Dynamics in photosynthetic transient complexes studied by paramagnetic NMR spectroscopy

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Chapter V

Concluding remarks

Many biological processes involve a balanced and highly organized network of specific proteins, which communicate with each other via physical interactions. Understanding the nature of these interactions is, therefore, a matter of primary importance. Cyt *f*-Pc complex is stabilized in its final orientation by both electrostatic and hydrophobic interactions. The aim of this thesis was to visualize the encounter state of Cyt *f*-Pc complex in order to understand the finely balanced hydrophobic and electrostatic interactions involved in the process of protein complex formation.

Encounter complex

The encounter complex model was initially introduced for small-molecules reactions[176,177] before being applied to macromolecular association theories.[178,179] In the diffusion-limited regime of protein association, the encounter complex can be defined as the intermediate formed after diffusion and subsequent interaction of the free proteins, which can potentially evolve to the final complex.[22] The formation of the encounter complex has long been considered to be mediated by long-range electrostatics. These interactions preserve the surface solvation of the individual proteins.[21] The model exhaustively describes complexes with electrostatic-assisted association and many examples have been reported.[23,28,30,171] The association between proteins with low charge complementarity could be theoretically described as driven by desolvation alone.[170] The addition of hydrophobic and electrostatic intercations to this model[71] indicated that the mechanism of association strictly depends on the surface properties of the specific proteins forming the complex. Our studies on the encounter state of *N-N*complex (Chapter III) and *N-Ph*complex (Chapter IV) provide novel experimental evidence that hydrophobic interactions participate to the formation and stabilization of the encounter complex as well as electrostatic interactions. In fact, the charge distribution on Pc results in the formation of a dipole that promotes the electrostatic pre-orientation of Pc with the hydrophobic patch towards the negatively charged Cyt *f*, allowing for the contact with the non-polar surfaces of Cyt *f* already in the encounter complex. The encounter and final state have similar energies and, therefore, the distinction between the encounter and the final complex seems to vanish. This new model for protein complex formation thus proposes that the encounter complex proceeds via a smooth and gradual transition to the final complex.

ET protein complexes

The inter-exchange between the two energetic states is highly consistent with the theory for which ET complexes exist in multiple active orientations.[20] In this regard, high dynamics were observed in several ET complexes.[28,30,72] The ET mainly depends on the distance between redox centers.[178] Once the redox centers reach a favorable distance from each other, ET can occur even between non-physiological redox partners[166] or between mutants of the physiological

partners.[7,8] The presence of multiple orientations seems to compensate for the low specificity by providing the necessary balance between association and dissociation required for efficient turnover in ET systems.[4] It is tempting to correlate the relative populations of encounter and final states to the biological function of the complex. In highly dynamic systems, such as ET complexes, the encounter state can be significantly populated[28] (Chapter III and IV) or the complex can even exist in a pure encounter state.[30] Instead, more specific systems, such as complexes involved in gene expression regulation,[23,141] seem to be also more static, having a greater tendency to prominently exist in a single, specific orientation, as required by the strict regulation to which these processes are subject.

Diamagnetic chemical shift perturbations analysis

NMR chemical shift perturbations (CSP) represent an extremely informative tool to study protein-protein interactions, especially for weak and dynamic complexes, such as that of Pc and Cyt *f*. In a typical experiment, the HSQC spectrum of ^{15}N-labeled Pc is monitored and the perturbations of the chemical shifts were recorded while increasing concentration of unlabeled Cyt *f*. The interaction with Cyt *f* causes changes on the surface of Pc, which affect the chemical shifts of the amide nuclei in the area involved in the complex formation, providing a residue-resolution map of the binding interface (Chapter IV). Titrations of Cyt *f* into Pc also provide a convenient way to establish the affinity and specificity of binding. From the CSP analysis it also was possible to establish the interference of spin labels on the *N-N*complex formation (Chapter II and III).

The degree of variability observed in the size of the chemical shifts among different complexes demonstrates to be a qualitative indication for intra-complex dynamics in electrostatic complexes.[15,31,32,72,73] A complex existing predominantly in a single orientation will yield large binding shifts as a result of desolvation and formation of specific, short-range interactions. In highly dynamic complexes, a significant fraction of the total population is present in the encounter state, consisting of multiple orientations that reduce the binding shifts by averaging.[9] Thus, the decrease of the average size of CSP in the *N-Ph*complex compared to those of *N-N*complex, supported the higher degree of dynamics suggested by PRE experiments and docking simulations (Chapter IV). This work has provided an interesting new observation, namely that the absolute CSP size observed in purely electrostatic complexes[30,31,72,73] is much smaller than that observed for *N-N*complex and *N-Ph*complex, which have shown to be dominated by hydrophobic as well as electrostatic interactions. The presence of hydrophobic interactions in the encounter complex implies the removal of water molecules that results in a big binding shift. The decrease of the CSP caused by dynamics is thus partially compensated by the effect of the desolvation of the encounter binding surfaces.

