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**Title:** Making the invisible visible: paramagnetic NMR and the transient protein complex
**Issue Date:** 2015-11-24
Combining multiple types of paramagnetic NMR data to study the cytochrome c – cytochrome c peroxidase encounter complex
ABSTRACT

The interaction between Cc and CcP is transient and highly dynamic making this complex an ideal candidate for studying the encounter complex. Previous pNMR studies demonstrated that the orientation of the complex found in the crystal structure, the stereo-specific state, accounted for only 70% of the lifetime of the complex in solution and the remainder was spent in a loosely associated encounter state. However, this model has previously only been fit to PRE data which, although exquisitely sensitive to minor states, is not ideal for determining the relative orientations of proteins within a complex. Here we use a CLaNP-5 probe coordinated to various Ln³⁺ ions to generate PRE as well as PCS and RDC, which do provide orientation information. Unfortunately, we find a poor fit between the theoretical and experimental data due to disruption of complex formation caused by steric and electrostatic repulsion between Cc and CLaNP-5. For two of the mutants, this likely results in a larger population in the encounter state as well as a larger spread in the number of Cc orientations than is accounted for in the existing model. Nevertheless, developing a model of the encounter complex using all three types of paramagnetic data remains an important goal for understanding how the complex behaves in solution and, therefore, this work should be repeated with CLaNP-7 or other less charged probes.
INTRODUCTION

The traditional view of protein complex formation as a simple two-step process has become outdated with the discovery in recent decades of the encounter complex. The encounter complex is a highly dynamic transition state along the binding trajectory in which the proteins pre-orient themselves for stereo-specific complex formation.\(^3\) The formation of the encounter complex is driven by long-range interactions such as electrostatic and desolvation forces.\(^4\) As the proteins sample each-others surfaces, the number of contacts between the two increases until optimal binding geometry is reached allowing short range interactions to form, such as vdW, hydrogen-bonding, hydrophobic contacts and specific salt bridges, and the complex proceeds to the tightly bound, stereo-specific state.\(^5\) ET complexes often make use of this intermediate, loosely associated encounter state to counterbalance their requirement for a high turnover rate while at the same time achieving a specific enough interaction to allow ET to occur.\(^5\)

The Cc-CcP complex is a well-studied model complex for ET. In the cell, Cc transfers two reducing equivalents to CcP, which are then used to catalyse the reduction of hydrogen peroxide to water.\(^135\) The crystal structure of this complex has been available since 1992 and shows the relative orientation of Cc on CcP in the stereo-specific state.\(^169\) In 2006, Volkov et al. attached MTSL to the surface of CcP and observed the resulting PRE effects in the NMR spectra of Cc. The orientation of the complex in solution was found to be close to that of the crystal structure but the stereo-specific state only accounted for 70% of the lifetime of the complex, with the remaining 30% spent in an encounter state.\(^1\) This was confirmed in a more extensive paramagnetic (pNMR) study by Bashir et al., in 2010.\(^8\) Later it was also found that the population in the encounter state could be shifted to as low as 10% or as high as 80% with targeted point mutations in the binding interface.\(^9\)

In 2014, Schilder et al., observed the complex from the other side by attaching MTSL on the surface of Cc and observed the resulting PRE effects in the NMR spectra of CcP (Chapter 4).\(^140\) The new data fit the previous 30% encounter/70% stereo-specific (30:70) model\(^1, 8\) well for most CcP residues but some discrepancies were found. This highlighted the importance of obtaining a comprehensive data set when working with pNMR data.\(^140\) All pNMR effects (PRE, RDC, PCS) represent an average over all the conformations present in the sample when there is fast interconversion between them on their respective NMR timescales. This makes visualization of the complex an ill-posed inverse problem,\(^273, 274\) in which many ensembles of solutions can be found to match the observed data.\(^3, 8, 10, 11, 13, 14, 25, 34-36\)
The only way to exclude false solutions is by obtaining additional data sets. This can be done using tags at different locations, using different types of paramagnetic centres or using different types of paramagnetic effects.\(^{(32, 33, 275-277)}\)

In 2014, Volkov et al. extended the available PRE data set by using EDTA-Mn\(^{2+}\) tags attached on the surface of Cc and observing the PRE effects in the NMR spectra of CcP while altering the oxidation state of the two proteins.\(^{(204)}\) When using RS CcP in the presence of ferric Cc (Cc\(_{\text{ox}}\)), the same as was done in previous studies, they found that data fit well to the 30:70 model.\(^{(1, 8)}\) Furthermore, they also measured PCS in the CcP spectra generated by the LS Fe\(^{3+}\) atom in ferrous Cc (Cc\(_{\text{red}}\)). Interestingly, for CcP(CN\(^{-}\))-Cc\(_{\text{red}}\), they found a slightly better match between the experimental and back-predicted PRE and PCS data when the population of the encounter state was increased to 40\%. This was likely due to subtle differences in the binding of Cc\(_{\text{red}}\) or Cc\(_{\text{ox}}\) as overall little difference was found between CcP(RS)-Cc\(_{\text{ox}}\) and CcP(CN\(^{-}\))-Cc\(_{\text{red}}\) complexes.\(^{(204)}\) Nevertheless, this demonstrates that additional information can be obtained by combining PCS and PRE data.

