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

Di Savino, A.

Chapter 6

General discussion and concluding remarks

General discussion and concluding remarks

Electrostatic interactions in protein complex formation. Understanding the process of the protein complex formation provides important insights into how protein complexes can be regulated. Electrostatic interactions can promote the recognition between proteins or between a protein and its ligand.[1–5] Electrostatics allow two proteins with opposite charges on their surface to be attracted and find each other in a very crowded environment, such as the inside of a cell. Electrostatic interactions drive the formation of the encounter complex, pre-orient the two proteins for binding[6] and minimize the area searched on the protein surface,[7] guiding the two proteins to the binding site. As a result, favorable electrostatic interactions accelerate the association rate of protein complexes.[8–11] Nevertheless, strong electrostatic interactions in the encounter complex can also disturb the binding to the stereospecific binding site slowing down the association.[12–16] This apparent contradiction is explained by the "Velcro model" introduced by McLendon in 1991.[17] He suggested that electron transfer proteins interact in a dynamic ensemble of orientations with similar energy, comparable to complementary sticky "Velcro" patches, rather than in a single static configuration. As a consequence, if the interactions between the proteins allow them to easily change orientation and sample different areas of the protein surface, a favorable electrostatic pathway can guide the proteins to bind to the stereospecific binding site. At the same time, many orientations with similar energy reduce specificity, i.e. there is competition between forming the stereospecific, active complex and the dynamic encounter complex. Second, if the electrostatic interactions between two "Velcro" patches are strong enough to fix the proteins in a sub-set of configurations, for example at low ionic strength, the formation of the stereospecific complex can also be hindered. These mechanisms allow the encounter complex to regulate the delicate equilibrium between high association rate and specificity, crucial for the biological function of transient complexes such as electron transfer protein complexes. In fact, the ET in the respiration or photosynthetic redox chains can be limited by the efficiency of protein interactions. Thus, electron transfer proteins achieve their efficiency through high association and high dissociation rates and a large population of encounter complex. The distance between the area sampled by the encounter complex and stereospecific binding site, and the presence of favorable electrostatic interactions leading to the latter determine the function of the encounter complex. If the encounter complex is close to the stereospecific binding site or a favorable electrostatic path leads to it, the encounter complex usually promotes the formation of the final and active complex and thus it is called productive.[10] If the encounter complex is far away from the stereospecific binding site and in absence of electrostatic pathways leading to it, the proteins will most probably dissociate without forming the active complex and the encounter complex is called futile.[18,19]

Characterization of the encounter complex by paramagnetic NMR spectroscopy. Although the importance of the encounter complex has been recognized by different studies,[1,20,21] it is still challenging to characterize it, due to its very elusive nature. Paramagnetic NMR, relaxation enhancement experiments (PRE) in particular, proved to be

an exquisite tool for investigating lowly populated states.[22] A paramagnetic tag attached to the protein surface causes relaxation of the nearby nuclear spins. The effect is measurable as a reduction in intensity of the resonances of the partner protein, enabling the observation of the surface sampled during the interactions (Chapter 1). We used the paramagnetic tag MTSL, which causes relaxation in a ~2.5 nm radius from the tag (Chapter 3 and 5). While the tagging protocol is apparently straightforward, the application of the technique to haem proteins turns out to be challenging due to the instability of the paramagnetic signal of the attached tag. The presence of the tag on the protein surface was routinely tested by mass spectrometry, indicating a 100 % efficiency of tagging, confirmation by EPR however gave much more variable results. The EPR measured a paramagnetic signal corresponding to a tagging ranging from 15 % to almost 90 % without any apparent differences in the conditions. It is unclear what causes the reduction of the MTSL, although it may be a consequence of chemistry catalyzed by the haem group in CcP. Mass spectrometry of CcP shows that older samples have undergone oxidations and Cc can become reduced after several hours in the presence of CcP, despite the apparent lack of electron donors. Since only a fraction of the paramagnetic signal remains stable after tagging, we recommend always testing the tagging efficiency with EPR measurements. The PREs are then corrected for the tagging efficiency of paramagnetic tag, which can be done only if the proteins interact in the fast exchange regime relative to the maximal PRE. Furthermore, a single tethered tag as MTSL can affect the PRE measurements due to its mobility. The linker of the tag can have different conformations causing a degree of averaging over the space of the spin label.[23] The tag may have a preferred orientation different from expected due to interactions with residues on the surface. This could result in a 'false' negative in which the absence of PREs is due the tag conformation on the protein surface and not lack of protein-protein interactions. For these reasons it is important to perform the experiment by tagging the protein on more sites close to the region of interest, to ensure the collection of consistent data. A solution could be to use a double armed paramagnetic tag as the CLaNP spin labels, but avoiding strongly charged probes that could interfere with the binding.[24]

