Intermolecular dynamics studied by paramagnetic tagging

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Abstract Yeast cytochrome c and bovine adrenodoxin form a dynamic electron transfer complex, which is a pure encounter complex. It is demonstrated that the dynamic nature of the interaction can readily be probed by using a rigid lanthanide tag attached to cytochrome c. The tag, Caged Lanthanide NMR Probe 5, induces pseudocontact shifts and residual dipolar couplings and does not perturb the binding interface. Due to the dynamics in the complex, residual dipolar couplings in adrenodoxin are very small. Simulation shows that cytochrome c needs to sample a large part of the surface of adrenodoxin to explain the small degree of alignment observed for adrenodoxin. The applied method provides a simple and straightforward way to observe dynamics in protein complexes or domain–domain mobility without the need for external alignment media.

Keywords Dynamics · Lanthanide tag · Encounter complex · Residual dipolar couplings · Paramagnetic NMR

Abbreviations
RDC Residual dipolar coupling
Cc Cytochrome c

Adx Adrenodoxin
PCS Pseudocontact shift
CLaNP-5 Caged Lanthanide NMR Probe 5
PRE Paramagnetic relaxation enhancement

Introduction

Protein dynamics is important for protein function and recognition. Solution NMR spectroscopy is a powerful tool to study protein dynamics on a timescale ranging from pico-seconds to seconds. 15N spin relaxation measurements combined with Lipario-Szabo analysis is now a standard NMR method to characterize the protein dynamics on the ps–ns timescale (Palmer 2004). Recently, relaxation dispersion has been developed as the method to characterize the protein dynamics in the µ–ms time scale (Korzhnev and Kay 2008). Residual dipolar couplings (RDCs) were initially proposed as a tool to provide long-range restraints for structure refinement. The RDC is also sensitive to motions spanning the ps–ms timescale. Thus, the dynamics information obtained from RDCs offer a view on dynamic processes that is complementary to other NMR spectroscopic approaches (Tolman and Ruan 2006; Lakomek et al. 2008; Lange et al. 2008).

RDCs can be measured when biomolecules are slightly aligned with the external magnetic field. For some metalloproteins, weak molecular alignment can be achieved at high fields due to their anisotropic magnetic susceptibility (Tolman et al. 1995; Banci et al. 1998). For other proteins, external alignment media have to be used (Tjandra et al. 1997; Tjandra and Bax 1997; Bax and Grishaev 2005). The interpretation of RDC data in the study of intermolecular dynamics is difficult when an external alignment medium is used, because the alignment tensors of the two molecules...
will be different for each relative orientation and the information obtained is only relative to the external magnetic field (Tolman and Ruan 2006). In the study of bimolecular interaction involving nucleic acids, the problem can be overcome by an elegant method in which an elongated RNA is used to obtain alignment, providing a fixed reference frame for RDC analysis (Zhang et al. 2007). For protein–protein complexes or protein domain–domain interactions with two rigid substructures, preferential alignment of one component and detection of RDCs on the other provides a more direct way for analysis of intermolecular dynamics, see Fig. 1 (Bertini et al. 2004; Rodriguez-Castañeda et al. 2006).

Lanthanide paramagnetic probes offer an attractive way to achieve such partial alignment (Otting 2008). Two-point attachment of the paramagnetic probe Caged Lanthanide NMR Probe 5 (CLaNP5) through cysteine disulfide bridges proved to be a good way to introduce large and well-defined paramagnetic effects by minimizing the dynamics of probe (Keizers et al. 2007; Keizers et al. 2008).

