by PCR with FWD and N200C_rev primers (Scheme D1 and Table D2), and the PCR product cut with *Xba*I and *Eco*RI. Simultaneous insertion of the two digested PCR fragments into the starting plasmid cleaved with *Xba*I and *Hin*dIII endonucleases yielded the desired N200C/C128A construct. All engineered plasmids have been verified by DNA sequencing.
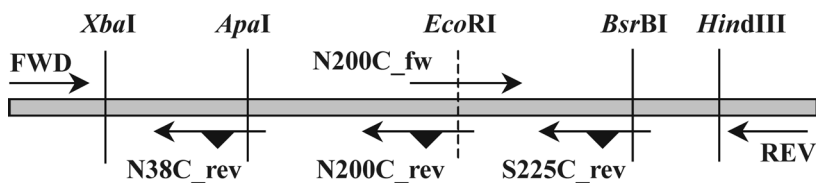
Scheme D1. Site-directed mutagenesis strategy for N38C, N200C, and S225C CcP. The primers are labelled and shown as arrows; filled triangles denote introduced mutations; the relevant restriction sites are indicated. The scheme is not drawn to scale.

Table D2. **Primers used in site-directed mutagenesis of CcP.** Introduced codon-changing (bold underlined) and silent (bold) mutations are indicated. The existing *Apa*I and *Bsr*BI sites in N38C and S225C primers, respectively, are shown in italics. For each mutant the primers' lengths and the restriction site created by the silent mutation are indicated in parentheses.

CcP

FWD (19 bp)

5'-GCC AAC TAT CGT CGT CAA G-3'

REV (21 bp)

5'-CCC TAA AGA CGC GCC CAG TCC-3'

N38C (43 bp)

rev   5'-CTA ATA *CGG GC*C CAT AGC CTA TAT AG**C A**GT CAT ATT CGT CAT C-3'

S225C (30 bp)

rev   5'-CAT GTA G*CC GCT C*TT **TGT** GTC CCA CTG TTC-3'

N200C (*Eco*RI)

fw (26 bp) 5'-CCA ATG A**A**T T**C**T ACT TGA ACT TGT TG-3'

rev (28 bp)5'-CAA GTA **G**AA **T**TC A**CA** GGT AAA GAC GTT G-3'

C128A (35 bp; *Mlu*I)[a]

fw   5'-GAT TCC ATG GAG A**GC** TGG **AC**G **C**GT CGA CAC GCC AG-3'

rev   5'-CTG GCG TGT CGA C**G**C **GT**C CA**G C**TC TCC ATG GAA TC-3'

S263C (39 bp; *Xba*I)[a]

fw   5'-GGA TTT TT**G** CAA AGC TTT TGA AAA ACT **T**C**T** **A**GA AAA CGG-3'

rev   5'-CCG TTT TC**T** A**GA** AGT TTT TCA AAA GCT TTG **C**AA AAA TCC-3'

T288C (32 bp; *Xho*I)[a]

fw   5'-CCA TTT ATT TTC AAG **TGC** C**T**C GAG GAA CAA GG-3'

rev   5'-CCT TGT TCC TC**G** A**GG CA**C TTG AAA ATA AAT GG-3'

T137C (39 bp; *Nci*I)[a]

fw   5'-CGC GTC GAC AC**C** CC**G** GAG GAT **TG**T ACC CCT GAC AAC GGG-3'

rev   5'-CCC GTT GTC AGG GGT A**CA** ATC CTC **C**GG **G**GT GTC GAC GCG-3'

[a] Primers used for WHOPS PCR

Appendix E

Input file for XPLOR-NIH 2.13 rigid-body docking of the Cyt c – CcP complex using PRE-derived distance restraints

