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Communication

# Photo-CIDNP NMR spectroscopy of a heme-containing protein

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**Abstract**

There are relatively few examples of the application of photo-CIDNP NMR spectroscopy to chromophore-containing proteins. The most likely reason for this is that simultaneous absorption of light by the photosensitiser molecule and the protein chromophore reduces the effectiveness of the photochemical reaction that produces the observed nuclear polarisation. We present details of experiments performed on the air-oxidised form of a small cytochrome, from the thermophilic bacterium *Hydrogenobacter thermophilus*, using both the wild-type protein and apo and holo forms of a double alanine *b*-type mutant. We show that, along with the apo state, it is possible to generate CIDNP in the air-oxidised form of the *b*-type mutant, but not in the corresponding *c*-type cytochrome. This finding is supported by control experiments using horse-heart cytochrome *c*.


*Keywords:* Photo-CIDNP; Cytochrome $c_{552}$

## 1. Introduction

Photo-CIDNP[3] is a powerful technique for studying the solvent accessibilities of certain aromatic amino acid residues on the surface of a protein [1–9]. The observed nuclear polarisation is generated by a photochemical reaction between a suitable photosensitiser, usually a flavin, and the side chains of solvent-accessible histidine, tyrosine, and tryptophan residues. Laser illumination generates an excited triplet-state flavin which then forms a spin-correlated radical pair with the amino acid side chain, either by electron transfer or hydrogen abstraction [10]. The radical pair undergoes coherent oscillations between singlet and triplet electronic spin states, modulated by hyperfine couplings in the radicals. It is these modulations which produce the non-Boltzmann nuclear spin populations detected in the intact, diamagnetic amino acid side chains following recombination of the radical pair [9]. The use of this technique to study chromophore-containing proteins has been limited due to absorption of the laser light by the chromophore, decreasing the number of triplet flavin molecules produced, and thus reducing the efficiency with which nuclear polarisation is generated. Hori et al. [11] have published a photo-CIDNP investigation of the air-oxidised forms of rabbit and bovine cytochrome $b_5$. This appears to be the only observation of photo-CIDNP enhancements of holo cytochrome proteins although the technique has been used to study the apo states of several cytochromes and cytochrome-derived peptides [12].

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[3] *Abbreviations used:* Photo-CIDNP, photo-chemically induced dynamic nuclear polarisation; NMR, nuclear magnetic resonance; FMN, flavin mononucleotide (oxidised).

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In this communication we present details of experiments performed using cytochrome $c_{552}$ from *Hydrogenobacter thermophilus*. Cytochrome $c_{552}$ is a small globular protein containing 80 amino acid residues with a molecular mass of 9.1 kDa, including the heme moiety. The wild-type $c_{552}$ protein binds the heme moiety covalently using a CXXCH sequence motif through thioether linkages involving Cys 10 and Cys 13, along with axial coordination of the heme iron by His 14 and Met 59 [13]. It has been shown previously that mutation of either of these cysteine residues has little effect on the physico-chemical properties of the protein [14]; replacement of both cysteine residues with alanine (C10A/C13A) results in a *b*-type cytochrome which binds the heme group non-covalently via axial ligation of the heme iron. Previous studies have shown that the holo *b*-type protein retains a native-like fold, but is reduced in stability relative to the *c*-type protein. This is indicated by a decrease of 40 K in the thermal denaturation temperature and a lowering of the unfolding midpoint in denaturant titration experiments [15–17]. It has also been shown that the holo C10A/C13A mutant slowly and spontaneously loses the heme group, and subsequently undergoes amyloid fibril formation over a period of several weeks [18]. The apo state of the protein is characterised by a decrease in helicity of 29% and an increase of 8.5% in the hydrodynamic radius, with respect to the holo form of the C10A/C13A mutant [16]. Both of these points and evidence from NMR spectroscopy [16,17] show that the apo state of the mutant can be characterised as a partially folded species with some molten-globule like characteristics which undergoes conformational fluctuations on a millisecond timescale. These observations are in close agreement with a similar investigation of the differences between the apo and holo forms of both cytochrome *c* [19] and myoglobin [20]. Addition of free heme to a solution of the apo C10A/C13A mutant protein results in the spontaneous formation of the *b*-type holo species, suggesting that in the apo state there is a nascent heme binding pocket [14,16]. This in is contrast to cytochrome *c* in which the addition of heme to the apo protein only results in partial restoration of the native structure [19]. An NMR-based comparison of the holo C10A/C13A mutant and the wild-type $c_{552}$ protein shows that there are only very minor differences in both helical secondary structure and amide protection, indicating that the holo mutant and wild-type proteins have similar tertiary folds [17].