In this study, CLaNP-5 is used to study the intermolecular dynamics in the complex of yeast cytochrome c (Cc) and bovine adrenodoxin (Adx). Adx is involved in steroid hormone biosynthesis by acting as an electron shuttle between NADPH-dependent adrenodoxin reductase and several cytochromes P450 (Grinberg et al. 2000). In vitro electron transfer from adrenodoxin reductase to Adx is often monitored by fast subsequent electron transfer from Adx to mitochondrial Cc. Yeast Cc is used as a model of P450 and the non-physiological complex between yeast Cc and bovine Adx is active in electron transfer. It is interesting that electron transfer in this complex is so rapid, because it is a non-physiological complex. Many complexes of electron transfer proteins have evolved to exhibit fast turn-over and show little specificity. For example, for the physiological complexes of cytochrome c and cytochrome c peroxidase (Volkov et al. 2006) and myoglobin and cytochrome b5 (Worrall et al. 2002; Liang et al. 2004) it has been shown that the encounter state, which by nature is an ensemble of non-specific, electrostatic complexes, represents a significant or even dominant fraction of the entire complex. A previous study on the complex of Cc and Adx showed that it too is very dynamic and can be considered as a pure encounter complex (Xu et al. 2008) and yet it is active in electron transfer. The study of the amplitude of the dynamic motions, i.e. the degree of surface sampling, will help to understand how such non-specific electron transfer complexes can still be active.

Large RDCs are observed for Cc when CLaNP-5 is attached. In the complex, very small RDCs are detected for Adx, suggesting averaging over multiple orientations of Adx relative to Cc due to large intermolecular dynamics.

Materials and methods

Site-directed mutagenesis

Mutant CcN56C/L58C was created by site-specific mutagenesis with the overlap extension method (Ho et al. 1989). The template DNA used for mutagenesis was pUC-cc containing the genes of yeast iso-1 Cc (C102T) and Cc heme lyase. The following four primers were used in three PCRs to amplify the mutated insert; M13 forward (5'0-CCCAGTCACGACGTTGTAAAACG-3'0), M13 reverse primer (5'0-AGCGGATAACAATTTCACACAGG-3'0), N56C/L58C forward primer (5'0-GCCAATATCAAGAAATGCGTGTGCTGGGACG-3'0) and N56C/L58C backward primer (5'0-GTCCCAGCACACGATTTCTTGATATTGGC-3'0). The product of the third PCR was purified and subcloned into the XmaI/HindIII site of the plasmid pUC-18. The DNA sequence of pUC-ccN56C/L58C was verified by sequencing.

Protein production and 15N labeling

The double mutant CcN56C/L58C was produced and purified using the same protocol as for wild type Cc (Xu et al. 2008). The final yield was 15 mg L-1 and 8 mg L-1 for the unlabeled and 15N labeled Cc mutant, respectively. The concentration of Cc mutant was determined using an extinction coefficient 27.5 mM-1 cm-1 at 550 nm for the reduced Cc mutant. The production of both unlabeled and 15N labeled Adx has been described (Worrall et al. 2003).
Paramagnetic probe attachment

The synthesis of Ln–CLaNp-5 probe has been described (Keizers et al. 2008). To attach Ln–CLaNp-5 to CcN56C/L58C, 600 µL 0.5 mM CcN56C/L58C was first treated with DTT (final concentration 5 mM) at room temperature for 2 hrs to remove possible dimers by reduction of disulfide bonds. A PD-10 column (GE Healthcare) was used to remove the DTT and bring the protein in the 3 mL buffer containing 20 mM sodium phosphate and 150 mM NaCl, pH 7.0. Seven to ten equivalents CLaNp-5 were added and the solution was stirred for 2 h at 4°C. The buffer was changed to 20 mM sodium phosphate, pH 7.0 before the solution was loaded on a HiTrap-SP column. The protein was eluted using a 350–700 mM NaCl gradient under an argon atmosphere to keep Cc in reduced state. Either ytterbium or lutetium containing CLaNp-5 was attached to the Cc double mutant. The extent of probe attachment to $^{15}$N CcN56C/L58C was estimated to be above 90% from mass spectrometry and from the absence of diamagnetic crosspeaks in the HSQC spectra of the paramagnetic samples.