The file is based on the published rigid-body docking algorithm[165] used in structure calculations of several protein complexes[165-168]. For more details see Chapter III and ref. 165.

```
set mess=off end
set echo=off end

!--------------- PARAMETERS & STRUCTURE -----------------------------

parameter @parameters.par end

structure @structure.psf end

eval ($inifile="coordinates.pdb")

coordinates @@$inifile

@learn.par

constraints fix (segid="CCP" or segid="SLA" or segid="SLB" or segid="SLC" or
segid="SLD") end

!------------- DEFINE VARIABLES -------------------------------------

eval ($a05 = 300)            !total nr cycles / $a53
eval ($a53 = 300)            !nr of steps
eval ($sqconst=0.2)    !general scaling
eval ($a55=300.0)            !TBATH
eval ($a59=250.0)            !pdb writing threshold
eval ($van=100.0)            !vdw writing threshold
eval ($a60=1.0)              !pdb lag factor
eval ($a301=1.0)             !VDW repel scale factor
eval ($sqexp=2)              !sqexponent in the NOE statement
eval ($scale=1.0)            !scale factor for restraints
eval ($low1=4.0)      !lower limit (restraints with lower limit only)
eval ($up1=100.0)     !upper limit (restraints with lower limit only)
eval ($low2a=12.5)    !lower limit (SLA restraints with upper limit only)
eval ($low2b=13.0)    !lower limit (SLB restraints with upper limit only)
eval ($low2c=12.3)    !lower limit (SLC restraints with upper limit only)
eval ($up2=4.0)       !upper limit (restraints with upper limit only)
eval ($low3=4.0)      !lower limit (restraints with both limits)
eval ($up3=4.0)       !upper limit (restraints with both limits)

!-------------- COUNTERS AND GLOBAL SETTINGS -----------------------

eval ($a48=$cpu*1e4)
set seed=$a48 end
eval ($a14=0)
eval ($a15=0)
```

```
eval ($a16=0)
eval ($a17=0)
eval ($a18=0)
eval ($a19=1)
eval ($a210=0)
eval ($a211=1)
eval ($nout1=0)                      !Number of structures output per run
eval ($min1=9999.0)
eval ($a01 = 1)                      !cycle counter
eval ($a54 = 0.01)                   !timestep in ps
eval ($a61 = $a54*$a53)              !time per cycle (ps)
eval ($a80 = $a61*$a05)              !total time (ps)
eval ($a58=30.0)                     !velocity factor
eval ($a56=1.5)                      !fbeta
eval ($a59a=0.01*$a59)               !vx-reset minimum

set display=coor.dat end            !write parameters

 display xx --------------------PARAMETERS---------------------------
 display xx startdate:         $DATE
 display xx starttime:         $TIME
 display xx ini.file:          $inifile
 display xx pc_rota:           cent=(0 0 0) axis(0 1 0) -20.0
 display xx pc_tran:           x+120.0 y+50.0 z-50.0 (update 30.12.2003)
 display xx timestep (ps):     $a54
 display xx nr of steps:       $a53         time /cycle (ps):    $a61
 display xx nr of cycles:      $a05         total time  (ps):    $a80
 display xx general scale:     $sqconst
 display xx TBATH:             $a55         velocity factor:     $a56
 display xx .pdb threshold     $a59
 display xx .pdb lag factor    $a60
 display xx Local mimim. impulse after 10 cycles with 'constant' Etot
 display xx ------------------------------------------------------------
 display
set display=OUTPUT end

flag exclude elec bond angl dihe impr include vdw noe end

vector do (fbeta=$a58) (segid="CC")
vector do (vx=$a56) (segid="CC")
vector do (vy=$a56) (segid="CC")
vector do (vz=$a56) (segid="CC")

set disp=ener.dat end
 display Energies for the output structures
 display file  Etot    VDW    NOE
 display --------------------------------------------------
set disp=OUTPUT end

set disp=toten.dat end
display Energies for all cycles
 display Cycle        Etot
 display --------------------------------------------------
set disp=OUTPUT end
```

