# Near-complete backbone resonance assignments of acid-denatured human cytochrome *c* in dimethylsulfoxide: a prelude to studying interactions with phospholipids

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## Abstract

Human cytochrome *c* plays a central role in the mitochondrial electron transfer chain and in the intrinsic apoptosis pathway. Through the interaction with the phospholipid cardiolipin, cytochrome *c* triggers release of pro-apoptotic factors, including itself, from the mitochondrion into the cytosol of cells undergoing apoptosis. The cytochrome *c*/cardiolipin complex has been extensively studied through various spectroscopies, most recently with high-field solution and solid-state NMR spectroscopies, but there is no agreement between the various studies on key structural features of cytochrome *c* in its complex with cardiolipin. In the present study, we report backbone $^{1}H$, $^{13}C$, $^{15}N$ resonance assignments of acid-denatured human cytochrome *c* in the aprotic solvent dimethylsulfoxide. These have led to the assignment of a reference 2D $^{1}H$-$^{15}N$ HSQC spectrum in which out of the 99 non-proline residues 87 % of the backbone amides are assigned. These assignments will be used in a subsequent interrupted H/D exchange strategy to map the binding site of cardiolipin on human cytochrome *c*.

**Key words:** human cytochrome *c*, apoptosis, cardiolipin, acid-denatured, DMSO

**Biological context**

Mitochondrial cytochrome *c* (cyt *c*) is a small (~ 12.5 kDa) soluble heme containing protein that plays a key role in life and death decisions of the cell (Huttemann et al. 2011). For life-sustaining energy production, cyt *c* acts as an electron carrier (redox protein) in the mitochondrial electron transfer chain (ETC) leading to the production of adenosine triphosphate (ATP). When problems caused by stress outweigh the benefits of sustaining viability of a cell, various signals can initiate the programmed cell death process through intrinsic (mitochondrial) type II apoptosis. In cells undergoing type II apoptosis, the phospholipid 1,3-bis(sn-3'-phosphatidyl)-sn-glycerol, commonly referred to as cardiolipin (CL) hijacks the ETC function of cyt *c* to create a protein-phospholipid complex in which cyt *c* is converted to a peroxidase enzyme through the dissociation of the axial Met80 heme ligand (Kagan et al. 2005). This new function of cyt *c* when bound to CL leads to the peroxidation of CL upon reaction of $H_2O_2$ with the cyt *c* heme, resulting in the dissociation of the complex. The modified CL then serves as a signal for downstream events that culminate in the permeabilisation of the outer mitochondrial membrane and subsequent release of pro-apoptotic factors, including cyt *c*, into the cytoplasm (Ow et al. 2008).

NMR spectroscopy is an amenable technique to study protein complexes, with complex interfaces being readily mapped through simple chemical shift perturbation experiments (Rajagopal et al. 1997). In recent times advances in site-specific isotope labelling methods and the development of paramagnetic probes have pushed the boundaries of conventional liquid-state NMR to encompass the study of large and dynamic complexes (Clore et al. 2007). The characterisation of phospholipid-bound soluble proteins is a challenge by liquid-state NMR. One approach that we are adapting to address this problem is to employ an interrupted H/D exchange method. This relies on the dissociation of the protein-phospholipid complex under the conditions that preserve the backbone amide protection patterns established in the complex by measuring the sample in an aprotic solvent, such as dimethysulfoxide (DMSO), preventing further H/D exchange. In this way information relating to the phospholipid binding site will be preserved and this can be extracted by comparing backbone amide protection factors in the absence of the phospholipid, creating an initial snapshot of the phospholipid binding site. A similar approach to determine the structure of amyloid fibrils (Hoshino et al. 2002), in protein-folding studies (Nishimura et al. 2005) and to delineate contact sites in a protein-protein complex (Dyson et al. 2008) have been reported.

Two recent NMR studies investigating the interaction of horse heart cyt *c* and CL have been reported. The first reports on a multidimensional magic angle spinning (MAS) solid-sate

NMR investigation with CL containing lipid-bilayers (Mandal et al. 2015) and the second is a liquid-state study with cyt *c* encapsulated in a CL-containing micelle (O'Brien et al. 2015). Surprisingly, both studies reveal only minimal chemical shift changes upon interaction with CL, implying no gross structural changes. This is opposed to FRET and other spectroscopic studies whereby large structural changes are inferred to occur on binding CL that drive the creation of the peroxidase form (Hanske et al. 2012; Hong et al. 2012; Muenzner et al. 2013). We aim to investigate the complex of human cyt *c* (hcyt *c*) and CL using a tailored interrupted H/D-exchange approach as outlined above. As a prelude to this we report here the near complete $^{1}H$, $^{15}N$, $^{13}C$ sequential assignments of acid-denatured hcyt *c* in DMSO.