Although PRE are extremely sensitive to minor states,\(^{(59)}\) they are not ideal for determining relative orientations within a protein complex (Chapter 5).\(^{(321)}\) Much better results can be obtained if PRE (distance information) are combined with PCS (distance and orientation information) and RDC (orientation information).\(^{(14, 31, 80, 104-108, 299-301)}\) Furthermore, the quality of pNMR results are also highly dependent on tag mobility as unwanted movement of the paramagnetic centre can lead to excess signal averaging and therefore an underestimation of the true signal.\(^{(325)}\) This can be limited by using the highly immobile, double-armed CLaNP-5-Ln\(^{3+}\).\(^{(85, 102, 326)}\) These probes will chelate any Ln\(^{3+}\) ion allowing for the fine-tuning of desired paramagnetic effects. For PCS and RDC, lanthanoids with anisotropic \(\Delta \chi\)-tensors such as holmium (Ho\(^{3+}\)) or thulium (Tm\(^{3+}\)) can be used, which produce measurable effects within approximately 10-40 Å\(^{(72)}\) and 15-70 Å\(^{(301, 327)}\) respectively. For PRE, an isotropic \(\Delta \chi\)-tensor is required as is the case for gadolinium (Gd\(^{3+}\)) which can produce measurable effects within approximately 20-45 Å.\(^{(301)}\) In combination, these ranges will allow for complete coverage of the entire Cc-CcP complex. Finally, as a diamagnetic control, lutetium (Lu\(^{3+}\)) is available.

In this study, we attach CLaNP-5-Ln\(^{3+}\) probes at four positions on the surface of CcP. First, we measured the CSP and determine the binding coefficients for the tagged protein. Then, using intramolecular PCS data, we determine the \(\Delta \chi\)-tensors and the positions of the paramagnetic centres. With this information, we determine the intermolecular PCS, RDC and PRE effects which are then compared to the existing 30:70 model of the complex that has
previously been fit to PRE data.\textsuperscript{(1, 8)} Unfortunately, we find that CLaNP-5 disrupts complex formation to some degree for all mutants and also appears to increase the population of the encounter complex for two of the mutants. This results in a poor fit between the experimental data and the theoretical models since the 30:70 model likely underestimates the spread of the Cc orientations sampled by this increased encounter state population. The strong positive charge of the probe is likely a contributing factor to this effect and therefore CLaNP-5 is not ideal for studying proteins whose interaction is driven predominately by electrostatics.\textsuperscript{(169, 328)} Future studies should be done with CLaNP-7 or other less charged probes.\textsuperscript{(329)}

**MATERIALS & METHODS**

**PROTEIN PURIFICATION**

The pET28aCcP plasmid\textsuperscript{(1)} was modified to contain double cysteine mutations at positions 24/28, 87/90, 212/214 and 249/253 using the Q5® Site-Directed Mutagenesis Kit (New England Biolabs) by Anneloes Blok. The CcP mutants were expressed and purified as published previously (Chapter 3).\textsuperscript{(139, 140, 142)} A pUC19 based plasmid containing the \textit{S. cerevisiae} iso-1-cytochrome \( c \) gene was used to express and purify Cc as described previously.\textsuperscript{(124, 125)}

**Ln\(^{3+}\)-CLaNP-5 ATTACHMENT**

The CLaNP-5-ln\(^{3+}\) tag containing either Lu\(^{3+}\), Ho\(^{3+}\), Tm\(^{3+}\) or Gd\(^{3+}\) as the Ln\(^{3+}\) was prepared as described previously.\textsuperscript{(102)} Approximately 20 mg of a double-cysteine CcP mutant was incubated for 1 hr at room temperature with 5 mM DTT in NMR buffer (20 mM NaPi, 100 mM NaCl) at pH 7. Meanwhile, 15 mL of NMR buffer was de-gassed and then bubbled with argon for 1 hr. The DTT was removed from the protein solution using a PD-10 column; as the protein eluted from the column, it was dripped into the degassed NMR buffer containing 5 molar equivalents of Ln\(^{3+}\)-CLaNP-5. The sample was incubated at 4 °C for at least 1 hr. Unlabeled protein, protein oligomers and surplus of Ln\(^{3+}\)-CLaNP-5 were removed using a gel filtration column (Superdex 75 10/300 GL, GE Healthcare). The tagging efficiency was determined to be \( \sim 90\% \) by comparing the intensities of diamagnetic residual peaks with PCS shifted peaks in the NMR spectra of CLaNP-5-Ho\(^{3+}\) and CLaNP-5-Tm\(^{3+}\) CcP.
Combining multiple types of paramagnetic NMR data to study the Cc-CcP encounter complex

NMR SPECTROSCOPY

NMR TITRATIONS

The binding constants were obtained for each CLaNP-5-Lu\(^{3+}\) tagged CcP double cysteine mutant by titrating a 5.2 mM stock of WT Cc into 400 uM \(^{15}\)N labelled CcP as described previously (Chapter 4).\(^{140}\) The average CSP (\(\Delta \delta_{\text{avg}}\)) were also derived and extrapolated to 100% bound as described previously.\(^{280}\)

PARAMAGNETIC NMR

The intramolecular paramagnetic effects for CLaNP-5-Lu\(^{3+}\), CLaNP-5-Ho\(^{3+}\) and CLaNP-5-Tm\(^{3+}\) tagged \(^{15}\)N-CcP double cysteine mutants were recorded using 2D BEST-TROSY-HSQC experiments\(^{279}\) on a Bruker AVIII HD spectrometer equipped with a \(^1\)H[\(^{13}\)C/\(^{15}\)N] TCI-cryoprobe operating at a Larmor frequency of 850 MHz at 293 K with 1024 and 100 complex points in the \(^1\)H and \(^{15}\)N dimensions respectively. The NMR buffer contained 20 mM NaPi, 100 mM NaCl, 6% \(D_2O\), pH 6.0. NMR spectra for mutant 87/90C were also recorded at 303 K for comparison. The intermolecular paramagnetic effects for CLaNP-5-Lu\(^{3+}\), CLaNP-5-Ho\(^{3+}\), CLaNP-5-Tm\(^{3+}\) and CLaNP-5-Gd\(^{3+}\) tagged CcP double cysteine mutants on \(^{15}\)N Cc WT were recorded using 2D HSQC\(^{330}\) experiments on a Bruker AVIII HD spectrometer equipped with a \(^1\)H[\(^{13}\)C/\(^{15}\)N] TCI-cryoprobe operating at a Larmor frequency of 850 MHz at 303 K with 1024 and 128 complex points in the \(^1\)H and \(^{15}\)N dimensions respectively. The intermolecular RDC was measured using IPAP-HSQC\(^{331}\) experiments on the same spectrometer with 1024 and 128 complex points in the \(^1\)H and \(^{15}\)N dimensions respectively. The NMR samples contained 300 \(\mu\)M of each protein in 20 mM NaPi, 100 mM NaCl, 6% \(D_2O\), pH 6.0. All data were processed using Topspin 3.2 (Bruker, Karlsruhe, Germany) and analysis was done using CCPN analysis 2.1.5.

