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The cytochrome *c* peroxidase and cytochrome *c* encounter complex: The other side of the story

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ABSTRACT

Formation of an encounter complex is important for efficient protein complex formation. The encounter state consists of an ensemble of orientations of two proteins in the complex. Experimental description of such ensembles inherently suffers from insufficient data availability. We have measured paramagnetic relaxation enhancements (PRE) on cytochrome *c* peroxidase (CcP) caused by its partner cytochrome *c* (Cc) carrying a spin label. The data complement earlier PRE data of spin labelled CcP, identifying several new interactions. This work demonstrates the need of obtaining as many independent data sets as possible to achieve the most accurate description of an encounter complex.

Structured summary of protein interactions:
CcP and **Cc** bind by nuclear magnetic resonance (View interaction)

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1. Introduction

1.1. The encounter complex and the inverse problem

Protein–protein complex formation requires an intermediary complex to form before the final, stereospecific state is reached. The formation of this encounter complex is driven by long-range charge–charge and hydrophobic interactions, resulting in a weakly associated complex in which the protein partners are free to rotate and reorient themselves. From there, the number of short-range interactions (van der Waals, hydrogen-bonding, hydrophobic interactions and salt bridges) between the pair is increased to form the stereospecific state [1].

The transient and highly dynamic nature of the encounter complex makes it difficult to observe and visualize. Because the encounter complex is comprised of a large number of transient, low energy and weakly interacting conformations, it is essentially invisible to many structural biology techniques. Paramagnetic nuclear magnetic resonance (NMR) spectroscopy provides a unique opportunity to study these highly dynamic complexes as the observed effects, from paramagnetic relaxation enhancement (PRE) in particular, are extremely sensitive for those lowly populated states in which the nucleus is closer to a paramagnetic centre than in the other state(s) [2].

The main drawback is that the PRE, like many other NMR observables, is an average over all the conformations present in the sample. This makes visualization of the complex an *ill-posed inverse problem* [3,4], in which many ensembles of solutions can be found to match the observed data [5–14]. In fact, the only result that can be determined conclusively is where the interaction does not occur. If a paramagnetic centre does not cause PRE on the partner, it can be concluded that the surface region around that centre is not sampled by the partner for a significant fraction of the lifetime of the complex. Therefore, by using paramagnetic probes at several locations on the protein's surface, an exclusion map can be generated [5–7,14–16]. The more restraints can be incorporated into the modelling calculations, the more refined the ensemble of structures becomes and the closer it will be to the true ensemble in the sample [17–21].

Abbreviations: Cc, cytochrome *c*; CcP, cytochrome *c* peroxidase; CSP, chemical shift perturbations; I_{para}/I_{dia}, intensity ratio; MTS, 1-acetoxy-2,2,5,5-tetramethyl-δ3-pyrroline-3-methyl)-methanethiosulfonate; MTSL, 1-oxyl-2,2,5,5-tetramethyl-δ3-pyrroline-3-methyl)-methanethiosulfonate; NaPi, sodium phosphate; PRE, paramagnetic relaxation enhancement; $\Delta\delta_{avg}$, average CSP

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1.2. The cytochrome c peroxidase–cytochrome c complex

Encounter complexes are highly populated in complexes that represent a compromise between specific binding and high-turnover. Therefore, electron transport complexes are ideal candidates for studying the encounter complex as they require binding specific enough to allow for electron transfer but weak and transient enough to accommodate very high turn-over rates [22]. The electron transfer complex between yeast *iso*-1-cytochrome *c* (Cc) and yeast cytochrome *c* peroxidase (CcP) is a well characterized system for studying the encounter complex. It spends approximately 30% of the time in the encounter complex [5,15], which can be shifted to as low as 10% or as high as 90% with point mutations near the binding interface [23].

The solution structure of the CcP–Cc encounter complex was determined in 2006 by Volkov et al. using PRE effects generated in the ^{15}N-HSQC spectra of Cc by MTSL spin labels attached at five locations on the surface of CcP [15]. Although both of these proteins contain a paramagnetic haem group, the effects produced by these are not suitable for studying the complex. Therefore, MTSL spin labels were used to generate PREs, which provided restraints for docking of the proteins. The study demonstrated that the complex spends approximately 70% of the time in the stereospecific state found in the crystal structure [24] and 30% in other orientations representing the encounter complex. The model of the latter was later refined by Bashir et al. in 2010 by expanding the initial data to include PRE restraints from MTSL attached at ten sites on CcP. Back-calculated data from a theoretical encounter complex, generated using an electrostatics based Monte Carlo method, was compared to the experimental PREs. The additional data obtained allowed for the complete mapping of the conformational space sampled; Cc was found to sample only 15% of the CcP surface during complex formation [5], in line with the results from earlier theoretical studies [25,26].

