

# Understanding protein complex formation: the role of charge distribution in the encounter complex

Di Savino, A.

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# **Chapter 5**

Enhancing the population of encounter complex affects protein complex formation efficiency.

Based on the research article:

Di Savino, A. and Ubbink, M. Enhancing the population of encounter complex affects protein complex formation efficiency, *Under revision*.

# Abstract

Optimal charge distribution is considered to be important for efficient formation of protein complexes. Electrostatic interactions guide encounter complex formation that precedes the formation of an active protein complex. However, disturbing the optimized distribution by introduction of extra charged patches on cytochrome c peroxidase does not lead to a reduction in productive encounters with its partner cytochrome c. To test whether a complex with a high population of encounter complex is more easily affected by suboptimal charge distribution, the interactions of cytochrome c mutant R13A with wild type cytochrome c peroxidase and a variant with an additional negative patch were studied. The complex of the peroxidase and cytochrome c R13A was reported to have an encounter state population of 80%, compared to 30% for the wild type cytochrome c. NMR analysis confirms the dynamic nature of the interaction and demonstrates that the mutant cytochrome c samples the introduced negative patch. Kinetic experiments show that productive complex formation is 5-7 fold slower at moderate and high ionic strength values for cytochrome c R13A but the association rate is not affected by the additional negative patch on cytochrome c peroxidase, showing that the total charge on the protein surface can compensate for less optimal charge distribution. At low ionic strength (44 mM), the association with the mutant cytochrome c reaches the same high rates as found for wild type cytochrome c, approaching the diffusion limit.

# Introduction

Electrostatic interactions play a major role in protein complex formation. The collision of free proteins in solution can result in early dissociation or lead to the formation of the stereospecific and active complex. Electrostatic interactions can promote the interactions between proteins, guiding the formation of an complex.<sup>1–5</sup> The encounter encounter complex is a very dvnamic ensemble of configurations in which proteins sample each other's surface.<sup>6</sup> It can lead to the formation of the stereospecific and active complex, in which case the encounter is productive.<sup>7</sup> In case of an early dissociation, the encounter is futile.8 The charge distribution on the surface is considered to be critical to enhance the number of encounters, productive by guiding the incoming partner to the binding site and reducing the surface area to be searched.9 Previous studies showed how charge mutations can affect the interactions between proteins. For example, on the complex formed by TEM1-β-lactamase (TEM1) and its inhibitor,  $\beta$ -



**Figure 5.1.** Mutations in the Cc:CcP complex. The crystal structure of the stereospecific complex formed by Cc (magenta ribbons) and CcP (green ribbons) is shown (PDB  $2PCC^{13}$ ). The heme groups are shown in salmon sticks, residue R13 is indicated in yellow spacefill representation, the Lys residues that were mutated to Glu to introduce additional negative charges in CcP\_B are indicated in red, and residue N87 of CcP mutated to cysteine for spin labelling in blue spacefill. The figure was made with Pymol<sup>12</sup>.

lactamase-inhibitor protein (BLIP), charge mutations can enhance the formation of the productive encounter complex resulting in a higher association rate ( $k_a$ ) but without lowering the dissociation rate. Alternatively, they can alter the encounter complex without any effect on the  $k_a$ , or improve the  $k_a$  without affecting the encounter complex.<sup>7</sup> Charge mutations can modify the balance of productive and futile encounter complexes, even if located far from the stereospecific binding site. The outcome of the mutation depends on

the optimization or interruption of favorable electrostatic 'pathways'.<sup>10</sup> The effect of the charged pathways was observed for the complex of cytochrome P450cam and putidaredoxin.<sup>11</sup>