**Materials and experiments**

*Sample preparation and NMR spectroscopy*

Over-expression and purification of uniformly-labelled $^{13}C$, $^{15}N$ wild-type hcyt *c* was carried out as previously described (Karsisiotis et al. 2015). Ferric hcyt c was prepared by the addition of 1.5-fold excess of $K_3[Fe(CN)_6]$ (Sigma) followed by removal of the excess $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ and desalted into 20 mM sodium phosphate pH 7 using a PD-10 column (GE-Healthcare). Samples were then concentrated using 5 kDa cut-off concentrators, acid-quenched with 0.3 % v/v trifluoracetic acid (TFA) (Acros Chemicals), flash frozen, freeze-dried overnight and stored at -20 $^{o}$C. Lyophilised samples were dissolved in 100 % DMSO (Sigma), supplemented with 0.2 % v/v TFA to aid solubility during resuspension, to a final volume of 500 μl and a concentration of ~ 1 mM. All NMR experiments were performed at 308 K and acquired on a Bruker 800 MHz spectrometer equipped with a 5 mm HCN inverse triple resonance z-axis gradient probe. Data were processed with either Topspin (Bruker Biospin) or the NMRPipe and NMRDraw package (Delaglio et al. 1995) or a combination of both programs. Data analysis was performed with the graphical NMR assignment program Sparky (Goddard and Kneller 2008) and the CCPNmr Analysis program (Vranken et al. 2005).

*Assignments strategy*

The NMR method we have been developing to investigate the interaction of hcyt *c* with CL is based around an interrupted H/D-exchange approach. To arrest H/D exchange the pH of the sample is acidified through the addition of TFA, which also acts as a counter-ion in enabling the freeze-quenched sample at a set-exchange time point, to be more easily dissolved in the aprotic solvent DMSO. As expected therefore, the amide proton chemical shift dispersion of

acid-denatured hcyt *c* in DMSO falls into a very narrow spectral width (Figure 1). This caused severe overlap in traditional sequence specific assignment experiment pairs, such as the 3D HNCACB/CBCA(CO)NH spectra. Therefore, experiments relying on carbonyl (*i*) and (*i-1*) connectivities, were additionally acquired, such as the HNCO/HN(CA)CO pair. However, these experiments are also susceptible to the narrow amide proton spectral width and were thus only used to corroborate existing assignment steps. An essential component of the assignment strategy to tackle resonance overlap in an unfolded protein was the use of the HNN experiment (Panchal et al. 2001). This provides (*i-1*), (*i*) and (*i+1*) connectivities and exploits the $^{15}$N chemical shift dispersions which are less affected in unfolded proteins, compared to folded proteins. Specifically, for any $^{1}$H-$^{15}$N plane intra-residual (*i*), sequential (*i-1*) and (*i+1*) $^{15}$N peaks are provided, thus connecting any amide with the amide nitrogen chemical shift of the preceding and the following residue. In addition, a very important secondary source of information is that, in these peak triplets, the sign of the diagonal (*i*) and sequential (*i-1*) and *i+1*) peaks is dependent on the nature of adjacent residues (glycines and prolines particularly) (Panchal et al. 2001). This feature provided additional sequence specific assignment checkpoints or starting points to use in conjunction with the traditional triple resonance experiment pairs. While the HNN experiment was particularly useful in the identification or verification of possible assignments, it was also susceptible to resonance overlap and poor spectral quality for some residues.

*Extent of resonance assignments*

Backbone amide proton and nitrogen resonance assignments were obtained for 87 % of the 99 non-proline residues in hcyt *c* (Figure 1). This compares to the 98 % of backbone amide resonances assigned in hcyt *c* under native conditions (Karsisiotis et al. 2015). Resonances which were not assigned in the present work are: K7, C17, T19, E66, Y67, Y74, I75, M80, K87, K88, E89, E90 and K100. These reflect either resonances not observed or severe overlap that cannot be resolved. The assignments for K8, H18, L68 and E69 have more ambiguity but they were included in the current dataset. This set of assignments are being used in conjunction with the development of an interrupted H/D exchange strategy to obtain both dynamic and structural information regarding the cardiolipin binding site on hcyt *c*. Encouragingly a good number of 2D $^{1}$H-$^{15}$N HSQC assignments, between 40 to 50, are transferable between data sets, subject to small chemical shift perturbations when the acid-quench and DMSO conditions vary. However, in the highly-overlapped region of the spectrum ($^{15}$N: 115-121 ppm and $^{1}$H:7.6-8.3 ppm), assignment transfer becomes difficult or impossible. Therefore, upon finalising