NMR spectroscopy

All NMR samples contained 20 mM potassium phosphate buffer, pH 7.4, 6% D$_2$O and one equivalent of ascorbic acid under an argon atmosphere to keep Cc in the reduced state. Three sets of samples were prepared comprising free $^{15}$N Cc with probe attached and the CLaNp-Cc/Adx (0.25 mM 1:1) complex in which either CcN56C/L58C or Adx was $^{15}$N labeled. Yb–CLaNp-5 was used in the paramagnetic samples and Lu–CLaNp-5 acted as a diamagnetic control. For pseudocontact shift measurements, $^1$H–$^{15}$N HSQC spectra were recorded at 14.1 T on a Bruker DMX 600 MHz spectrometer equipped with a TCI-Z-GRAD cryoprobe operating at 293 K. For $^1$H–$^{15}$N RDC measurements, $^1$H–$^{15}$N IPAP-HSQC (Ottiger et al. 1998) spectra were acquired at 21.1 T on a Bruker 900 MHz spectrometer equipped with a TCI-Z-GRAD cryoprobe. The RDC was obtained through the measurement of the splitting difference in the $^{15}$N doublet of amide resonances between the samples containing paramagnetic Yb and diamagnetic Lu. NMR data were processed in Azara (www.bio.cam.ac.uk/azara), and analyzed in Ansig for Windows (Helgstrand et al. 2000).

PCS and RDC analysis

The crystal structure of yeast iso-1 Cc (PDB entry: 1YCC (Louie and Brayer 1990)) was used for PCS and RDC analysis. Hydrogens were added in XPLOR-NIH (Schwieters et al. 2003) according to the standard geometry parameters. A set of pseudo-atoms representing the magnetic susceptibility tensor frame was added for the fit of PCSs and RDCs using ParaAstraints in XPLOR-NIH (Banci et al. 2004). The equation used for the tensor calculation is:

$$\text{PCS} = \frac{1}{12\pi r^3} \left[ \Delta \chi_{ax} (3 \cos^2 \theta - 1) + \frac{3}{2} \Delta \chi_{rh} \sin^2 \theta \cos 2\Omega \right]$$

where $r$, $\theta$, and $\Omega$ are the polar coordinates of the nucleus with respect to the principal axes of the $\chi$-tensor and $\Delta \chi_{ax}$ and $\Delta \chi_{rh}$ are the axial and rhombic components of the $\chi$-tensor, respectively. The position of the lanthanide was determined by energy minimization of the PCS energy term and a term that restrained the metal to 6–10 Å from the Cx of cysteines 56 and 58 of Cc (Keizers et al. 2008). The lowest-energy structure was used for fitting of RDCs using the FRUN algorithm in the ParaAstraints suite. The equation used for RDC alignment tensor determination is:

$$D^{\text{ass}} = \frac{B_0^2 \gamma_i \gamma_j h}{15kT16\pi^3 r_{ij}^3} \left( \Delta \chi_{ax} (3 \cos^2 \theta - 1) + \frac{3}{2} \Delta \chi_{rh} \sin^2 \theta \cos 2\Omega \right)$$

where $B_0$ is the applied magnetic field, $\gamma_i$ and $\gamma_j$ are the gyromagnetic ratios of nuclei $i$ and $j$ respectively, $r_{ij}$ is the $i$–$j$ inter-nuclear distance (1.02 Å for amide N–H bonds) (Jeffrey 1992), $\theta$ and $\Omega$ determine the inter-nuclear vector orientation with respect to the principle axes of the $\chi$-tensor, $T$ is the absolute temperature, and $k$ and $h$ are the Boltzmann and Planck constants, respectively.