Because of their biological functions, electron transfer (ET) proteins often form transient complexes and the charge distribution on their surface is highly optimized to have favorable electrostatic interactions, leading to efficient protein complex formation. These complexes usually have low affinity (K<sub>D</sub> in the µM-mM range), a consequence of the association and dissociation rate constants both being high. Protein complexes with highly optimized electrostatic interactions have k<sub>a</sub> values that approach the limit set by translational diffusion.<sup>1</sup> The ET complex formed by cytochrome c (Cc) and cytochrome c peroxidase (CcP) from baker's yeast (Saccharomyces cerevisiae) is one of the most studied and best characterized ET complexes. The formation of the complex is guided by electrostatic interactions between the positively charged binding site on Cc and the negatively charges on CcP.<sup>13–17</sup> The encounter state represents 30% of the complex, while 70% is in the stereospecific complex and Cc samples merely 15% of the surface of CcP.<sup>18,19</sup> Several studies have shown that mutations in the interface between Cc and CcP strongly affect association,<sup>20-22</sup> so we wondered how important the optimized charge distribution is for the efficiency of the ET reaction. To test this, negative patches were added distant from the stereospecific binding site on CcP, enlarging the surface sampled by Cc in the encounter state. Interestingly, the new negative charges create productive encounters, slightly enhancing the  $k_a$ , even if located far from the stereospecific binding site (<sup>23</sup> and <sup>24</sup>). This suggests that the overall charge on CcP surface is more important than the charge distribution. We considered that this could be a consequence of the stability of the stereospecific state as compared to the encounter state. To test this idea, for the current work, we turned to a mutant of Cc, R13A, reported to form a complex with Cc with 80% population of the encounter state.<sup>25</sup> Arg13 has been shown to be a hot-spot in the interactions of the stereospecific complex and its mutation to Ala shifts the balance toward the encounter state and reduces the affinity by 30 fold.<sup>25,26</sup> This mutant was used to study the interaction with native CcP (CcP\_A) and a variant with eight additional negative charges on the side of CcP relative to the stereospecific binding site (CcP\_B, Figure 5.1) $(^{23}$ and <sup>24</sup>). Chemical shift perturbation (CSP) analysis and paramagnetic relaxation enhancement (PRE) experiments show that the complex is highly dynamic and that Cc visits the new negative patch in CcP B. Kinetic experiments yield reduced association rates for the mutant Cc at moderate and high ionic strength values, but surprisingly, the addition of the negative patch does not reduce the number of productive encounters. At low ionic strength, the mutant Cc associates as fast as wild type (wt) Cc with CcP. The results are discussed in the context of the importance of charge optimization for complex formation.



**Figure 5.2.** CSP upon complex formation. a) Comparison between the average CSP for the complexes CcP\_A:Cc R13A (in red), CcP\_B:Cc R13A (in black), CcP\_A:wt Cc (in red shading) and CcP\_B:wt Cc (in grey).<sup>23</sup> The black crosses indicate residues for which data are not available; b) CSP map for <sup>15</sup>N Cc R13A in complex with CcP\_A (left) and CcP\_B (right). The surface model of Cc (PDB 2PCC<sup>13</sup>, haem in salmon sticks) is colored according to the code: residues with  $\Delta \delta_{avg} \ge 0.06$  ppm are red, 0.04-0.06 ppm are orange, 0.02-0.04 ppm are yellow, <0.02 ppm are blue, the residues for which no data are available are light gray and residue R13 is black (See Table S5.1 for the list of red, orange and yellow residues).

## Results

*The Cc R13A – CcP interaction is predominantly in the encounter state.* Binding to CcP causes considerable changes in the amide chemical shifts of Cc.<sup>27</sup> These changes are thought to be caused predominantly by the stereospecific complex. In this state, Cc is partly desolvated and engages in specific interactions, whereas in the encounter complex Cc is thought to remain solvated and assume multiple orientations.<sup>28</sup> Cc R13A was shown to shift the equilibrium between encounter and stereospecific states toward the former.<sup>25</sup> In line with those findings, the chemical shift perturbations (CSP) observed for the <sup>15</sup>N Cc R13A binding to CcP\_A are much smaller than those for wt Cc (Figure 5.2a, red bars). Also upon binding to CcP\_B the CSP remain overall smaller for Cc R13A than for wt Cc, indicating that the complex is mainly in the encounter state. Cc R13A interacts with the same surface to CcP\_A and CcP\_B (Figure 5.2b) but some differences can be observed. The CSP pattern shows several larger perturbations when binding to CcP\_B (in the region of residues 5-10 and around residue 90), suggesting the formation of several more specific interactions.