The holo mutant and wild-type proteins both contain a heme group whose oxidation state affects the properties of the proteins and consequently their NMR spectra. In the reduced state of the protein the heme iron is present as low-spin, diamagnetic Fe(II) ($S = 0$), while in the air-oxidised protein it is low-spin paramagnetic Fe(III) ($S = 1/2$). Only small changes in the observed chemical shifts between the oxidised and reduced states of horse-heart cytochrome *c* have been reported for protons which are not in close proximity of the heme group [20–25], indicating that the structural effects of the paramagnetic centre and its influence on the NMR line widths are likely to be minimal. It would therefore be of interest to compare the CIDNP spectra of the oxidised and reduced forms of the holo C10A/C13A mutant and the wild-type cytochrome $c_{552}$. However, to date, it has not been possible reliably to produce the reduced forms of these proteins under conditions compatible with photo-CIDNP measurements, maintaining the oxidised form of the flavin photosensitiser. Indeed, it has been reported recently that cytochrome $c_{552}$ undergoes autoxidation in the absence of excess amounts of reductant [26]. All photo-CIDNP experiments described in this communication were performed on the air-oxidised species.

The presence of the heme group also results in the characteristic blood-red colour of aqueous solutions of the protein which, at the concentrations and wavelengths (488 and 514 nm) used to record the CIDNP spectra, have a similar optical density to the flavin solution used as the CIDNP photosensitiser, being at most a factor of two larger than that of FMN. The similarity in optical density at these wavelengths means that a significant portion of the light entering the sample is not absorbed by the flavin, leading to significantly smaller nuclear polarisations than would be observed in the case of the apo state of the protein.

## 2. Results and discussion

Of the 80 amino acids in cytochrome $c_{552}$, there are six which are potentially amenable to study by photo-CIDNP NMR spectroscopy: His 14, Tyr 25, Tyr 32, Tyr 41, Trp 54, and Trp 75. Fig. 1 shows the solution state NMR structure of the wild-type protein [13], with these six side chains and the heme moiety with its cysteine linkages and axial ligands highlighted. In the native state His 14 is involved in axial ligation of the heme moiety and in a π–π stacking interaction with Tyr 32. Interestingly, the native form of the wild-type protein contains a high density of hydrophobic residues in its core [27]. This has been postulated to be responsible both for the thermostability of the protein and for the compact nature of the apo state of the C10A/C13A mutant [16]. The formation of compact hydrophobic cores is a common structural feature of partially folded proteins [28–32].

Table 1 shows the solvent accessibility of the residues of interest. These data were calculated using the program Naccess [33,34], using the wild-type protein structure (PDB File: 1AYG [13]). Solvent accessibilities can be used to aid the interpretation of CIDNP spectra, since the formation of a radical pair which is a prerequi-

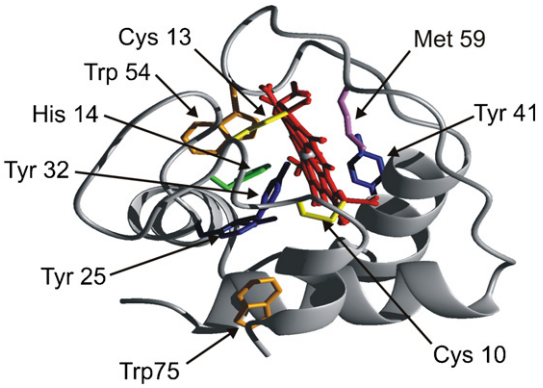


Fig. 1. The NMR structure of cytochrome $c_{552}$ from *H. thermophilus* (PDB File: 1AYG [13]). The potentially CIDNP-observable residues are highlighted with tyrosine in blue, tryptophan in orange, and histidine in green. The heme group is drawn in red, with the cysteine-thioether links (Cys 10 and Cys 13) shown in yellow. The heme coordinating methionine (Met 59) is also shown in violet. The figure was drawn using the MOLMOL program [40] and rendered using POVRAY (http://www.povray.org).