(*continued*)

```
!------------------ DEFINE RESTRAINTS ----------------------------------

noe
 NRES=30000
(continued)

end

 @noeA.xpl               ! Defines restraints
 @noeB.xpl
 @noeC.xpl
 @noeD.xpl

!-------------------- DYNAMICS RUN & OUTPUT ----------------------------

parameter

 @nbfix.xpl

 nbonds
  cutnb=8.5
  inhi=0.25
  ctofnb=7.5
  ctonnb=6.5
  repe1=0.6
  NBXMod=-2
  rexp=2
  irex=2
  rcon=$a301
  wmin=1.5
 end

end

constraints
 interactions (segid="CCP")(segid="CC")
end

energy end

while ($a01 LE $a05) loop calc

 display cycle $a01
 dynamics rigid
  dt=$a54
  group=(segid="CC")
  dynmode=TCOU
  tbath=$a55
  nprint=50
  nstep=$a53
  NTRFRQ=0
end

eval ($a17=$a16)               !write .pdb structures
 eval ($a16=$a15)              !write energy data of each structure
 eval ($a15=$a14)
 eval ($a14=$ENER)
 eval ($abc=$VDW)
```

```
if ($a14<$a15) then
  if ($a14<$a16) then
   if ($a14<$a17) then
    if ($a18>$a60) then
     if ($a14<$a59) then
      if ($abc<$van) then
       eval ($nout1=$nout1+1)
       eval ($pdb="structure_"+encode($nout1)+".pdb")
       write coord output=$pdb end
       if ($a14<$min1) then
         eval ($ref=$nout1)
       end if
       eval ($min1=min($a14,$min1))
       pick bond (segid="CCP" and name FE) (segid="CC" and name FE) geom
       eval ($a50=$RESULT)

       set disp=ener.dat end
        display $nout1 $a14    $VDW    $NOE
       set disp=OUTPUT end

       set disp=coor.dat end
        display Cycle: $a01    File#: $a19    Fe-Fe: $a50    temp: $TEMP
        display Etot: $a14            Evdw:  $VDW            Enoe:  $NOE
        display -----------------------------------------------------------
       set disp=OUTPUT end

       eval ($a18=0)
       eval ($a19=$a19+1)
      end if
     end if
    end if
   end if
  end if
 end if
end if
eval ($a18=$a18+1)

set disp=toten.dat end
 display $a01 $a14
set disp=OUTPUT end

if ($TEMP>200000.0) then            !corrects excessive temperature
 eval ($a45=RAND()+0.01)
 vector do (vx=$a56*$a45*rand()) (segid="CC")
 vector do (vy=$a56*$a45*rand()) (segid=CC")
 vector do (vz=$a56*$a45*rand()) (segid="CC")
end if

if ($a210=10) then                  !impulse to escape local minimum
 if ($ENER>$a59a) then
  eval ($a212=$ENER/$a211)
  if ($a212>0.8) then
   if ($a212<2.0) then

    eval ($a213=100*(rand()-0.5) )
    eval ($a214=100*(rand()-0.5) )
    eval ($a215=100*(rand()-0.5) )
    vector do (x=x+$a213) (segid="CC")
    vector do (y=y+$a214) (segid="CC")
    vector do (z=z+$a215) (segid="CC")
```

(*continued*)

```
     vector do (vx=50) (segid="CC")
     vector do (vy=50) (segid="CC")
     vector do (vz=50) (segid="CC")

     set disp=coor.dat end
      display cycle $a01: impulse: $a213, $a214, $a215 to x,y,z; v=50
     set disp=OUTPUT end

    end if
   end if
   eval ($a211=$ENER)
   else
   eval ($a211=1)
  end if
  eval ($a210=0)
 end if
 eval ($a210=$a210+1)
 eval ($a01 = $a01 + 1)

end loop calc

!----------------------FINISH-----------------------------------

!-------------------------- Calculating rmsds ----------------------

vector idend ( store9 ) ( name ca or name n or name c )  !backbone selection
eval ($ref_file="structure_"+encode($ref)+".pdb")

set display=rms.dat end
 display Backbone pairwise RMSD from the lowest energy strucutre ($ref_file)
 display  file           rmsd
 display ----------------------------------------------------------------
set disp=OUTPUT end
 coor disp=comp @@$ref_file
eval ($count1=0)

while ($count1 < $nout1) loop fill

 evaluate ($count1=$count1+1)
 evaluate ($file="structure_"+encode($count1)+".pdb")
 coor @@$file
 coor sele=(recall 9) fit end
 coor sele=(recall 9) rms end
 eval ($b1 = $result)

 set display=rms.dat end
  display  $file            $b1

end loop fill

 set display=OUTPUT end

set echo=true end
stop
```