*Cc R13A binds the new patch on CcP\_B.* To establish whether the introduction of the added negative charges in CcP\_B influence the encounter complex with Cc R13A, PRE experiments were performed. CcP\_A and CcP\_B were tagged with the spin label MTSL after mutating residue N87, which is located close to the added negative patch (Figure 5.1), to cysteine. The tag causes enhanced relaxation for nuclear spins in <sup>15</sup>N labelled Cc R13A



**Figure 5.3.** Probing new interactions with PRE NMR. The PREs are shown for amide nuclear spins of Cc R13A in presence of CcP\_A (in red) or CcP\_B (in black), tagged with MTSL at Cys87, which is located close to the negative patch added on CcP\_B. The error bars are indicated as shaded regions and represent the propagated  $2 \times$  SD errors of the raw data. The observed PREs were divided by 0.15 to correct for the fraction of CcP that was paramagnetic (see text).

that come within ~2.5 nm of the tag during the interactions with CcP. EPR experiments show that only 15% of the MTSL remained paramagnetic during the tagging reaction (and remains stable afterward). We have observed before that MTSL gets partly reduced during the reaction with CcP so routinely the paramagnetic fraction is established by EPR. The interaction of Cc with CcP is very fast on the NMR timescale so all Cc molecules sample many CcP molecules rapidly, averaging the effect of interactions with diamagnetic and paramagnetic CcP molecules. Figure 5.3 presents the observed PRE for the amides of Cc R13A upon interacting with CcP\_A and CcP\_B labelled at Cys87 with MTSL. The PREs have been corrected for the 15% paramagnetic labelling by dividing the observed PREs by 0.15. In CcP\_A significant PRE signal was observed for only one aminoacidic residue, indicating that Cc R13A does not interact with the surface close to N87. Upon complex formation with CcP\_B, large PREs are observed, indicating that Cc R13A visits the region with the extra charges in proximity of the paramagnetic tag on CcP\_B. Clearly, the encounter complex includes the new patch on CcP\_B, in line with the findings for wt Cc.<sup>23</sup>

*Cc R13A reacts more slowly with CcP.* To determine whether the R13A mutation in Cc influences the association rate constant with CcP, it was measured as a function of the ionic strength using stopped-flow measurements. Following the work of Miller et al. (1994),<sup>29</sup> the association rate constant can be measured by observing ET from  $Cc(Fe^{2+})$  to CcP compound I (CpdI). The ET rate constant is high, so the observed second order rate constant is a lower-limit estimate of the association rate constant, as explained in detail in a



**Figure 5.4.** Rate of association ( $k_a$ ) between the Cc and CcP variants. The  $k_a$  values, plotted as a function of the square root of the ionic strength, were obtained from the simulations of the stopped flow kinetics. Errors were calculated as the standard deviation between replicates and simulations performed at different CcP concentrations (see Materials and Methods of <sup>24</sup> for details).

previous paper.<sup>24</sup> The association rate constant for wt Cc to CcP\_A and CcP\_B is strongly dependent on the ionic strength (Figure 5.4, data reproduced from ref <sup>23</sup> and <sup>24</sup>), due to the favorable electrostatic interactions between the Cc and CcP surfaces.<sup>30–34</sup> Following the same approach, the association rate constant ( $k_a$ ) was measured for Cc R13A and CcP\_A or CcP\_B. In a wide range of ionic strength values, from 122 mM (11 mM<sup>1/2</sup> on the axis of Figure 5.4) to 730 mM (27 mM<sup>1/2</sup>), the  $k_a$  of the complexes formed by Cc R13A are 5-7 fold lower than for the complexes formed by wt Cc (Tables S5.2, S5.3 and S5.4). However, curiously, at the lowest ionic strength tested, 44 mM (7 mM<sup>1/2</sup>) the  $k_a$  of the Cc R13A complexes reaches the same level as those for the complexes formed by wt Cc. Before, the surprising observation was made that the introduction of a strong negative patch on CcP distant from the stereospecific binding site did not lower the  $k_a$  for wt Cc, but rather increased it somewhat.<sup>24</sup> Similarly, for Cc R13A, we observe that the formation of the ET active complex is not slowed down by the additional charges on CcP\_B, as compared to CcP\_A.

# Discussion

Volkov *et al.*<sup>25</sup> showed that the Cc mutation Arg13 to Ala drastically increases the population of the encounter complex with CcP, from 30% to 80%. Also, the binding constant (K<sub>B</sub>) decreases from  $1.9 * 10^5$  M<sup>-1</sup> of the wt complex to  $0.06 * 10^5$  M<sup>-1</sup> for Cc R13A bound to CcP. We previously reported that the addition of a negative patch on a side of the binding site of CcP (CcP\_B) enlarges the area sampled by Cc in the encounter complex. Since the k<sub>a</sub> for Cc and CcP\_B is slightly higher than that for the wt complex, it was concluded that the added charges result in more productive encounters.<sup>23</sup> The aim of the present study was to investigate to what degree the delicate balance between stereospecific complex and encounter complex influences the association between Cc and CcP by comparing the interactions between CcP with wt Cc and Cc R13A.