Overall charge vs. charge distribution. This thesis represents a follow-up of the previous research done on the Cc:CcP complex. The encounter complex formed by Cc and CcP was characterized with PRE and successfully simulated using Monte Carlo simulations based exclusively on electrostatic interactions.[25] Other studies have described the importance of the electrostatic interactions in the Cc:CcP complex,[26,27] supporting the idea that the distribution of the charges on the protein surface is optimized by evolution to perform the most efficient reaction. This work challenged that assumption, testing the effect of modifying the charge distribution on the surface of CcP, the subsequent effects on the encounter complex with Cc, and consequently on the association rate and activity of the protein complex. Five CcP variants were designed, which combine the addition of new negative patches with the neutralization of the charges in the stereospecific binding site (Chapter 4). Monte Carlo simulations showed that Cc is strongly attracted to the added negative patches, particularly in absence of the native (wt) charges in the binding site,

almost completely shifting the encounter complex to the added patches (Chapters 3 and 4). PRE experiments on the complexes formed by wt CcP and the CcP variant with added negative charges on a side of the binding site (CcP_B) confirmed that Cc samples the new negative patch (Chapters 3 and 5). Although PRE experiments were not performed on the other CcP variants because of their low yields, it is reasonable to assume that they would have provided similar results. Stopped flow experiments enabled us to test how the new encounter complexes affect the association rate with Cc, showing that the addition of a new negative patch slightly enhances the k_a of the complex, no matter how far the new charges are from the stereospecific binding site (Chapters 3 and 4). Hence the new encounter complexes consist, at least in part, of productive encounters. The increase in the k_a is not very large and it is not known how many additional encounters form at the new negative patch, thus it cannot be determined what fraction is productive. The neutralization of the charges in the stereospecific binding site strongly reduces the association rate also in presence of the added negative patches (Chapter 4). Whereas the added charges contribute to productive encounters in the presence of the charges in the stereospecific binding site, they are futile in the absence of these charges. Therefore, presence of the wt charges in the binding site is the most important condition to determine if the new encounter complexes are productive or futile (Chapter 4). A second element to consider is the ionic strength at which the association rate is measured. Although the effect measured in this work is small, at low ionic strengths the electrostatic attraction to the new patch can be strong enough to hold Cc away from the binding site for longer, slowing down the association rate of the complex and its activity (Chapter 4).[12–16] In summary, this work proves that, in presence of the wt charges in the binding site of CcP, the overall charge of the protein surface is more important than the specific distribution of the charges (Chapter 3 and 4). Unfortunately, we were not able to obtain crystal structures of the CcP mutants to ascertain that the mutations did not disturb the structure of the protein. Nonetheless, all the CcP variants conserved the UV-vis spectrum of the wt CcP, correctly formed the CpdI intermediate and were able to perform the reaction with Cc. This reassures us that, although it is not possible to exclude small changes in the three-dimensional structure, these did not affect the integrity of CcP. However, it is possible that small modifications of the protein surface in proximity of the stereospecific binding site could slow down the formation of the active complex with Cc.

Changing the balance between stereospecific and encounter states. The next step in our research was to investigate how the charge distribution on the protein surface affects the association rate of the complex when the balance between stereospecific and encounter complex is heavily shifted to the latter (Chapter 5). The wt Cc:CcP complex exists as 70% stereospecific complex and 30% encounter complex.[25] It was previously suggested that the CSP provide a good indication of the dynamics in a protein complex.[28–30] In fact, the CSP are mainly caused by the stereospecific complex, in which the protein complex is mostly in a stable orientation. On the contrary, in the encounter complex the two proteins sample many different orientations, which are averaged in the CSP, resulting in small chemical shifts. The CSPs measured for the complexes Cc:CcP_A and Cc:CcP_B are almost