Simulation of the dynamics

The coordinates of the Ln–Cc structure and Adx (PDB entry 1AYF (Müller et al. 1998)) were used for simulations to explore the minimal sampling space required for averaging of the intermolecular RDCs. Similar to what was described before (Xu et al. 2008), the relative diffusional movement of the proteins in the native complex was decomposed into two types of rotations. One is the rotation of Adx around its own center of mass (wobbling motion) and the other is the rotation of Adx around the Cc, resulting in the translational movement over its surface. The heme metal center of Cc was used as the rotating center for the latter movement. Each rotation can be further decomposed into the rotations around the X, Y and Z axes. Six angle variables are thus used to describe the rotation ranges. The initial orientation of two proteins was generated in a way that N16 of Cc is in close contact with L80 of Adx. The initial distance of the geometrical center of Adx to the
metal alignment center was set to 42 Å. This brought the proteins together in an orientation consistent with chemical shift perturbation data. The effect of averaging over the six rotating and wobbling axes was evaluated by generating 100 sterically allowed orientations randomly within a given angle range. The averaged intermolecular RDCs were calculated for amide protons of Adx in this ensemble and compared with the observed RDCs for Adx in the complex. By stepwise increments of the angle ranges the amplitude of motion in the simulation was increased. By varying the ranges of the six angle variables independently, the contribution to each of them to the averaging effect could be established.

Results

Probe attachment on Cc

Two surface-exposed residues of Cc, N56 and L58 were mutated to cysteines for probe attachment. These residues are located in the region of a short β-strand (V57-W59), at the backside of Cc with respect to the heme edge, which is the site involved in binding to various interaction partners (Ubbink and Bendall 1997; Worrall et al. 2001; Crowley et al. 2002; Banci et al. 2003; Volkov et al. 2005). The attachment of the probe to a site far from the interaction interface should cause little perturbation of the complex. The side chains of the two residues are 5–10 Å apart, an optimal distance for attachment of the two arms of the CLaNP probe. Via two disulfide bridges formed between the cysteine residues and the arms of the Ln-probe, the latter was rigidly attached to the protein surface. The molecular weight of the purified product of Lu–CLaNP-15N Cc (13547.9 ± 1 Da) agrees with the theoretical molecular weight (13548.2 Da), confirming that both leaving groups are removed and the probe is attached via two disulphide bridges. The yield of the entire labeling procedure, including purification is about 50%.

Magnetic susceptibility tensor determination

The 15N-HSQC of Lu–Cc is similar to that of wt Cc except for a few residues in close proximity to the sites of mutation, indicating that probe attachment does not cause large perturbations of the structure. For Yb–Cc PCSs were caused by the interaction between the unpaired electrons of the lanthanide ion and the nuclei of Cc and were measured by comparison of HSQC spectra for samples with diamagnetic Lu and paramagnetic Yb–CLaNP-5. A single set of PCSs was observed for Yb–Cc as compared to Lu–Cc. For each backbone amide 1H and 15N pair far from the metal site, similar PCSs are expected (in ppm) for the 1H and 15N nuclei. This leads to the unambiguous assignment of some backbone amides at the initial stage, allowing magnetic susceptibility tensor determination and subsequent assignment of more residues in an iterative procedure. The largest PCS (>2 ppm) was detected for the amide proton of W59 of Cc (Fig. 2 and Table S1). In total, 73 amide protons of reduced Cc with probe attached were assigned and used for magnetic susceptibility tensor (Δχ) determination by fitting to the crystal structure of yeast Cc (1YCC). The sizes of the axial and rhombic components (Δχax and Δχrh in 10^{-32} m^3) are 8.3 ± 0.2 and 3.1 ± 0.2 respectively. Figure 3 shows a correlation plot of experimentally observed and theoretically predicted PCS. The good fit (Q factor: 0.047) supports the finding that CLaNP is rigid relative to the frame of the protein.