DATA ANALYSIS

PCS AND RDC ANALYSIS

Using the intramolecular PCS data, the \(\Delta \chi\)-tensors for CLaNP-5-Ho\(^{3+}\)/Tm\(^{3+}\) tagged CcP were determined in Numbat\(^{96}\) using the measured PCS values and the structure of CcP from the crystal structure of the complex (PDB entry 2PCC)\(^{169}\) to which HN atoms were added using Xplor-NIH.\(^{282, 283}\) For the intermolecular PCS data, the measured PCS values were extrapolated to 100% bound Cc and the position of the Ln\(^{3+}\) ions were fixed to the
coordinates determined during the intramolecular $\Delta\chi$-tensor calculations. The intermolecular $\Delta\chi$-tensors were determined in Numbat\(^{(96)}\) using the extrapolated PCS values and the structure of Cc from the crystal structure of the complex (PDB entry 2PCC).\(^{(169)}\) The Q-values for the intramolecular PCS were calculated according to equation 5.2 as described previously (Chapter 5).\(^{(8, 321)}\) The RDCs were measured directly from the IPAP-HSQC spectra (in ppm) and were first converted to Hz and then extrapolated to 100% bound Cc. An error of $\pm 6$ Hz was extrapolated from the error in peak peaking. The theoretical values were predicted by Numbat\(^{(96)}\) based on the PCS based intermolecular the $\Delta\chi$-tensors.

Since no data were recorded for CLaNP-5-Ho\(^{3+}\) at positions 24/28 and 249/253, the $\Delta\chi_{ax}$ and $\Delta\chi_{th}$ values were estimated based on the ratios between these values for Ho\(^{3+}\) and Tm\(^{3+}\) at positions 87/90 and 212/214. The Ho\(^{3+}\) and Tm\(^{3+}\)$\Delta\chi$-tensors were then used to calculate the theoretical paramagnetic effects (PCS and RDC) and the distances between the amide protons of Cc and the Ln\(^{3+}\) atom for the stereo-specific complex. The same was done for each of the 1701 orientations of Cc observed in the MC ensemble used to describe the encounter complex before.\(^{(8)}\) Orientations in the ensemble in which the lanthanoid approached any of the Cc Cα atom to less than 6 Å were excluded, because they were taken to represent a penetration of Cc by the CLaNP-5-Lu\(^{3+}\). For mutants 24/28, 87/90, 212/214 and 249/253 this resulted in 2, 0, 13 and 3 exclusions out of 1701 orientations, respectively. To model the 30% encounter/70% stereo-specific complex, the back-calculated values for each were combined; $r^{-6}$ averaging was used for combining the distances. Molecular manipulations and distance measurements were done using XPLOR-NIH.\(^{(282, 283)}\)

**PRE ANALYSIS**

The intensity ratio of the amide resonances in the spectra of the paramagnetic (Gd\(^{3+}\)) and diamagnetic (Lu\(^{3+}\)) samples ($I_{para}/I_{dia}$) was measured and normalized as described previously.\(^{(8)}\) The paramagnetic contribution to the transverse relaxation rate, $R_{2,para}$, was calculated as described previously (Chapter 4).\(^{(8, 77, 140)}\) The average $R_{2,dia}$ value was used with a large error margin for those amides for which an $I_{para}/I_{dia}$ could be measured but for which the line width of the diamagnetic peak could not be obtained. For the amide peaks that disappeared in the paramagnetic spectrum, an upper limit for $I_{para}$ was set to two standard deviations of the noise level of the spectrum.\(^{(140)}\) The calculated $R_{2,para}$ values were then converted into distances as described previously,\(^{(8)}\) see equation 4.1, with a Cc fraction bound of 0.784, 0.727, 0.735 or 0.765 for positions 24/28, 87/90, 21/214 and 249/253, respectively. The value of $\tau_c$ for the complex was previously estimated to be 16 ns.\(^{(1, 8)}\)
RESULTS & DISCUSSION

EFFECTS OF PROBE ATTACHMENT

Four double cysteine mutations were generated on the surface of CcP in a large ring around the stereo-specific binding site: N24C/L28C, N87C/K90C, K212C/E214C and K249C/N253C. By attaching CLaNP-5-Ln3+ probes at these positions, the entire complex could be observed using pNMR (Figure 7.1). Previous pNMR studies observed strong PRE effects on CcP when SLs were attached at positions 38C, 200C and 288C. Two of these double cysteine mutations are very close to the attachment sites in previous studies; 24/28 is close to 288C and 249/253 is close to 200C, while 38C falls roughly between 24/28 and 87/90 (Figure 7.1A).