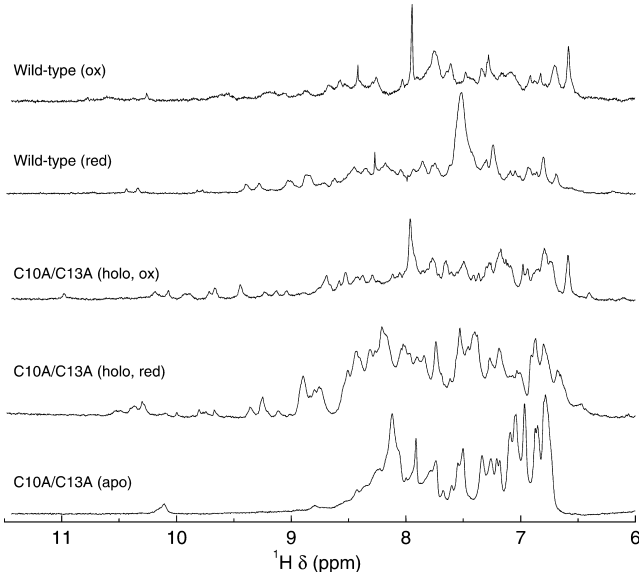


Fig. 2. Partial 600 MHz $^1$H NMR spectra of the C10A/C13A mutant and wild-type proteins. The solutions contained 200 μM protein and 200 μM FMN in 90% $H_2O$/10% $D_2O$, and a trace of dioxane to provide a chemical shift reference at 3.75 ppm. The wild-type and C10A/C13A mutant were expressed in *Escherichia coli* using the methodology described in [15]. The apo protein was prepared as described in [16]. The reduced state samples were prepared using an excess of sodium dithionite. The sample temperature was maintained at 298 K using a Peltier thermoelectric device [41]. All NMR data were recorded using a spectral width of 8000 Hz and acquired with 4096 complex points. The data were apodised with 2 Hz exponential line broadening prior to Fourier transformation. The spectra were processed using the NMRPipe package [42].

site for the generation of the nuclear polarisation, requires the excited triplet-state flavin and the amino acid side chain to come into close proximity.

The aromatic and NH region of the proton NMR spectra of both forms of the mutant protein and the wild-type protein is shown in Fig. 2. The spectra of the wild-type protein and the holo state of the mutant (upper and central traces in Fig. 2, respectively) show reasonably sharp, resolved resonances, indicative of small folded proteins. Comparing these with the corresponding reduced state spectra in Fig. 2, it is evident that the influence of the paramagnetic heme group is small. However, the apo state (lower trace in Fig. 2) shows decreased chemical shift resolution compared to the heme-containing proteins. Since, as described above, the apo state of the mutant has some molten-globule like character, this is attributed to an ensemble of conformers interconverting on a millisecond timescale [16,20,31].

The upper trace of Fig. 3 shows the CIDNP spectrum of the wild-type protein, for which little, if any, polarisation can be seen. Similar experiments performed using the air-oxidised form of horse-heart cytochrome *c* also fail to show any nuclear polarisation. Interestingly, CIDNP can be observed if the horse-heart cytochrome *c* is reduced to the diamagnetic state using sodium dithionite, followed by diafiltration under anaerobic conditions and addition of degassed FMN (data not shown). The exact reasons for these differences between the oxidised and reduced forms of the protein, which only differ in optical density by a factor of two, remain unclear. However, it is possible that quenching of the triplet flavin by the heme moiety is more efficient in the air-oxidised forms of the *c*-type cytochromes, than it is in the *b*-type. There is also the possibility that coupling between the paramagnetic heme group and one or both of the transient radicals interferes with the coherent singlet-triplet interconversion in the radical pair. One can speculate that subtle changes in the electronic properties of the heme group arising from the different bonding to the protein may be responsible.