The CSP analysis shows that Cc R13A forms a much more dynamic complex with both CcP\_A and CcP\_B, than does wt Cc, in line with a more populated encounter state. The Cc R13A CSP patterns obtained with the two variants of CcP are not the same, contrary to the finding for wt Cc.<sup>23</sup> In the latter, the stereospecific complex is more populated and the main contributor to the CSP. The CSP differences observed for Cc R13A indicate differences in the interactions with CcP\_A and CcP\_B. This observation is further supported by the PRE results that show clearly that the region with the new negative patch in CcP\_B is visited by Cc R13A. It does not do so in CcP\_A, in accord with the Monte Carlo simulations reported in ref. <sup>25</sup>, which showed that the encounter complexes of wt Cc and Cc R13A with CcP\_A are very similar and comprise the surface area of CcP around the stereospecific binding site.

To test to what degree the increased dynamics in the complexes formed by Cc R13A influence the formation of the active complex, the  $k_a$  for binding to CcP\_A or CcP\_B was determined. The favorable electrostatic interactions between Cc R13A and the CcP variants cause a strong ionic strength dependence of  $k_a$ . At an ionic strength of 122 mM and higher,

the  $k_a$  values for the complexes formed by Cc R13A are 5-7 fold lower than for wt Cc at the same ionic strength. Surprisingly, at the lowest ionic strength tested, 44 mM, the  $k_a$  of the complexes formed by Cc R13A and either CcP variants reaches the  $k_a$  of the complexes formed by wt Cc. So, whereas the rate constant plateaus at low ionic strength for wt Cc, the one for Cc R13A monotonically increases.

The plateau reached for wt Cc can have various reasons. It could be that monopole-dipole interactions may come into play<sup>35,36</sup> at low ionic strength that work against each other and causing the leveling off of the  $k_a$ . However, this effect is observed for CcP\_A and CcP\_B and even for CcP\_D, which has a strong new negative patch at the far end from the stereospecific binding site.<sup>24</sup> These three variants have widely different dipoles, yet all show the same behavior. Alternatively, the diffusion limit may be reached, so increasing charge interactions further does not result in faster association. A third explanation is that the encounter complex, which is dominated by electrostatic interactions is most favorable at low ionic strength and thus, the balance between encounter and stereospecific binding site has been proposed before.<sup>37–40</sup> It is not trivial to determine which of the latter two explanations is applicable here. We used simulation of complex formation to evaluate ranges that the  $k_a$  values can assume.

$$\operatorname{Cc}(\operatorname{Fe}^{2+}) + \operatorname{CpdI} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \operatorname{Encounter \ complex} \underset{k_{-2}}{\overset{k_2}{\twoheadrightarrow}} \operatorname{Stereospecific \ complex} \underset{\longrightarrow}{\overset{k_{et}}{\to}} \operatorname{Cc}(\operatorname{Fe}^{3+}):\operatorname{CpdII} \qquad \text{reaction 1}$$

The association reaction can be described by reactions 1, in which CpdII refers to compound II, the second intermediate in the reduction of hydrogen peroxide by CcP. Cc and CcP associate to form the encounter complex, which can evolve to the stereospecific complex in which ET can occur. At I = 122 mM, the  $K_B$  value, the population of the encounter complex and the ka values have been reported for both wt Cc and Cc R13A in complex with CcP\_A,(<sup>24,25</sup>, this work) restraining the microscopic rate constants in reaction 1. Details are given in the supporting information (Text S5.1) and Table S5.5 gives estimated values. Simulation shows that the 7-fold lower  $k_a$  value observed for Cc R13A is attributable to a combination of a lower  $k_1$  and the lower population of the stereospecific complex (implying that the second equilibrium is more to the left). Cc R13A lacks the positive side chain of Arg13 in the binding interface, so a lowered  $k_1$  is to be expected, because of weaker electrostatic interactions. The k<sub>a</sub> observed for Cc R13A at 44 mM can be found by increasing  $k_1$  and lowering  $k_{-1}$  without changing the population of the encounter state. For wt Cc, it could be that the k<sub>1</sub> obtained at I = 122 mM ( $2 \times 10^9$  M<sup>-1</sup>s<sup>-1</sup> for CcP A) represents the diffusion limit and cannot increase further at I = 44 mM. If it does increase, it must be accompanied by a substantially increased population (from 30% to >90%) of the encounter state to obtain the experimental k<sub>a</sub> value, which is nearly the same as the one at I = 122 mM (Table S5.5). In summary, these simulation data do not allow to favor one of these explanations. This analysis shows that the lower association rate constant of Cc R13A

is due to reduced electrostatic interactions and less favorable binding at the stereospecific complex. At low ionic strength this difference with wt Cc is eliminated, because of the plateau reached by  $k_a$  of wt Cc.