The CLaNP-5-Ln3+ probe is relatively large and can therefore potentially cause steric interference during complex formation. Furthermore, the net charge of attached probe is +3 which is not ideal for a complex that is itself highly charged (CcP is negative and Cc is positive) and relies mainly on electrostatics to drive the interaction. Therefore, WT Cc was titrated into a solution of CLaNP-5-Lu3+ tagged 15N-CcP and the CSP caused by binding were measured. Several resonances shifted in the spectra indicating a fast exchange binding process, as is expected for the Cc-CcP complex. The binding constants \( K_B \) were determined by fitting the CSP curves for several amide resonances to a 1:1 binding model as described previously (Chapter 4)\(^{(140)}\) (Figure 7.2). For the WT complex, the \( K_B \) is \( 2 \times 10^5 \) M\(^{-1}\)\(^{(60, 140, 183)}\). The calculated \( K_B \) values for CLaNP-5-Ln3+-CcP range from \( 1.0 \times 10^5 \) M\(^{-1}\) for position 24/28 to \( 0.5 \times 10^5 \) M\(^{-1}\) for position 87/90 indicating that the presence of the probe is interfering somewhat with complex formation either through steric interferences or electrostatic repulsion. This difference in binding is taken into account during data analysis since all pNMR effects are extrapolated to 100% bound. The range in the magnitude of \( \Delta \delta_{\text{avg}} \) is indicative of the degree of dynamics in the complex and therefore the relative amount of the complex in the encounter state\(^{(9, 15, 27, 332)}\). The \( \Delta \delta_{\text{avg}} \) ranges from approximately -0.75 to 0.62 for position 24/28. For positions 212/214 and 249/253 the \( \Delta \delta_{\text{avg}} \) ranges from approximately -0.75 to 0.50 indicating perhaps a slight increase in the encounter state population but the strongest effect is seen for position 87/90 with a range from only -0.29 to 0.39. Therefore, it is possible that the populations of stereo-specific and encounter state may be shifted and this will need to be taken into consideration when comparing the experimental data to the 30:70 model.\(^{(3, 8)}\)
Chapter 7

**FIGURE 7.1** A) Experimentally determined locations of Ln\(^{3+}\) ion (green sphere) within the CLaNP-5 probe attached to double cysteine mutations at positions 24/28, 87/90, 212/214 and 249/253 on the surface of CcP (grey; C-terminus in light blue; N-terminus on the back-side) with respect to the binding site of Cc (semi-transparent pink cartoon) (PDB-entry 2PCC).\(^{(169)}\) B) Molecular structure of CLaNP-5 with a coordinated lanthanoid ion.\(^{(102)}\) C) 3D model of CLaNP-5 attached via two sulphur atoms (yellow) and containing a coordinated lanthanoid ion (green sphere).\(^{(102)}\)

**FIGURE 7.2** Chemical shift perturbations for selected CcP residues in the \(^1\)H or \(^{15}\)N dimension during titration with WT Cc. The titrations were done for CLaNP-5-Lu\(^{3+}\) CcP attached at positions 24/28 (A), 87/90 (B), 212/214 (C) or 249/253 (D). The curves were fitted globally to a 1:1 binding model and the solid lines show the best fit when using a shared \(K_B\) value. These experiments were done in 20 mM NaPi, 100 mM NaCl (pH 6.0) at 293 K.
Combining multiple types of paramagnetic NMR data to study the Cc-CeP encounter complex

FIGURE 7.3 Chemical shift perturbation map for WT Cc with $^{15}$N-CcP C128A$^{140}$ (A) or with CLaNP-5-La$^{3+}$ attached at positions 24/28 (B), 87/90 (C), 212/214 (D) or 249/253 (E) with the lanthanoid ion shown as a green sphere. The CSP are colour coded on a surface model of CeP in the stereo-specific complex (PDB-entry 2PCC). Cc is shown in cyan ribbon with the haem group in red sticks. CSP were extrapolated to 100% bound CeP. Residues with $\Delta\delta_{av}$ ≥ 0.06 ppm are red, 0.04-0.06 ppm are orange, 0.02-0.04 ppm are yellow, 0-0.02 ppm are blue and with no data are grey.
Using the calculated $K_b$, the average amide shifts, $\Delta \delta_{ave}$, were extrapolated to 100% bound CcP and mapped onto the surface of CcP (Figure 7.3). Despite the differences in the binding constants for CLaNP-5-Lu$^{3+}$-CcP in comparison to WT, relatively minor differences are observed in the CSP maps. The strongest CSP are located at the stereo-specific binding interface for all double mutants as expected and only a few extra weak effects are observed toward the back of CcP. This effect is strongest for 87/90, which has the lowest $K_b$ value of $0.5 \times 10^5$ M$^{-1}$. But this effect is not directly correlated to the $K_b$ values for the other double mutants, as 24/28 also shows more weak CSP on the back of CcP ($K_b=1.0 \times 10^5$ M$^{-1}$) that do either 212/214 ($K_b=0.6 \times 10^5$ M$^{-1}$) or 249/253 ($K_b=0.8 \times 10^5$ M$^{-1}$). Nevertheless, these data indicate that the presence of the CLaNP-$5$ probe is interfering to some degree with formation of the complex.

**INTRAMOLECULAR DATA ANALYSIS**

Intramolecular PCS were measured for CLaNP-$5$-Ho$^{3+}$ at positions 87/90 and 212/214 and for CLaNP-$5$-Tm$^{3+}$ at all four positions and the $\Delta \chi$-tensors were calculated (Table 7.1).

<table>
<thead>
<tr>
<th>Position</th>
<th>Ho$^{3+}$ $\Delta \chi_{ax}$ (10$^{-32}$ m$^3$)</th>
<th>Ho$^{3+}$ $\Delta \chi_{th}$ (10$^{-32}$ m$^3$)</th>
<th>Tm$^{3+}$ $\Delta \chi_{ax}$ (10$^{-32}$ m$^3$)</th>
<th>Tm$^{3+}$ $\Delta \chi_{th}$ (10$^{-32}$ m$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24/28</td>
<td>-24.7 ± 1.3</td>
<td>-4.4 ± 0.5</td>
<td>49.4 ± 0.3</td>
<td>16.7 ± 0.8</td>
</tr>
<tr>
<td>87/90</td>
<td>-28.0 ± 0.5</td>
<td>-9.5 ± 0.2</td>
<td>57.6 ± 1.1</td>
<td>36.0 ± 0.7</td>
</tr>
<tr>
<td>87/90 (30 °C)</td>
<td>-29.0 ± 0.9</td>
<td>-8.8 ± 0.4</td>
<td>57.6 ± 1.3</td>
<td>36.5 ± 0.9</td>
</tr>
<tr>
<td>212/214</td>
<td>29.6 ± 1.9</td>
<td>9.4 ± 0.8</td>
<td>54.7 ± 2.8</td>
<td>40.2 ± 2.1</td>
</tr>
<tr>
<td>249/253</td>
<td>-23.8 ± 1.2</td>
<td>-5.0 ± 0.5</td>
<td>47.6 ± 1.1</td>
<td>18.8 ± 1.3</td>
</tr>
</tbody>
</table>