The goal of the present study was to view the CcP–Cc encounter complex from "the other side" and validate the previously determined ensemble. The NMR resonances of the backbone amides of CcP (34.2 kDa) were assigned, which then allowed us to observe both chemical shift perturbations (CSP) and PRE effects in the NMR spectrum of CcP that were generated in the presence of spin-labelled Cc. We observe many effects similar to those previously reported for the complex as well as several novel interactions. These results show the importance of extending the available set of restraints as far as possible to increase the accuracy of an encounter complex description.

2. Material and methods

2.1. Sub-cloning of yeast CcP

The gene construct for *Saccharomyces cerevisiae* CcP C128A [15] was sub-cloned into a pET28a(+) vector. The gene was amplified using PCR with a 5′ primer containing a *Pci*I site (resulting in MSKT as the first four amino acids) and a 3′ primer containing an *Xho*I site. The fragment was cloned into a pET28a(+) vector cut with *Xho*I and *Nco*I, which are compatible with *Pci*I, yielding pET28aCcP. The sequence of the insertion was verified by DNA sequencing.

2.2. Expression and purification of CcP

The pET28aCcP plasmid was used to express and purify CcP in a protocol adapted from Refs. [27,28] with changes for labelled protein expression and the use of phosphate buffers, see Supplementary Methods for details. The concentration of CcP was determined using UV–Vis spectroscopy at ε_{408nm} = 98 mM^{-1}cm^{-1} and the coordination of the haem group was determined using several absorbance ratios [29].

2.3. Protein expression and purification of Cc

A pUC19 based plasmid containing the *S. cerevisiae* iso-1-cytochrome *c* gene was used to express and purify Cc as described previously [30,31]. The wild type (WT) protein and mutant V28C [9] were used. The concentration of Cc was determined using UV–Vis spectroscopy and ε_{410nm} = 106.1 mM^{-1}cm^{-1} [31]. The standard yield was approximately 20 mg/L in rich media for both WT and V28C Cc.

2.4. Spin-labelling

Samples of V28C Cc were labelled with either MTS [1-acetoxy-2,2,5,5-tetramethyl-δ3-pyrroline-3-methyl)-methanethiosulfonate] or MTSL [1-oxyl-2,2,5,5-tetramethyl-δ3-pyrroline-3-methyl)-methanethiosulfonate] (Toronto Research Chemicals, North York, ON, Canada) as described previously [15], see Supplementary Methods for details. The labelling efficiency was determined by mass spectroscopy to be essentially 100%.

2.5. NMR spectroscopy

2.5.1. CcP assignment

CcP appears to be stable at 20 °C for only 4–5 days, so several samples were required for the backbone assignment experiments. A large sample of 400 μM triple labelled [^{15}N, ^{13}C, ^{2}H] CcP was prepared in 20 mM sodium phosphate (NaPi), 100 mM NaCl, 6% D_2O, pH 6.0 and then aliquoted into several identical samples. A full set of protein amide backbone assignment experiments were recorded and processed at the Biomolecular Magnetic Resonance facility, Goethe University, Frankfurt. The data was processed using Topspin 3.1 (Bruker, Karlsruhe, Germany) and spectral assignment and analysis was done using CCPN analysis 2.1.5 [32]. See Supplementary Methods for details. NMR assignments have been submitted to the BMRB under entry number 19884.

2.5.2. Titration experiments

To obtain binding constants, 1.7–2.5 mM stocks of WT or MTS-V28C Cc were titrated into 400 μM double labelled [^{15}N, ^{2}H] CcP in 20 mM NaPi, 100 mM NaCl, 6% D_2O, pH 6.0. 2D BEST-TROSY-HSQC experiments [33] were recorded 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. Spectra were recorded at intervals of 0.2:1 Cc:CcP until a final ratio of Cc:CcP of 2.0:1 was reached. All data were processed using Topspin 3.2 (Bruker, Karlsruhe, Germany) and analysis was done using CCPN Analysis 2.1.5.

The average CSP ($\Delta\delta_{avg}$) were derived as described previously [34]. With the derived binding constants, it was calculated that 98% of WT or 99% V28C Cc was bound to CcP, in the sample with a 2:1 ratio of Cc:CcP. Therefore, in order to obtain $\Delta\delta_{avg}$ extrapolated to the 100% bound form, the respective $\Delta\delta_{avg}$ values were divided by 0.98 or 0.99. The chemical shift titration curves were analyzed with a two-parameter, non-linear least squares fit using a one-site binding model as described previously [35]. The fitting was done using OriginPro 8.5 (OriginLab, Northampton, USA).

2.5.3. Paramagnetic experiments

NMR samples contained 400 μM double labelled [^{15}N, ^{2}H] CcP in 20 mM NaPi, 100 mM NaCl, 6% D_2O, pH 6.0 with either 120 μM or 290 μM MTS(L)-V28C Cc. 2D BEST-TROSY-HSQC experiments were recorded and processed as described for titration

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