Paramagnetic NMR

The presence of a paramagnetic center in a protein can be a valuable source of structural information by affecting the chemical shifts and the nuclear relaxation rates of the observed nucleus in a distance-dependent manner.[180] The possibility to introduce a paramagnetic centre via site-directed labeling has extended the application of paramagnetic NMR vastly. The pseudocontact shifts caused by the intrinsic paramagnetic haem iron of ^{N}Cyt *f*, provides sufficient restraints for the determination of an ensemble of orientations of ^{Ph}Pc in the final $^{N\text{-}Ph}$complex (Chapter IV). The size of PCS is strongly related to the distance between the observed nucleus and the iron and depends also on the magnetic susceptibility anisotropy tensor. As a result, the presence of multiple orientations in a complex will reduce the average PCS, yielding a qualitative correlation between the PCS size and the degree of dynamics within the complex.[32] Indeed, the in $^{N\text{-}Ph}$complex the haem induces smaller PCS than in the $^{N\text{-}N}$complex, similarly to what was observed for the CSPs. Both reflect the larger dynamics within the cross complex (Chapter IV).
Paramagnetic relaxation enhancement is a well-established method for classical structural determination studies.[181] The sensitivity of PRE to lowly populated states makes it a versatile technique to investigate dynamic processes involved in complex formation.[96] The attachment of spin labels at several locations on the Cyt *f* surface allowed for the detection of the diffusive encounter states of $^{N\text{-}N}$complex and $^{N\text{-}Ph}$complex to be characterized (Chapter III and IV, respectively). The PRE patterns clearly indicated that both ^{N}Pc and ^{Ph}Pc are pre-oriented towards ^{N}Cyt *f* during the search of the specific binding site. The accurate maps of the binding interface of the encounter complexes obtained from PREs provided residue-resolution information on the region directly explored by the encounter complex.
To characterize the entire complex, both encounter and final states, a single method is inadequate. PRE and PCS provide complementary views that together help to obtain a more accurate picture of the complex.

Computational methods to study protein interactions

The field of structural biology is receiving immense benefits from the parallel development of computational methodologies to predict protein docking. In a nutshell, these methods use the coordinates of the unbound proteins to obtain computationally a model of the bound complex on the basis of either experimental data[182] or theoretical assumptions.[183-185]
Structural information, as gained by X-ray crystallography or NMR spectroscopy, can be explicitly considered and treated as active and driving force for the docking.[154,186] Recent advances in the use of paramagnetic NMR data[174] allow new data-driven docking techniques to determine the solution structures, even of large molecules and complexes[187,188] and transient protein-protein complexes.[26] NMR restraint-guided docking has been used to determine the final orientation of $^{N\text{-}N}$complex (Chapter II) and $^{N\text{-}Ph}$complex (Chapter IV) on the basis of observed

PREs and PCSs, respectively. Because many protein complexes exist and function as a dynamic ensemble, there is a growing need to be able to model such ensembles even though the interpretation of experimental data is not straightforward. The challenge of visualizing a dynamic encounter complex on the basis of experimental PREs was elegantly addressed by Prof. Clore and coworkers by representing one of the interaction partner as an ensemble of conformers, which is docked simultaneously to the single copy of the other protein.[141] This approach was thus used for the visualization of the encounter state on the basis of the experimental PREs both for the $^{N\text{-}N}$complex and $^{N\text{-}Ph}$complex (Chapter III and IV, respectively). In both encounter complexes, Pc was found in contact with the non-polar surfaces of Cyt *f*, strongly suggesting that hydrophobic interactions indeed contribute to the encounter complex. The encounter complexes thus produced using PRE driven ensemble docking were similar for location and distribution. The encounter of the $^{N\text{-}Ph}$complex resulted to have a more diffuse nature, reflecting the higher degree of dynamics with respect to the $^{N\text{-}N}$complex. Because the observed PRE is a population weighted average of all species present in solution,[84] many possible docking solutions can correspond to the observed PREs, limitating thus the accuracy of the method to furnish a high-resolution picture of the encounter complex.

Softwares for theoretical prediction of protein complex structure and association are mainly based on shape complementarity,[189] electrostatics[57,109] and solvation terms.[190] The primary methods for the computational study of protein association, such as BD and MC, simulate the complex formation on the basis of the electrostatic properties of the individual proteins. On the assumption that the electrostatic forces dominate the encounter complex formation, MC simulations were used to obtain a structural description of the encounter complex of $^{N\text{-}N}$complex (Chapter III) and $^{N\text{-}Ph}$complex (Chapter IV). MC approach did not produce encounter complexes in agreement with the experimental PREs, indicating that electrostatic interactions are not dominant in these Cyt *f*-Pc complexes. Still, MC simulations also provided evidence for the electrostatic pre-orientation of Pc in both complexes, in qualitative agreement with CSP and PRE interaction maps. Thus, the MC approach was confirmed to be a powerful tool to evaluate the contribution of electrostatic forces in complex formation.