The resulting $\Delta \chi$-tensors had very similar $\Delta \chi_{ax}$ and $\Delta \chi_{th}$ values at the different positions. For Ho$^{3+}$, $\Delta \chi_{ax}$ was approximately $-28.5 \times 10^{-32}$ m$^3$ and $\Delta \chi_{th}$ was approximately $9.4 \times 10^{-32}$ m$^3$ which was slightly larger (although in similar proportion) to the theoretical values of $18.5 \times 10^{-32}$ m$^3$ and $5.8 \times 10^{-32}$ m$^3$, respectively. Note that for positions 24/28 and 249/253, no data for Ho$^{3+}$ was recorded so the values listed are estimates based on the experimentally determined values for the other two positions (see materials and methods). It is important to note that although using these estimates to fit the intermolecular PCS and PRE results in a reasonable fit to the experimental data (discussed below), these estimates do not take into account any
differences in the orientation of the Ho$^{3+}$ $\Delta \chi$-tensor in comparison to that of Tm$^{3+}$ and so caution must be used when interpreting any results based on these estimates.

The $\Delta \chi_{ax}$ and $\Delta \chi_{rh}$ values for Tm$^{3+}$ were much larger than those for Ho$^{3+}$, as expected, but they also showed more variation. For positions 24/28 and 249/253, the values for $\Delta \chi_{ax}$ and $\Delta \chi_{rh}$ were very similar at approximately $48 \times 10^{-32}$ m$^3$ and $18 \times 10^{-32}$ m$^3$, respectively, while the values for positions 87/90 and 212/214 were approximately $57.5 \times 10^{-32}$ m$^3$ and $35 \times 10^{-32}$ m$^3$, respectively. The $\Delta \chi_{ax}$ values were similar to previously reported for CLaNP-5-Tm$^{3+}$ ($\Delta \chi_{ax}=55.3 \times 10^{-32}$ m$^3$; $\Delta \chi_{rh}=6.9 \times 10^{-32}$ m$^3$) but all tensors showed significantly more rhombic character. However, values in which the proportion of $\Delta \chi_{ax}$ and $\Delta \chi_{rh}$ were roughly equal to each other have also been reported ($\Delta \chi_{ax}=21.9 \times 10^{-32}$ m$^3$; $\Delta \chi_{rh}=20.1 \times 10^{-32}$ m$^3$). These differences are likely due to small conformational changes in the CLaNP-5 structure, affecting the coordination to the Ln$^{3+}$, as a result of slightly different protein structures at each of the binding sites.

The backbone amide resonance assignments for CcP were obtained at 20 °C but those available for Cc were obtained at 30 °C. Generally it is not a problem to switch between these two temperatures when doing NMR experiments, depending on which of the binding partners are being studied. However, this study required that the intra- and intermolecular PCS/RDC data be comparable, which could potentially be problematic since the magnitude of an anisotropic $\Delta \chi$-tensor is temperature dependent. For example, the PCS would be expected to be approximately 3% larger at 20 °C than at 30 °C. Of course, it would be possible to simply record all data at the same temperature but changing the temperature can shift the resonances in the spectra, making the assignments unclear. In order to assess how much difference a 10 °C change in temperature would cause in the $\Delta \chi$-tensor, the PCS were measured for CLaNP-5-Ho$^{3+}$/Tm$^{3+}$ at position 87/90 at both 20 °C and 30 °C (Table 7.1). At 30 °C, $\Delta \chi_{rh}$ was 7.2% smaller but the $\Delta \chi_{ax}$ was 3.7% larger for Ho$^{3+}$ and the difference was only 1.0% for both values for Tm$^{3+}$. In fact, the values for both metals were the same within error, indicating that any affect from temperature is not likely significant in comparison to the error in calculating the $\Delta \chi$-tensors.

For all the $\Delta \chi$-tensors, the back-predicted PCS were compared to the experimentally determined PCS (Figure 7.4).
FIGURE 7.4 Back-calculated PCS plotted against experimentally observed PCS for CLaNP-5-Ho$^{3+}$/Tm$^{3+}$ attached to double cysteine mutants of CcP at positions 24/28 (C), 87/90 (A, D), 212/214 (B, E) or 249/253 (F). The linear best fit is shown in red and a y=x line is shown in blue. The $\Delta\chi$-tensor was calculated with Numbat$^{(96)}$ using intramolecular PCS and the CcP structure in the crystal structure of the complex with Cc (PDB entry 2PCC$^{(169)}$).
Combining multiple types of paramagnetic NMR data to study the Cc-CcP encounter complex

**FIGURE 7.5** Comparison of the back-calculated (red line) and experimentally observed (blue dots) PCS on CcP amide hydrogen atoms for the Ho\(^{3+}\) and Tm\(^{3+}\) \(\Delta\chi\)-tensors of CcP with CLaNP-5 attached to double cysteine mutants of CcP at positions 24/28 (C), 87/90 (A, D), 212/214 (B, E) or 249/253 (F). The difference between the two is plotted in grey. The average error for the PCS was ±0.002 ppm. The \(\Delta\chi\)-tensor was calculated with Numbat\(^{(96)}\) using intramolecular PCS and the CcP orientation in the crystal structure of the complex with Cc (PDB entry 2PCC\(^{(169)}\)).
When the back-calculated and experimentally observed PCS are plotted against each other for all mutants, a good correlation is obtained.

Similar results were seen when the back-calculated and experimentally observed PCS were compared for all CcP residues (Figure 7.5). Overall, the fit between the back-calculated and experimentally observed intramolecular PCS was very good for all positions. There were small deviations in the PCS for some residues but they were spread across the sequence and no obvious areas of strong mismatch could be identified. As expected, the largest predicted PCS occurred closest to the respective attachment site for each CcP mutant. The position of the paramagnetic centre used during the subsequent intermolecular Δχ-tensor calculations was fixed at the position determined during these intramolecular Δχ-tensor calculations. These Δχ-tensors were also used to back-predict pNMR data for different models of the complex (discussed below).