identical, suggesting that the added charges on CcP_B do not affect the equilibrium between stereospecific and encounter complex (Chapter 3). The mutation of the Arg13 to Ala in the binding site of Cc was shown to increase the encounter complex population from 30 % to 80 %, causing a clear reduction of the CSP.[31] Similarly, the CSP measured for the Cc R13A:CcP_B complex are significantly smaller compared to the wt complex but slightly larger than for the Cc R13A:CcP_A complex, suggesting the presence of somewhat more specific interaction (Chapter 5). PRE experiments confirmed that similar to wt Cc (Chapter 3), also Cc R13A visits the negative patch added on CcP_B and not on CcP_A, meaning that the added charges enlarged the surface sampled in the encounter state. However, in this case the effect on the association rate is dominated by the R13A mutation on Cc rather than the added patch on CcP_B. In fact, both the CcP variants tested in complex with Cc R13A present a 5-7 fold decrease in the association rate compared to wt Cc at the same ionic strength. Simulations show that the lower k_a derives from a combination of factors, a lower rate of encounter complex formation between Cc and CpdI caused by the loss of the positively charged side chain on residue 13, and the lower population of stereospecific complex (Chapter 5). The increase in the association rate in presence of CcP_B compared to CcP_A is small and only observed at ionic strengths lower that 120 mM. This study correlates the occupancy of the encounter complex population with the efficiency in the protein complex formation, showing that the delicate balance between encounter and stereospecific complex can be critical for the activity of ET complexes (Chapter 5).

Experimental considerations. Both experimental and computational data were used in this work to study the encounter complex. Although the results of the two approaches were qualitatively consistent, it was not possible to gain more precise, quantitative information. Studying the wt Cc:CcP complex, Bashir *et al.* correctly predicted the encounter complex based on only electrostatic interactions. Furthermore, combining Monte Carlo simulations and PRE data, they were able to calculate the relative populations of the stereospecific and encounter complex.[25] Unfortunately, the approach failed when applied to the Cc:CcP_B complexes (Chapter 3) since it was not possible to correctly fit the experimental data. Furthermore, despite the fact that PRE experiments confirmed the Monte Carlo simulations, showing that Cc visits the regions with the added negative charges, it is surprising to measure faster association rates than in the wt complex, regardless of where these charges are positioned (Chapter 4). In fact, the Monte Carlo simulations show such strong interactions between Cc and the new patches on CcP that a reduction in the association rate of the complex would be expected. However, the Monte Carlo simulations do not take into account the hydrophobic interactions that often have the role of stabilizing protein complexes, including the Cc:CcP complex.[21,32–39] Furthermore, the simulations assume that all the residues introduced by the mutations in the CcP are negatively charged. It should be considered that the clusters of charged residues can have considerably higher pK_a values compared to the theoretical values of amino acids, making the patch less charged then expected at pH 6. This implies that the Monte Carlo simulations could overestimate the

negative charge of the introduced patches. Despite the quantitative discrepancies in the data acquired by experimental and computational methods, the simulations remain a precious tool for the interpretation of experimental data or to build a hypothesis which can be tested by experiments.

Conclusions. This thesis shows that the encounter complex has a critical role not only for the first steps of protein complex formation but for the final activity of the complex. Furthermore, this work gives additional evidence that it is possible to improve the efficiency of the protein complex through charge mutations also far away from the stereospecific binding site (Chapters 3 and 4).[11]

All data reported here and in many other studies are acquired through *in vitro* experiments, performed in ideal, diluted solutions, under specific conditions, which can be far from the *in vivo* environment. As electrostatic interactions and the encounter complex are involved in the formation of protein complexes in living cells, experiments in crowded conditions and new approaches to perform *in vivo* studies could give an important contribution in understanding the mechanisms regulating the protein complex formation in the cell.[40] Our understanding of the encounter complex is still limited. For example, the different biological roles of the encounter complex still need to be explored. Recently, it was suggested that futile encounter complexes could have an important role in the regulation of enzyme activity by forming competitive encounter complexes.[41] Furthermore, we still cannot define the timescale of proteins dynamics within the encounter complex. At the lower end is the rotational correlation time of the free proteins (low ns), if assumed that they are not slowed down much in the encounter complex. At the high end is the lifetime of the complex (> 100 μs). It is likely that the proteins in the encounter complex have much freedom and rotate rapidly but strong electrostatic patches may keep them on certain parts of the surface of the partner for prolonged times. Also the height of the energy barrier between encounter state and stereospecific complex is generally unknown. Experimental techniques that can cover this time window to study motions in the encounter complex are few. Although its extreme dynamic nature makes it challenging to investigate, a deep understanding of how the encounter complex affects the protein complex would give an important contribution in the field of the protein-protein interactions.

References

(1) Schreiber, G., Haran, G., and Zhou, H. X. (2009) Fundamental aspects of protein-protein association kinetics. *Chem. Rev. 109*, 839–860.

(2) Ubbink, M. (2012) Dynamics in transient complexes of redox proteins. *Biochem. Soc. Trans. 40*, 415–418.