# Conclusions

Our CSP data confirm that the mutation of the Arg 13 of Cc to Ala enhances the dynamic component in the binding with the CcP variants. While it was shown that the binding mode of wt Cc with CcP\_A and CcP\_B is the same,<sup>23</sup> the interactions of Cc R13A with CcP\_B differ more from those with CcP\_A and are somewhat more specific. Paramagnetic relaxation enhancement (PRE) experiments showed that, similarly to what shown for the Cc:CcP\_B complex,<sup>23</sup> the added charges enlarge the surface of CcP visited by Cc R13A. The new patch disturbs the optimized charge distribution on the surface of CcP, yet it does not result in less productive encounters because the association rate constant is not reduced. Stopped flow experiments were used to evaluate the influence of the higher population of encounter state on the association rate between Cc R13A and the CcP variants. At ionic strength values above 122 mM (11 mM<sup>1/2</sup>), the k<sub>a</sub> exhibits a 5-7 fold reduction compared to wt Cc, indicating that the population of the encounter state has a role in balancing dynamics and specificity in the protein complex formation, and thus can be critical for the efficiency of ET.

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# **Supporting Information**

# **Materials and Methods**

# **Sample Preparation**

S. cerevisiae Cc and CcP and their variants Cc R13A and CcP B, were expressed and purified as previously described.<sup>1-5</sup> The EPR and the PRE experiments were performed as previously reported.<sup>3,4,6</sup> For PRE analysis, two sets of R<sub>2</sub>dia values were obtained: one was obtained using CCPN analysis version 2.4.0 and the second one fitting the resonances to a glore line shape using FuDA (kindly previously provided by Dr. D. Fleming Hansen, University College London). The two sets of R<sub>2</sub>dia values were averaged to obtain the final R<sub>2</sub>dia, and their standard deviation was reported as error. The PREs were corrected for the percentage of paramagnetic signal of the MTSL tagged CcP (15% for both CcP A and CcP B, see Results) and for the fraction of Cc bound to CcP. The percentage of Cc R13A bound to CcP was calculated using the previously reported binding constant.<sup>7</sup> The assignment of the <sup>15</sup>N Cc R13A HSOC was obtained comparing the spectrum with the assignment of the wt Cc spectrum<sup>8-10</sup> (BMRB 17845). Several residues, mostly around the mutation site, could not be assigned. The chemical shift perturbations (CSP) were measured by overlaying the HSOC spectra of Cc R13A bound to CcP tagged with the diamagnetic tag MTS to the spectrum of free Cc R13A. The average CSP were calculated as previously reported.11

# **Kinetic experiments**

The stopped flow experiments, the simulations of the kinetics and the analysis of the data was performed as previously described.<sup>12</sup> The t-test analysis for significance in difference of ka values is given in Tables S5.3 and S5.4.

# Text S5.1. Simulation of the rates for the Cc:CcP\_A and CcR13A:CcP\_A complexes

The model for the association reaction is given by reaction 1. The observable is the absorbance change associated with the oxidation of ferrous Cc within the complex with  $Cc.^{12}$ 

$$\operatorname{Cc}(\operatorname{Fe}^{2+}) + \operatorname{CpdI} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \operatorname{Encounter \ complex} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \operatorname{Stereospecific \ complex} \underset{\longrightarrow}{\overset{k_{et}}{\to}} \operatorname{Cc}(\operatorname{Fe}^{3+}):\operatorname{CpdII} \qquad \text{reaction 1}$$

The rates constants in reaction 1 for CcP\_A in complex with wt Cc or Cc R13A were simulated using equation S1:

$$k_a = \frac{k_1 k_2 k_{et}}{k_{-2} k_{-1} + k_{-1} k_{et} + k_2 k_{et}}$$
equation S1

Equation S1 is derived by assuming pseudosteady conditions for the two intermediate states, the encounter complex and the stereospecific complex.