**INTERMOLECULAR PCS**

Intermolecular PCS were measured for Cc in the presence of CLaNP-5-Ho³⁺/Tm³⁺ tagged CcP. As expected, there were fewer peaks in the paramagnetic spectra than the diamagnetic due to increased relaxation for nuclei close to the paramagnetic centre. Line-broadening due to chemical exchange may have also occurred. For a given nucleus in Cc, the CcP-bound and free forms will each have a characteristic resonance frequency. The magnitude of the difference between these frequencies, Δω, in comparison to the exchange rate, k_ex, defines the type of chemical exchange occurring; chemical exchange can be classified as fast (k_ex ≫ Δω), intermediate (k_ex ≈ Δω) or slow (k_ex ≪ Δω). (309) The Cc-CcP complex generally experiences fast-exchange on the NMR timescale and therefore each nucleus will appear as a single peak in the spectrum, representing a weighted average of the two resonance frequencies. However, the use of strong paramagnetic centres, such as Tm³⁺, can potentially shift some nuclei into the intermediate or slow exchange regimes resulting in two separate peaks in the paramagnetic spectrum, as Δω is the sum of the difference in the chemical shifts and the PCS. While the first is a relatively small effect, the PCS can be quite large, shifting the exchange regime for that nucleus to intermediate or even slow exchange. (335) However, most observed PCS were ≤ 0.5 ppm, still in the fast or fast-intermediate exchange regime. The measured PCS values were extrapolated to 100% bound Cc and the Δχ-tensors were calculated (Table 7.2). The most notable difference when compared to the intramolecular Δχ-tensors (Table 7.1) is that all the values are much lower for the intermolecular Δχ-tensors (Table 7.2). The Cc-CcP
Combining multiple types of paramagnetic NMR data to study the Ce-CeP encounter complex

TABLE 7.2 Intermolecular PCS derived $\Delta\chi$-tensor values for Ho$^{3+}$ and Tm$^{3+}$ tensors for CLaNP-5 bound double cysteine mutants of yeast CeP C128A calculated with Numbat (96) using PCS obtained from a $[^{15}\text{N},^{1}\text{H}]$-BEST-TROSY-HSQC spectrum taken at 20 °C and the CeP crystal structure (PDB entry 2PCC (169)).

<table>
<thead>
<tr>
<th>Position</th>
<th>Ho$^{3+}$ $\Delta\chi_{ax}$ (10$^{-32}$ m$^3$)</th>
<th>Ho$^{3+}$ $\Delta\chi_{rh}$ (10$^{-32}$ m$^3$)</th>
<th>Tm$^{3+}$ $\Delta\chi_{ax}$ (10$^{-32}$ m$^3$)</th>
<th>Tm$^{3+}$ $\Delta\chi_{rh}$ (10$^{-32}$ m$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24/28</td>
<td>-10.7 ± 0.4</td>
<td>-3.3 ± 0.4</td>
<td>35.8 ± 1.4</td>
<td>8.7 ± 1.4</td>
</tr>
<tr>
<td>87/90</td>
<td>-11.1 ± 2.0</td>
<td>-1.9 ± 0.7</td>
<td>25.8 ± 1.8</td>
<td>8.8 ± 1.1</td>
</tr>
<tr>
<td>212/214</td>
<td>5.4 ± 2.4</td>
<td>2.3 ± 1.1</td>
<td>20.4 ± 1.5</td>
<td>3.1 ± 1.1</td>
</tr>
<tr>
<td>249/253</td>
<td>-14.5 ± 0.9</td>
<td>-5.3 ± 0.6</td>
<td>37.1 ± 0.9</td>
<td>2.5 ± 0.7</td>
</tr>
</tbody>
</table>

complex is known to partially exist in a highly dynamic encounter state that consists of multiple conformations in solution. Since the observed PCS represent an average over all Ce conformations present in the sample, lower observed PCS and therefore lower $\Delta\chi_{ax}$ and $\Delta\chi_{rh}$ values are expected. The presence of the encounter complex contributes to a poorer fit between the predicted and experimentally observed PCS (Figure 7.6) because the predicted PCS values are based only on the stereo-specific orientation of the complex. However, the CSP data suggest that the presence of the tag is also disrupting complex formation to some degree and disturbances to the stereo-specific complex would also contribute to this poorer fit. The intermolecular PCS data also showed that it is beneficial to use both lanthanoids to obtain complete coverage of a protein complex; Ho$^{3+}$ can provide information for nearby residues, whose peaks are completely broadened out by Tm$^{3+}$, while Tm$^{3+}$ can provide long-distance information beyond the limit of Ho$^{3+}$. In order to evaluate the fit to the 30:70 model (1, 8) of the complex, the intermolecular PCS was plotted against back-predicted PCS values for a complex 100% in the stereo-specific state, 100% in the encounter state or a 70%/30% combination of the two using the $\Delta\chi$-tensors obtained with intramolecular data (Table 7.1) (Figure 7.7). As mentioned above, the PCS represents an average of all conformations present in the sample; so nuclei sample a larger space in ensembles of conformation than in a stereo-specific structure, resulting in smaller PCS due to averaging of the $(3\cos^2\theta-1)$ factor (Equation 2.7). On the other hand, the PCS also depends on the distance between the observed nucleus and the paramagnetic centre ($r^{-3}$), so orientations that bring the nucleus closer to the paramagnetic centre may cause the average PCS to become larger. Both effects can be observed when comparing the back-predicted PCS for a 100% stereo-specific complex with those for a 100% encounter complex (Figure 7.7). Although none of the intermolecular PCS fit the back-calculated data for the 100% stereo-specific or the 30:70 model particularly well, the Tm$^{3+}$ data for position 24/28 are the closest fit to the 30:70 model.
FIGURE 7.6 Back-calculated PCS plotted against experimentally observed PCS on Cc for CLaNP-5-Ho$^{3+}$/Tm$^{3+}$ attached to double cysteine mutants of CcP at positions 24/28 (C), 87/90 (A, D), 212/214 (B, E) or 249/253 (F). The linear best fit is shown in red and a y=x line is shown in blue. The $\Delta\chi$-tensor was calculated with Numbat$^{96}$ using intermolecular PCS the CcP orientation in the crystal structure of the complex with Cc (PDB entry 2PCC$^{109}$) and the Ln$^{3+}$ position as determined from the intramolecular PCS.
FIGURE 7.7 Analysis of intermolecular PCS data observed for Cc amide hydrogen atoms in a 1:1 complex with CLaN5-Ln3+ tagged CcP at positions 24/28 (A, B), 87/90 (C, D), 212/214 (E, F) or 249/253 (G, H). The experimental PCS (black dots) are compared to the average back-calculated PCS are also shown for 100% stereo-specific complex (purple line), 100% encounter complex (green line) or 30% encounter/70% stereo-specific complex (blue line). The Ho3+ Δχ-tensors used for back-calculation for positions 24/28 and 249/253 are estimates (red *). The average error for the PCS was ±0.003 ppm.
The experimental PCS for the remaining positions show significant averaging in the values with effects that are mostly similar to or even weaker than those predicted for a complex 100% in the encounter state. The results suggest that the presence of the CLaNP-5 probe is disrupting stereo-specific complex formation and could also be causing an increase of the population in the encounter state. The very low experimental PCS values indicate that much more averaging is occurring in solution than is represented by any of these models, including the 100% encounter complex model, which could be explained by an under-sampling of the true breadth of Cc orientations present in the encounter complex.