(3) Yang, J., Zeng, Y., Liu, Y., Gao, M., Liu, S., Su, Z., and Huang, Y. (2020) Electrostatic interactions in molecular recognition of intrinsically disordered proteins. *J. Biomol. Struct. Dyn.* Taylor and Francis Ltd.

(4) Zhou, H. X., and Pang, X. (2018) Electrostatic interactions in protein structure, folding, binding, and condensation. *Chem. Rev.* American Chemical Society.

(5) Clore, G. M. (2014) Interplay between conformational selection and induced fit in multidomain protein-ligand binding probed by paramagnetic relaxation enhancement. *Biophys. Chem.* Elsevier B.V.

(6) Matthew, J. B., Weber, P. C., Salemme, F. R., and Richards, F. M. (1983) Electrostatic orientation during electron transfer between flavodoxin and cytochrome *c*. *Nature 301*, 169–171.

(7) Adam, G., and Delbrück, M. (1968) Reduction of dimensionality in biological diffusion processes, in *Structural Chemistry and Molecular Biology* (A. Rich, N. D., Ed.), p 198. W. H. Freeman and Co., San Francisco.

(8) Harel, M., Cohen, M., and Schreiber, G. (2007) On the dynamic nature of the transition state for protein-protein association as determined by double-mutant cycle analysis and simulation. *J. Mol. Biol. 371*, 180–196.

(9) Worrall, J. A. R., Reinle, W., Bernhardt, R., and Ubbink, M. (2003) Transient protein interactions studied by NMR spectroscopy: The case of cytochrome *c* and adrenodoxin. *Biochemistry 42*, 7068–7076.

(10) Andrałojć, W., Hiruma, Y., Liu, W. M., Ravera, E., Nojiri, M., Parigi, G., Luchinat, C., and Ubbink, M. (2017) Identification of productive and futile encounters in an electron transfer protein complex. *Proc. Natl. Acad. Sci. U. S. A. 114*, E1840–E1847.

(11) An, S. Y., Kim, E.-H., and Suh, J.-Y. (2018) Facilitated protein association via engineered target search pathways visualized by paramagnetic NMR spectroscopy. *Structure 26*, 887-893.e2.

(12) Meyer, T. E., Zhao, Z. G., Cusanovich, M. A., and Tollin, G. (1993) Transient kinetics of electron transfer from a variety of *c*-type cytochromes to plastocyanin. *Biochemistry 32*, 4552–4559.

(13) Watkins, J. A., Cusanovich, M. A., Meyer, T. E., and Tollin, G. (1994) A “parallel plate” electrostatic model for bimolecular rate constants applied to electron transfer proteins. *Protein Sci. 3*, 2104–2114.

(14) Suh, J. Y., Tang, C., and Clore, G. M. (2007) Role of electrostatic interactions in transient encounter complexes in protein-protein association investigated by paramagnetic relaxation enhancement. *J. Am. Chem. Soc. 129*, 12954–12955.

(15) Hazzard, J. T., McLendon, G., Cusanovich, M. A., and Tollin, G. (1988) Formation of electrostatically-stabilized complex at low ionic strength inhibits interprotein electron transfer between yeast cytochrome *c* and cytochrome *c* peroxidase. *Biochem. Biophys. Res. Commun. 151*, 429–434.

(16) Bernal-Bayard, P., Molina-Heredia, F. P., Hervás, M., and Navarro, J. A. (2013) Photosystem i reduction in diatoms: As complex as the green lineage systems but less

efficient. *Biochemistry 52*, 8687–8695.

(17) McLendon, G. (1991) Control of biological electron transport via molecular recognition and binding: The "velcro" model, in *Long-Range Electron Transfer in Biology*, p 159. Springer Berlin Heidelberg, Berlin, Heidelberg.

(18) Harel, M., Spaar, A., and Schreiber, G. (2009) Fruitful and futile encounters along the association reaction between proteins. *Biophys. J. 96*, 4237–4248.

(19) Fawzi, N. L., Doucleff, M., Suh, J. Y., and Clore, G. M. (2010) Mechanistic details of a protein-protein association pathway revealed by paramagnetic relaxation enhancement titration measurements. *Proc. Natl. Acad. Sci. U. S. A. 107*, 1379–1384.

(20) Ubbink, M. (2009) The courtship of proteins: Understanding the encounter complex. *Febs Lett. 583*, 1060–1066.

(21) Gerlach, G. J., Carrock, R., Stix, R., Stollar, E. J., and Aurelia Ball, K. (2020) A disordered encounter complex is central to the yeast Abp1p SH3 domain binding pathway. *PLoS Comput. Biol. 16*.