The central trace in Fig. 3 is the CIDNP spectrum of the heme-containing holo variant of the C10A/C13A mutant. Nuclear polarisation is observed albeit with a low signal-to-noise ratio due to the absorption of a por-

Table 1
Relative static accessibility calculations

| Residue | His 14 | Tyr 25 | Tyr 32 | Tyr 41 | Trp 54 | Trp 75 |
|---|---|---|---|---|---|---|
| Relative accessibility (%)[a] | 0.4 | 0.1 | 13.0 | 39.7 | 14.0 | 21.4 |

[a] Calculations were performed on the wild-type cytochrome $c_{552}$ using the solution-state NMR structure (PDB File: 1AYG [13]). The values were calculated with the Naccess program [33,34], using a probe radius of 1.4 Å and are quoted relative to the accessibility calculated for an extended conformation of the tripeptide AXA, where X is the residue of interest.

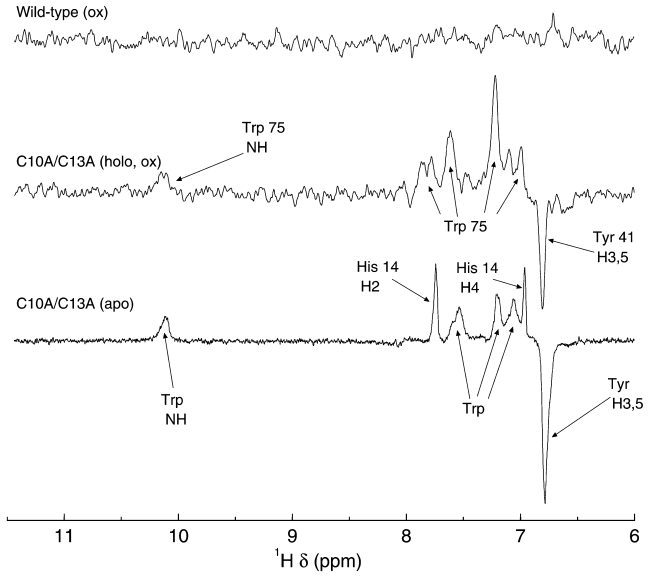


Fig. 3. Partial 600 MHz $^{1}$H photo-CIDNP spectra of the C10A/C13A mutant and wild-type proteins. The solutions used were the same as those in Fig. 2. The apo state spectrum was apodised with 2 Hz exponential line broadening prior to Fourier transformation, while the holo state and wild-type spectra were apodised using 10 Hz line broadening. The samples were illuminated using light from a Spectra-Physics Stabilite 2016-05 argon ion laser, operating in multiline mode, with a power output of 5 W at the laser head (principal wavelengths at 488 and 514 nm). The light was guided to the sample via of a 1 mm diameter optical fibre (Newport Optics, F-MMC), held 4 mm above the radiofrequency coil by the use of a coaxial insert (Wilmad, Model WGS-5BL) [43]. The light was chopped into pulses of 100 ms duration by a spectrometer-controlled mechanical shutter (NM-Laser products, 200 FNC). The spectra shown are the difference between 16 scans with laser pulses and 16 dark scans, hence only polarised resonances are observed.