Probe induced RDC

At 21.1 T RDCs as large as 9 Hz were detected for Cc attached to the Yb-probe (Table S2). The alignment tensor was determined using 64 RDCs of N–H pairs of Cc. Figure 4a shows the correlation for experimental and back-calculated RDCs by fitting to the crystal structure of Cc (Q factor 0.263). The correlation is acceptable given the error margins (1.5 Hz for this 23 kDa complex) and the fact that the crystal structure was not refined using the RDC data. The fitted alignment tensor is 6.71 and 2.0 (in 10^{-32} m^3) for axial and rhombic components, respectively. The size of the axial component derived from RDC data is 19% smaller than the one from the PCSs. The correlation between observed RDCs and RDCs

![Fig. 2 Overlay of 15N–1H HSQC spectra of 15N-labeled CcN56C/L58C attached to Yb–CLaNP-5 (red) or Lu–CLaNP-5 (Black). The spectra were recorded at 293 K and 14.1 T](image-url)
predicted with the PCSs derived tensor is shown in Fig. 4b. The Q factor for this fit is 0.287, only slightly larger than that of the fit based on the RDC derived tensor. Thus, it could be that the difference between the $\Delta y$ values derived from RDCs and PCSs is not significant. Alternatively, the discrepancy could be attributable to internal dynamics, because RDCs are more sensitive to the internal dynamics than PCSs. Such dynamics may represent slight mobility of the probe, but can also be caused by mobility of amide groups (Bouvignies et al. 2005).

The interaction of Ln–Cc with Adx

The interface of Ln–Cc determined from chemical shift perturbations is similar to that in the native complex (Table S3 in supplementary material). A titration experiment of unlabeled Adx to $^{15}$N Lu–Cc yielded a $K_a$ value 2.1 ($\pm 0.7) \times 10^9$ M$^{-1}$ for binding of Ln–Cc to Adx (Fig. 5). This value is slightly smaller than that for the wild type Cc/Adx complex, $4.0 \pm 1.0 \times 10^4$ M$^{-1}$ (Worrall et al. 2003), possibly due to the small temperature difference of the measurements. The NMR titration for the complex with wt Cc was conducted at 285 K, whereas 293 K was used in the present study. Using the $^{15}$N labeled Cc attached to CLaNP-5, both PCSs and RDCs were measured for Cc in the complex Cc/Adx. In the 1:1 complex of CLaNP5-Cc/Adx, the observed PCSs and RDCs for Cc are the weighted average of free and complex states. A comparison of PCSs and RDCs for free Yb–Cc and Yb–Cc in the complex (Tables S1 and S2) indicates that the magnetic susceptibility tensor does not change upon complex formation.

Small paramagnetic effects on Adx in the Cc/Adx complex

When Ln–Cc interacts with $^{15}$N Adx, the paramagnetic effects observed for Adx are proportional to the fraction of Adx bound in the complex. At the ratio of 1:1 Adx/Ln–Cc (0.25 mM), 61% Adx is in the complex state. A few residues of Adx in the 1:1 complex show small intermolecular PCSs. An attempt to model the orientation of two proteins with these PCSs failed. According to the Eq. 1, only small PCSs are expected for a few residues of Adx even in the single-orientation complex because the Ln metal center is relatively far (>35 Å) from most of the residues of Adx. RDCs on the other hand are independent of the amide–Ln distance. If the degree of alignment of Adx in the complex is the same as for Yb–Cc, RDCs of 4–5 Hz are expected, and when extrapolated to the 100% bound state, values up to 9 Hz are predicted. The largest extrapolated RDC observed for Adx in the 100% bound state is about 2 Hz (Fig. 6), which is much smaller than the RDCs determined for Cc and below the significance level (1.5 Hz error margin $\times 1/0.61$ for extrapolation to 100% bound = 2.5 Hz). The striking difference of RDC values detected for Adx and Cc in the complex is in accord with the intermolecular dynamics between Cc and Adx in the complex.