exchange method conditions, a new set of triple resonance experiments is required, but the current assignments greatly speed up assignments of the new dataset. Alternatively, assignments can be transferred through a $^{1}H$-$^{15}N$ HSQC titration between the initial and final conditions. Finally, a factor that greatly enhances the usability of the current assignment set is that the proposed site(s) of CL interaction does not encompass the residues for which assignments are missing (O'Brien et al. 2015).

*Data bank deposition*

Backbone ($H^{N}$, N, CA, CB CO) resonance assignments have been deposited in the BioMagResBank database under the accession number 26973.

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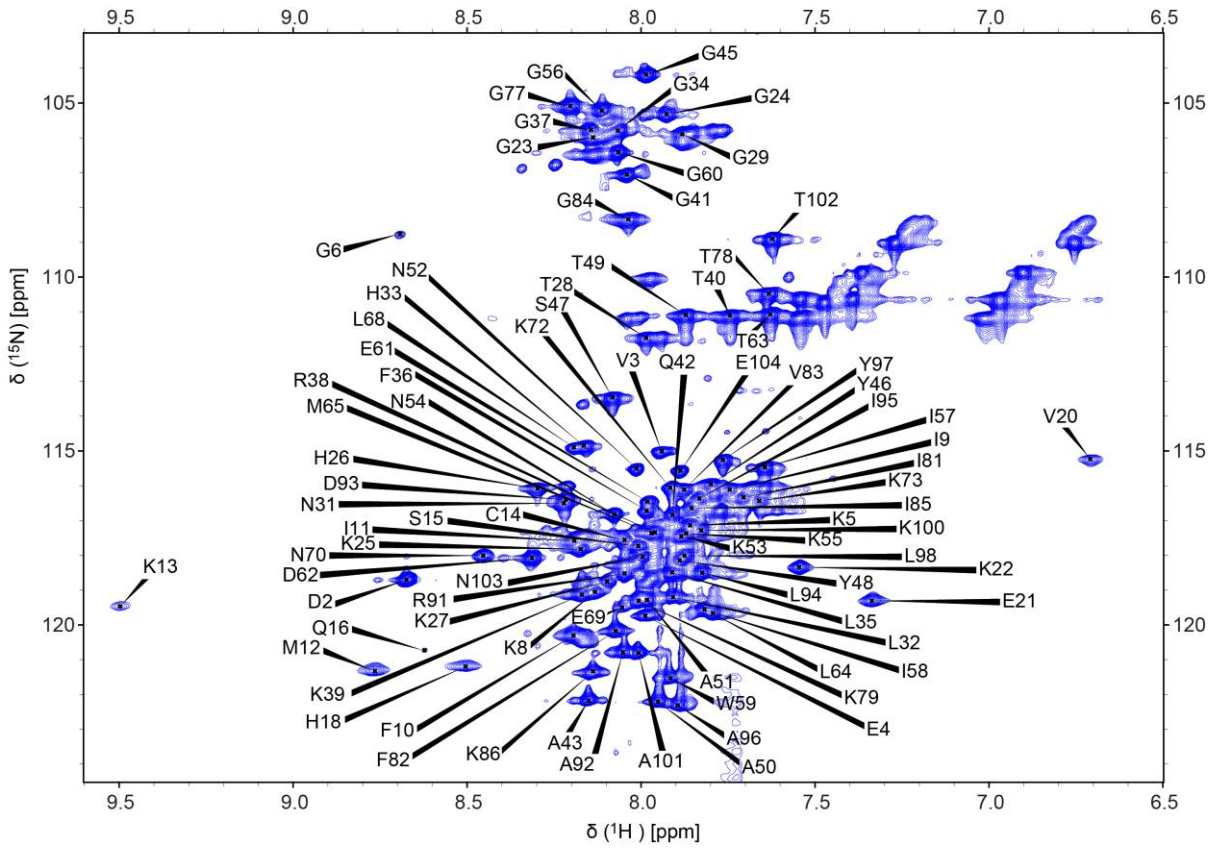


**Figure 1:** [$^{1}$H, $^{15}$N]-HSQC spectrum (308 K) of acid-denatured hcyt *c* in the aprotic solvent DMSO. The resonance assignments of the backbone HN resonances are indicated.