tion of the incident laser light by the heme group. Given that photo-CIDNP spectra are much less crowded than the corresponding NMR spectra, it should be possible to assign the observed polarised signals to specific amino acid residues in the protein. Also, since the NMR structure of the wild-type protein is known, the complete assignment is also available albeit for the reduced form. This information can be used as a guide in assigning the CIDNP spectrum, especially for residues far removed from the heme centre in the case of the holo proteins. The assignment of the observed resonances can be made by reference to the static accessibility data and by comparison with chemical shift assignments obtained from three-dimensional (3D) $^{15}$N-edited TOCSY–HSQC and NOESY–HSQC experiments on $^{15}$N-labelled reduced state holo C10A/C13A protein [17] and the wild-type $c_{552}$ [13]. These 3D NMR experiments have enabled the backbone amide protons and the indole NH protons of the tryptophan residues to be assigned. The similarity of the chemical shift of the indole NH of Trp 75 in the reduced state (10.23 ppm), to the broad singlet seen at 10.11 ppm (Fig. 3) allows this resonance to be assigned accordingly. It then follows that a number of absorptive signals in the aromatic region from 8.0 to 6.8 ppm can be assigned to the indole ring system of the same tryptophan residue. The second tryptophan residue, Trp 54, is not expected to be polarised, since, according to the static accessibility calculations (Table 1), it is not significantly exposed to the solvent. However, the number and intensity of the signals in the aromatic region would seem to indicate that there may be some contribution from Trp 54 in addition to Trp 75.

Tyrosine residues in photo-CIDNP spectra of proteins are characterised by the emissive phase of the polarisation produced for the H3,5 ring protons. The static accessibilities in Table 1 indicate that the strong emissive signal at 6.81 ppm is most probably Tyr 41. The other tyrosine residues have much lower solvent accessibility and are unlikely to be able to undergo the electron transfer reaction required to produce CIDNP. His 14 is not observed as it is involved in axial ligation of the iron and interaction with other hydrophobic residues in and around the heme binding pocket, such as Tyr 32.

Lacking a heme group, the apo state is colourless and therefore presents no problems for the generation of nuclear polarisation. The photo-CIDNP spectrum of the apo mutant is shown as the bottom trace of Fig. 3, with a clearly increased signal to noise ratio compared to the holo C10A/C13A mutant. The assignments of the apo state are not available. However, the chemical shifts of the signals seen in the CIDNP spectrum of this state are close to the random coil chemical shift values of the Trp, Tyr, and His aromatic protons [35]. We can therefore conclude that in the CIDNP spectrum we are observing at least one of both tryptophan residues (Trp 75 and maybe also Trp 54) and one or more of the tyrosine residues in cytochrome $c_{552}$ (Tyr 41 and maybe also Tyr 25 and Tyr 32). There is only one histidine in the protein and so the singlets observed at 7.75 and 6.96 ppm, characteristic of the H2 and H4 ring protons of histidine, can be assigned to His 14, which lies in the heme binding pocket. As described above, previous studies have postulated that this pocket is at least partially formed in the expanded apo state [16]. This therefore suggests that the nascent binding pocket is occupied transiently by an excited triplet-state flavin molecule which produces the observed nuclear polarisation.

## 3. Conclusions

In this communication we have demonstrated that it is possible to generate photo-CIDNP in a heme-containing protein, namely the holo state of the C10A/C13A mutant of cytochrome $c_{552}$, but not in the wild-type protein. The reasons for this difference are probably related to the different electronic properties of the prosthetic

heme group, which may influence quenching of the triplet flavin, or interaction of the heme group with the transient radicals involved in the radical pair reaction.

Comparison of the CIDNP spectra of the apo and holo C10A/C13A mutants shows that there are some similarities between the two proteins, indicating that a compact fold is adopted by the apo state which closely resembles that of the native state for the holo protein. Nuclear polarisation of the ring protons of His 14 in the apo state of the C10A/C13A mutant indicates that the absence of the heme moiety from the binding pocket may, in some conformations, allow its transient occupation by an excited flavin molecule. These results corroborate previous experiments which suggest that the heme binding site is nascently formed in the apo protein [16].

Having demonstrated that under certain conditions, CIDNP can be detected in heme-containing proteins, there is the possibility of using photo-CIDNP to probe the folding of these proteins and to study the ligand binding properties. The coupling of the photo-CIDNP methodology to rapid mixing techniques within the NMR magnet has been used previously to study protein folding in real-time [8,36,37]. Ligand-induced folding in conjunction with NMR, and photo-CIDNP, has been applied to a number of systems including bovine α-lactalbumin [4,38,39] and such methods could be used to study structural changes in the *b*-type cytochromes associated with heme incorporation.

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