The macroscopic  $K_D$  is given by:

$$K_D = \frac{k_{-1}k_{-2}}{k_1k_2}$$
 equation S2

The fraction of the encounter  $f_{enc}$  complex is given by:

$$f_{enc} = \frac{k_{-2}}{k_2 + k_{-2}}$$
equation S3

At I = 120 mM,  $k_a$ ,  $K_D$ , and  $f_{enc}$  have been determined for wt Cc and Cc R13A in complex with wt CcP (CcP\_A).(<sup>5,7,12</sup>; this work) These values were defined as targets to simulate by varying  $k_1$ ,  $k_{-1}$ ,  $k_2$  and  $k_{-2}$ .  $k_{et}$  was set to 50,000 s<sup>-1</sup> (<sup>12</sup>) and assumed to be independent of ionic strength. The  $K_D$  defines the ratio of  $k_2$  and  $k_{-2}$  but not their absolute values. It was assumed that Cc reorients fast within the encounter complex, on a timescale of the rotational diffusion constant (low ns range), but this choice has little effect on the results.

**Table S5.1.** CSP classes used in the maps. The resonances of <sup>15</sup>N CcR13A in complex with CcP\_A and CcP\_B are listed in descending order (from left to right) according to the shift intensity in ppm. The residues with CSP <0.02 ppm are omitted as their shift intensity is considered not significant.

# CcP\_A:CcR13A:

≥0.06 ppm	Lys 86	Gln 16	His 33	Lys 4	Gly 77	Lys 72	Leu 9	Met 80
0.04-0.06 ppm	Lys 89	Lys 79	Asp 90	Lys 5	Thr 78	Thr 8		
0.02-0.04 ppm	Ser 2 Glu 88 Asn 70 Leu 58	Asp 93 Val 57 Lys 87 Gly 1	Cys 17 Gln 42 Lys 100 Leu 68	Val 28 Lys 73 Ala 7 Arg 91	Thr 19 Gly 6 Ala 3 Gly 41	Tyr 48 Glu -3 Phe 82	Val 20 Lys -1 Leu 98	Tyr 74 Leu 94 Lys 54
no data	Thr 12 Pro 76	Arg 13 Ala 81	Cys 14 Gly 83	Pro 25 Gly 84	Pro 30	Asn 31	Met 64	Pro 71

# CcP\_B:CcR13A:

≥0.06 ppm	Thr 8 Leu 85 Gln 16	Lys 89 Lys 72 Phe 10	Lys 86 Lys 5	Met 80 His 33	Gly 6 Lys 11	Leu 94 Lys 79	Asp 93 Ser 2	Gly 77 Thr 19
0.04-0.06 ppm	Arg 91 Asn 92	Lys 73	Lys -1	Asn 70	Asp 90	Leu 98	Thr 78	Gly 1
0.02-0.04 ppm	Lys 87 Gly 34 Asp 60 Glu 21 Ile 95	Ile 53 Tyr 48 Glu -3 Asp 50 Asn 52	Val 20 Val 57 Lys 99 Glu 66	Thr 69 Ala 7 Tyr 67 Ala 43	Cys 17 Leu 15 Gly 41 Ala 0	Leu 68 Val 28 Ala 3 Trp 59	Tyr 74 Phe -2 Thr 96 Lys 54	Ile 75 Thr 49 Leu 58 Thr 102
no data	Leu 9 Pro 71	Thr 12 Pro 76	Arg 13 Ala 81	Cys 14 Phe 82	Pro 25 Gly 83	Pro 30 Gly 84	Asn 31 Glu 88	Met 64

**Table S5.2** Association rate constants ( $M^{-1}s^{-1}$ ) for CcP\_A and CcP\_B in complex with Cc and Cc R13A. The rate constants and error are calculated as described in <sup>12</sup>.