(22) Clore, G. M., and Iwahara, J. (2009) Theory, practice, and applications of paramagnetic relaxation enhancement for the characterization of transient low-population states of biological macromolecules and their complexes. *Chem. Rev. 109*, 4108–4139.

(23) Jeschke, G. (2013) Conformational dynamics and distribution of nitroxide spin labels. *Prog. Nucl. Magn. Reson. Spectrosc.*

(24) Keizers, P. H. J., Desreux, J. F., Overhand, M., and Ubbink, M. (2007) Increased paramagnetic effect of a lanthanide protein probe by two-point attachment. *J. Am. Chem. Soc. 129*, 9292–9293.

(25) Bashir, Q., Volkov, A. N., Ullmann, G. M., and Ubbink, M. (2010) Visualization of the encounter ensemble of the transient electron transfer complex of cytochrome *c* and cytochrome *c* peroxidase. *J. Am. Chem. Soc. 132*, 241–247.

(26) Gabdoulline, R. R., and Wade, R. C. (2001) Protein-protein association: investigation of factors influencing association rates by Brownian dynamics simulations. *J. Mol. Biol. 306*, 1139–1155.

(27) Northrup, S. H., Boles, J. O., and Reynolds, J. C. (1988) Brownian dynamics of cytochrome *c* and cytochrome *c* peroxidase association. *Science (80-.). 241*, 67–70.

(28) Prudêncio, M., and Ubbink, M. (2004) Transient complexes of redox proteins: structural and dynamic details from NMR studies. *J. Mol. Recognit. 17*, 524–539.

(29) Hom, K., Ma, Q. F., Wolfe, G., Zhang, H., Storch, E. M., Daggett, V., Basus, V. J., and Waskell, L. (2000) NMR studies of the association of cytochrome b5 with cytochrome *c*. *Biochemistry 39*, 14025–14039.

(30) Shao, W., Im, S. C., Zuiderweg, E. R. P., and Waskell, L. (2003) Mapping the binding

interface of the cytochrome *b*5-cytochrome *c* complex by nuclear magnetic resonance. *Biochemistry 42*, 14774–14784.

(31) Volkov, A. N., Bashir, O., Worrall, J. A. R., and Ubbink, M. (2009) Binding hot spot in the weak protein complex of physiological redox partners yeast cytochrome *c* and cytochrome *c* peroxidase. *J. Mol. Biol. 385*, 1003–1013.

(32) Kim, Y. C., Tang, C., Clore, G. M., and Hummer, G. (2008) Replica exchange simulations of transient encounter complexes in protein-protein association. *Proc. Natl. Acad. Sci. U. S. A. 105*, 12855–12860.

(33) Van de Water, K., van Nuland, N. A. J., and Volkov, A. N. (2014) Transient protein encounters characterized by paramagnetic NMR. *Chem. Sci. 5*, 4227–4236.

(34) Scanu, S., Foerster, J. M., Ullmann, G. M., and Ubbink, M. (2013) Role of hydrophobic interactions in the encounter complex formation of the plastocyanin and cytochrome *f* complex revealed by paramagnetic NMR spectroscopy. *J. Am. Chem. Soc. 135*, 7681–7692.

(35) Sugase, K., Dyson, H. J., and Wright, P. E. (2007) Mechanism of coupled folding and binding of an intrinsically disordered protein. *Nature 447*, 1021-U11.

(36) Camacho, C. J., Weng, Z., Vajda, S., and DeLisi, C. (1999) Free energy landscapes of encounter complexes in protein-protein association. *Biophys. J. 76*, 1166–1178.

(37) Camacho, C. J., Kimura, S. R., DeLisi, C., and Vajda, S. (2000) Kinetics of desolvation-mediated protein-protein binding. *Biophys. J. 78*, 1094–1105.

(38) Camacho, C. J., and Vajda, S. (2002) Protein-protein association kinetics and protein docking. *Curr. Opin. Struct. Biol. 12*, 36–40.

(39) Rajamani, D., Thiel, S., Vajda, S., and Camacho, C. J. (2004) Anchor residues in protein-protein interactions. *Proc. Natl. Acad. Sci. U. S. A. 101*, 11287–11292.

(40) Phillip, Y., and Schreiber, G. (2013) Formation of protein complexes in crowded environments-From in vitro to in vivo. *FEBS Lett. 587*, 1046–1052.

(41) Kale, S., Strickland, M., Peterkofsky, A., Liu, J., and Tjandra, N. (2019) Model of a kinetically driven crosstalk between paralogous protein encounter complexes. *Biophys. J. 117*, 1655–1665.