Modelling of the intermolecular dynamics

A simulation was performed to explore the magnitude of the intermolecular motions in the complex. The movement
of the proteins within the complex was decomposed into six different rotations (see Materials and methods section). Within a set sampling space, all sterically allowed conformations were randomly sampled. In this way, ensembles of the protein complexes were generated and compared with the observed chemical shift perturbations and RDCs. In any single-orientation conformation of Ln–Cc/Adx, the predicted largest RDCs for amide protons of Adx are as large as ±8–9 Hz, similar to those detected for Cc. The insignificant RDCs observed for Adx in the complex are the direct result of intermolecular dynamics, which averages the N–H bond orientations relative to the reference frame. The simulation suggests that the rotation of Adx around Cc (translational movement of Adx over the Cc surface) hardly reduces the averaged RDC values on Adx (Figure S1 in supplementary material). This is in contrast to the large averaging effect of the same movement on PCS originating from heme center in the previous study (Xu et al. 2008). The rotation of Adx around its own geometrical center can lead to a large change of N–H internuclear vector orientation (h and x in Eq. 2) with respect to the principal axes of the magnetic susceptibility tensor, thus averaging the RDC to an insignificant value. In an ensemble with a set of maximal rotation angles of 90, 120 and 40 degrees of rotations around the geometrical center of Adx for x, y and z rotations, respectively, the intermolecular RDC can be averaged out. In this ensemble, Cc needs to sample a large surface area of Adx (Fig. 7a). The sampling space in this ensemble agrees well with the chemical shift perturbation data and is also consistent with the degree of dynamics suggested by our previous study using both PCSs derived from the Cc heme and paramagnetic relaxation enhancement (PRE) mapping from spin labeling (Xu et al. 2008).

Discussion

Paramagnetic metal ions provide NMR effects such as PRE, PCS and RDC due to self-orientation at high magnetic field. These effects can be converted into distance or angular restraints which are useful not only for structure determination, but also for the study of dynamics. PRE is isotropic and inversely dependent on the metal–nucleus distance \(1/r^6\) dependence. Therefore, PRE is powerful to detect minor populations in a heterogeneous system (Volkov et al. 2006; Tang et al. 2006; Tang et al. 2007). PCS depends on the metal–nucleus orientation and distance, falling off with \(r^3\). The magnitude of metal-induced RDCs is not dependent on metal–nucleus distance, thus each of these observables provides a different view on the dynamics in the system. PCSs and RDCs exhibit averaging for all motions occurring on a time scale faster than \(\sim 10^{-2}\) s.
For a two-component system (two domains or proteins) aligned with an external medium, the presence of intermolecular dynamics may be induced from differences between the alignment tensors experienced by the two components (Braddock et al. 2002; Ulmer et al. 2002). Failure to observe such a difference, however, does not exclude the possibility of dynamics. If the components tumble entirely independently the two tensors are only determined by shape or charge properties of each of the two components. If, on the other hand, the components interact, both tensors will differ for each relative conformation of the two components. Contrary to this, metal-induced alignment provides a fixed reference alignment frame for one component, enabling direct detection of motions of the other component. This method already proved useful to obtain information on dynamics of domain–domain interactions of metallo-proteins (Bertini et al. 2004, 2007). To extend this method to other proteins without metal sites, a paramagnetic tag needs to be attached rigidly to the protein surface.

CLaNP-5 has been shown to exhibit minimized internal dynamics through attachment via two disulfides bridges (Keizers et al. 2007, 2008). In this study, the Yb–CLaNP-5 was rigidly attached to the backside of reduced, diamagnetic Cc to obtain large PCSs and RDCs, with much smaller paramagnetic effects observed for Adx in the dynamic complex. Among all Ln metals, Yb is classified as moderately paramagnetic, with a small broadening effect. More highly paramagnetic Ln metals (Dy, Tb, Tm) which induce larger PCSs and RDCs (Keizers et al. 2008), can provide more extensive datasets for accurately modeling the 3-D sampling space of the dynamic complex. Although in this study two metalloproteins were involved, this method should be generally applicable to other protein complexes or multi-domain proteins without metal-binding sites, because the heme and FeS cluster were not used in this study in any way.

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