	CcP_	A:Cc	CcP_	B:Cc	CcP_A:	CcR13A	CcP_B:	CcR13A
I (mM)	ka	st.dev.	ka	st.dev.	$\mathbf{k}_{\mathrm{a}}$	st.dev.	ka	st.dev.
752	6.2E+06	6.0E+05	6.7E+06	8.3E+04	1.3E+06	3.8E+04	1.1E+06	2.5E+04
622	8.2E+06	3.3E+05	1.4E+07	1.7E+06				
502	2.0E+07	1.2E+06	2.8E+07	2.6E+06				
392	4.4E+07	4.5E+06	6.2E+07	9.4E+06				
292	1.3E+08	1.5E+07	2.2E+08	3.1E+07	2.4E+07	2.9E+06	2.4E+07	2.4E+06
192	7.7E+08	8.6E+07	7.5E+08	1.3E+08				
122	1.7E+09	3.0E+08	2.0E+09	2.4E+08	2.3E+08	1.3E+07	3.2E+08	2.5E+07
44	1.7E+09	2.9E+08	1.4E+09	3.2E+08	1.6E+09	1.7E+08	1.7E+09	1.4E+08

Table S5.3. p-values obtained from the t-test performed by comparing the rate of association (k(II)) of the Cc:CcP complexes at ionic strength
values from 44 mM to 752 mM. The grey boxes indicate p-values > 0.016, meaning that the difference in the rates of association is considered not
o be significant. Bonferroni correction was applied using $p < 0.05$ for individual comparisons and $n = 3$ for the number of comparisons for each
ariant.

1					I
CcP_A:Cc/CcP_B: CcR13A	6.7E-08	1.4E-11	1.3E-05	8.8E-01	CcP_A:CcR13A/CcP_B: CcR13A
CcP_A:Cc /CcP_A: CcR13A	1.0E-07	6.7E-12	8.7E-06	3.8E-01	CcP_B:Cc /CcP_B:CcR13A
CcP_A:Cc/CcP_B:Cc	3.3E-01	1.3E-06	7.6E-02	4.5E-02	CcP_B:Cc/CcP_A: CcR13A
I (mM)	752	292	122	44	I (mM)

CcP_A:CcR13A/CcP_B: CcR13A	2.4E-04	1.0E+00	7.2E-06	5.6E-01
CcP_B:Cc /CcP_B:CcR13A	2.5E-08	2.4E-10	5.4E-07	2.8E-02
CcP_B:Cc/CcP_A: CcR13A	2.5E-08	2.1E-10	4.2E-07	1.0E-01
I (mM)	752	292	122	44

Table S5.4. Nu	umber of independent	simulations p	performed t	to calculate	the rate	constants k(	II) and
used in t-test.							

I (mM)	CcP_A:Cc	CcP_B:Cc	CcP_A:CcR13A	CcP_B:CcR13A
752	12	8	8	8
622	7	8		
502	12	16		
392	8	15		
292	14	14	10	10
192	12	12		
122	8	8	12	8
44	8	8	8	10

	wtCc:CcPA 11 mM <sup>1/2</sup>	Target	CcR13A:CcPA 11 mM <sup>1/2</sup>	Target	wtCc:CcPA 7 mM <sup>1/2</sup>	Target	CcR13A:CcPA 7 mM <sup>1/2</sup>	Target
k1 (M <sup>-1</sup> S <sup>-1</sup> )	2.00E+09		8.00E+08		3.00E+09		1.95E+09	
k.1 (s <sup>-1</sup> )	2.30E+04		3.10E+04		2.50E+03		2.50E+03	
$k_{2}\left(s^{4}\right)$	6.00E+07		1.70E+07		5.00E+06		1.70E+07	
$k_{2}$ (s <sup>-1</sup> )	2.60E+07		7.00E+07		7.00E+07		7.00E+07	
$k_{et}$ (s <sup>-1</sup> )	5.00E+04		5.00E+04		5.00E+04		5.00E+04	
$\mathbf{K}_{\mathbf{D}}\left(\mathbf{M}\right)$	4.98E-06	5.00E-06	1.60E-04	1.66E-04	1.17E-05		5.28E-06	
$k_{a}\left(M^{-1}s^{-1}\right)$	1.67E+09	1.66E+09	2.25E+08	2.26E+08	1.76E+09	1.74E+09	1.62E+09	1.62E+09
fraction encounter	0.30	0.30	0.80	0.80	0.93		0.80	
time $E>S$ , $k_2$ . <sup>1</sup> (ns)	16.67		58.82		200.00		58.82	
$k_a(R13A)/k_a(wt)$			0.14	0.14			0.93	0.93

Table S5.5. Estimated rate constants for the Cc:CcP\_A and CcR13A:CcP\_A complexes at 122 mM (11 mM<sup>1/2</sup>) and 44 mM (7 mM<sup>1/2</sup>) ionic strength.

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