**INTERMOLECULAR RDC**

RDC are relatively small and therefore difficult to measure accurately. Tm$^{3+}$ can be used to generate large RDC, \( \geq 20 \text{ Hz} \), but this comes at the cost of severe line-broadening for many peaks. However, RDC are not dependent on the distance between the probe and the observed nucleus so they can be very useful for obtaining orientation information over an entire protein complex. The intermolecular RDC were measured for Cc in the presence of CLaNP-5-Tm$^{3+}$ CcP (Figure 7.8). Like PCS, RDC also represent an average of all conformations in solution and a large degree of averaging was observed for positions 87/90 and 212/214, with the experimental RDC values being of similar magnitude to those predicted for a 100% encounter complex. Interestingly, in contrast to positions 87/90 and 212/214 which fit the 100% encounter complex model best, the magnitude of the RDC for positions 24/28 and 249/253 was much closer to those predicted for the 30:70 model or the 100% stereo-specific complex. However, unlike the PCS, which can be very large, especially for Tm$^{3+}$, and easy to distinguish, the RDC can be small and difficult to measure with good precision. This is reflected in the large errors (\( \pm 6 \text{ Hz} \)) for the measured RDC values, which makes it difficult to distinguish exactly which model the RDC fit best. For position 249/253, the PCS data also showed limited averaging and fit the 30:70 model much better than the 100% encounter complex model suggesting a difference in the encounter/stereo-specific populations for those positions compared to positions 87/90 and 212/214. The data for position 24/28 also fit the 30:70 well for most residues with limited averaging of the PCSs; The fit is less good for residues 66-76 and 89-95 but the PCS for these nuclei were very large and only a few data points were could be measured.
Combining multiple types of paramagnetic NMR data to study the Cc-CeP encounter complex

**PARAMAGNETIC RELAXATION ENHANCEMENT**

Although RDC and PCS have the advantage of providing orientation information, PRE are exquisitely sensitive to minor states and therefore ideal for studying encounter complexes despite only providing distance information. Here we used CLaNP-5-Gd$^{3+}$ attached at the four positions on CcP to generate long-range PRE effects on Cc. Three of the CLaNP-5 attachment sites used in this study are close to the sites used in previous studies: 24/28 is close to 288C, 249/253 is close to 200C and while 38C falls roughly between 24/28 and 87/90.$^{(1,8)}$
The $I_{\text{para}}/I_{\text{dia}}$ ratios obtained with CLaNP-5-Gd$^{3+}$ were similar to those observed previously at the nearby positions (Figure 7.9). Notably, position 24/28 experienced two areas of strong PRE near residues 69 and 89, which was also seen for 288C, around residues 75 and 82. For position 249/253, five areas experienced strong PRE (near residues 15, 29, 35, 50 and 80), four of which were similar to those seen for 200C (near residues 15, 25, 35 and 80).

The distance between the CLaNP-5 probes on CcP and the backside of Cc was less than 50 Å for all probe attachment sites. Due to the strength of the PRE generated by Gd$^{3+}$, almost all Cc residues experienced some degree of relaxation enhancement resulting in essentially complete coverage of Cc, which can be seen in the PRE maps (Figure 7.10).

**FIGURE 7.9** $I_{\text{para}}/I_{\text{dia}}$ values (black diamonds) for Cc amide protons in the presence of CcP with CLaNP-5-Gd$^{3+}$ attached at position 24/28 (A), 87/90 (B), 212/214 (C) or 249/253 (D). The data were obtained with 78.4%, 72.7%, 73.5% or 76.5% CcP bound, respectively. The standard deviation of the noise level was determined for each spectrum and was then used in the calculation of the propagated error (black bars) for each $I_{\text{para}}/I_{\text{dia}}$ value.
Combining multiple types of paramagnetic NMR data to study the Cc-CcP encounter complex

**FIGURE 7.10** PRE map for Cc in a 1:1 complex with CLaNP-5-Gd³⁺ tagged CcP at positions 24/28 (A), 87/90 (B), 212/214 (C) or 249/253 (D). The PRE effects are colour-coded on a surface model of Cc in complex with CcP (grey ribbon; haem group in black sticks) (PDB-entry 2PCC)(5) and the Gd³⁺ ion in green. Residues with Γ₂, para ≥ 200 s⁻¹ are red, 200 s⁻¹< Γ₂, para < 100 s⁻¹ are orange, 10 s⁻¹< Γ₂, para < 100 s⁻¹ are yellow, Γ₂, para ≤ 10 s⁻¹ are blue and with no data are grey
Previous pNMR studies of the Cc-CcP complex used nitroxide based SLs to generate the PRE effects. These SLs only generate measurable effects between approximately 14-24 Å, while the range for Gd$^{3+}$ is approximately 20-45 Å, resulting in almost complete coverage of Cc. While the strongest effects are always located on the side of Cc facing the binding interface, which is consistent with the CSP map (Figure 7.3), the localization of the strongest effects (shown in red in both figures) has shifted to the residues nearest the CLaNP-5 probe.

The PRE effects were converted to distances between the affected residues and the paramagnetic centres and these were compared to back-predicted distances for three models of the complex (Figure 7.11). As discussed above, the PCS and RDC data contain additional orientation information that is clearly not described well by the 30:70 model of the complex. Although the fits are not great, as discussed below, there is a better fit overall between the PRE derived distances and back predicted distances for the 30:70 model in comparison to the PCS and RDC data, which shows how insensitive PRE is to differences in orientation (Figures 7.7 & 7.8).

The best fit is seen for position 249/253 which fits the 30:70 model very well with the exception of residues 34-36 and 95-105 (Figure 7.11 D). The Tm$^{3+}$ PCS and RDC data for position 249/253 also had a reasonably good fit to the back-predicted data for the 30:70 model, although the location of the strongest PCS were shifted slightly (Figure 7.7 G, H). Since limited averaging of the RDC was observed (Figure 7.8 D), these differences were likely due to changes in the stereo-specific complex caused by the presence of the CLaNP-5 probe. Similar results were seen for position 24/28. There was no significant averaging of the RDC (Figure 7.8 A) and the Tm$^{3+}$ PCS fit the back-predicted data for the 30/70 model well; the fit appears to be less good for residues 65-80 and 85-95 but their predicted PCS were very large and therefore very few data points could be obtained for these nuclei (Figure 7.7 B). The PRE data also fit the 30:70 model well although several residues between 19-55 were much closer to the paramagnetic centre than predicted (Figure 7.11 A). These results indicate that the probe is causing some minor disruptions to the stereo-specific complex.

In contrast, the data for position 87/90 suggest that the encounter state is being disrupted and its population is also likely increased. Both the PCS and RDC experience significant averaging (Figures 7.7 C, D and 7.8 B) and the PRE data show experimental distances for almost all nuclei that are less than predicted by any of the models (Figure 7.11 B). If the presence of the CLaNP-5 probe causes slight repulsion and rotation of Cc, these small differences in the distances to the paramagnetic probe will be measured by the extremely
Combining multiple types of paramagnetic NMR data to study the Cc-CcP encounter complex

FIGURE 7.11 Analysis of intermolecular PRE data observed for Cc amide nitrogen atoms in a 1:1 complex with CLaNPs-5-Ln3+ tagged CcP at positions 24/28 (A, B), 87/90 (C, D), 212/214 (E, F) or 249/253 (G, H). The experimental distances (black dots connected by red line) are compared to the theoretical distances for a 100% stereo-specific complex (purple line), a 100% encounter complex (green line) or a 30% encounter/70% stereo-specific complex (blue line).

sensitive PRE. The PRE map also shows a weaker interaction between the proteins, with Cc being pushed away from the CLaNPs-5 attachment site (Figure 7.10 B). This likely indicates a greater proportion of the complex in the loosely associated and highly dynamic encounter state where Cc is sampling more of the surface of CcP than expected.

Similarly, for position 212/214 a significant degree of averaging of the PCS and RDC data as well as a very poor fit to the back-predicted data was observed (Figures 7.7 E, F and 7.8 C). The fit of the PRE data for this position was also poor; although the overall pattern of effects matches that of the 30:70 model relatively well, several regions (residues 1-13, 68-70, 93-97) are further away from the paramagnetic centre than predicted, matching more closely to the stereo-specific state, while others (residues 19-26, 48-55, 64, 77-82) are much closer than
predicted, matching more closely to the encounter state (Figure 7.11 C). Placing the probe at this position clearly interferes with complex formation and also appears to increase the population of the encounter complex.

In conclusion, the presence of the CLaNP-5 probe is disrupting complex formation to a greater or lesser degree for all double mutants. This is likely due to steric and electrostatic interference with Cc preventing the stereo-specific complex from forming, particularly for positions 24/28 and 249/253. The net positive charge of CLaNP-5 is not ideal when working with such highly charged proteins whose interaction is driven predominately by electrostatics.\(^\text{169, 328}\) For positions 87/90 and 212/214, the presence of the probe is also altering the encounter complex and likely increasing its population. Therefore, the existing models underestimate the spread of the Cc orientations sampled by this altered encounter state resulting in very poor fits to the PCS and RDC data, which are orientation sensitive. However, the PRE data fit the 30:70 model reasonably well, with some exceptions, demonstrating how insensitive PRE are to protein orientations, as we also concluded in Chapter 5.\(^\text{321}\)

Nevertheless, a new model that combines all three types of paramagnetic information will likely produce a much more accurate picture of how the Cc-CcP complex behaves in solution and so this work should be repeated with CLaNP-7 or other less charged probes.\(^\text{329}\) It would also be useful to combine the current Gd\(^{3+}\) generated PRE data with the previous nitroxide radical generated PRE data since the range in which these two paramagnetic centres generate effects are complementary: 20-45 Å and 14-24 Å, respectively. However, combining all of these effects cannot easily be done in the currently available protein docking software which presents a large technical challenge. Furthermore, although additional data sets are ideal for combating the ill-posed inverse problem,\(^\text{273, 274}\) this will require significant computational capacity.
Combining multiple types of paramagnetic NMR data to study the Cc-CeP encounter complex