Mapping the cytochrome c_{553} interacting site using ^{1}H and ^{15}N NMR

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Abstract Cytochrome c_{553} is the electron transfer partner of formate dehydrogenase and of [Fe]-hydrogenase, two metalloenzymes essential in the metabolism of sulfate reducing bacteria. These two enzymes contain a 'ferredoxin-like' domain which presents 30% identity with *Desulfovibrio desulfuricans* Norway ferredoxin I. This was chosen as a model for the 'ferredoxin-like' domain involved in the electron transfer reaction with cytochrome c_{553}. 1D NMR titration of complex formation gave us the stoichiometry (1:1) and the dissociation constant of the complex (K_d $\sim 3\times10^{-6}$ M). 2D heteronuclear NMR experiments were performed to analyze the ^{1}H and ^{15}N chemical shift variations that are induced by the protein-protein recognition. This is the first mapping of the interaction site on a *c*-type cytochrome, using heteronuclear NMR.

Key words: Cytochrome c_{553}; Ferredoxin; ^{1}H and ^{15}N NMR; Protein-protein interaction

1. Introduction

Sulfate reducing bacteria are anaerobic organisms able to reduce sulfate, sulfite or thiosulfate to sulfide. For growth, these bacteria use a number of substrates such as lactate, formate, hydrogen, ethanol, pyruvate, fumarate or malate. The electron carriers associated with this anaerobic metabolism have been extensively studied from the primary dehydrogenase, such as lactate or formate dehydrogenase, to the terminal electron acceptor, sulfite or sulfate reductase. The electron transfer chains associated with these reactions are characterized by the presence of low oxidoreduction potential cytochromes. The monohemic cytochrome c_{553} is similar to mitochondrial and photosynthetic cytochromes. The two axial ligands are methionine and histidine residues as generally found in the monohemic cytochromes *c*. However, the oxidoreduction potential is surprisingly low (40 mV). We are currently examining structural features of this molecule important in determining its reduction potential, and we have demonstrated that the low oxidoreduction potential of this cytochrome is correlated with the high exposure of the heme to the solvent [1]. The electron exchange of this molecule has been studied with two oxidoreduction partners, formate dehydrogenase [2] and [Fe]-hydrogenase [3].

Desulfovibrio desulfuricans Norway (*Dd*N) ferredoxin I presents 60% homology and 30% identity with the 'ferredoxin-like' domain of *Desulfovibrio vulgaris* Hildenborough (*Dv*H) [Fe]-hydrogenase and 27% identity with the N-terminal sequence of *Escherichia coli* formate dehydrogenase β subunit. Kinetic measurements have established that *Dd*N ferredoxin I reduces *Dv*H cytochrome c_{553} in the presence of pyruvate dehydrogenase and pyruvate [4]. For these reasons, we chose *Dd*N ferredoxin I as a model for the 'ferredoxin-like' domain of the two enzymes in order to study the general features of the protein recognition. We have applied a diversified approach using 1D and 2D heteronuclear NMR to map the interacting site on the cytochrome c_{553}.

2. Material and methods

2.1. Protein purification

Ferredoxin I was purified from *Dd*N as previously reported [5]. *Dv*H cytochrome c_{553} was expressed and purified from *Desulfovibrio desulfuricans* G200 as previously described [6]. The ^{15}N labeling of cytochrome c_{553} was obtained from 3 l of recombinant *D. desulfuricans* G200 (pRC41) grown on Postgate C medium where yeast extract was replaced by ^{15}N-Celtone powder [7].

2.2. NMR samples

For heteronuclear NMR experiments the labeled cytochrome c_{553} was concentrated to 0.1 mM, in potassium phosphate buffer 30 mM, pH 5.9, 10% D_2O. Ferredoxin was concentrated to 10 mM in 30 mM phosphate buffer pH 5.9, on an Amicon microconcentrator (Centricon). In these conditions the two proteins are at the pH previously used for proton assignment of cytochrome c_{553}. Titration of complex formation was obtained by addition of a ferredoxin aliquot to the cytochrome sample.

2.3. NMR spectra

NMR spectra were recorded on a Bruker Avance DRX 500 spectrometer at 296 K. The NMR spectra were processed with UXNMR and Aurelia software provided by Bruker. 1D NMR spectra were obtained with a water line presaturation during the relaxation delay. 2D ^{1}H-^{15}N HSQC spectra were recorded using a watergate pulse sequence in the TPPI mode. The spectral widths are 10 000 Hz for ^{1}H and 2534 Hz for ^{15}N. 2048 data points in t_2 and 32 transients for each 256 t_1 were used. ^{1}H chemical shifts were referenced to the H_2O resonance calibrated at 4.792 ppm at 296 K, relative to the sodium trimethylsilyl-2,2,3,3-($2H_4$) propionate. ^{15}N chemical shifts were referenced indirectly using the above ^{1}H reference and the gyromagnetic ratio (0.101329118) [8]. The ^{1}H and ^{15}N chemical shifts of oxidized cytochrome c_{553} previously reported [7,9] were used for the resonance assignments in 1D and 2D experiments.

2.4. Chemical shift analysis

Parameters of complex formation were determined by calculating the relative induced shifts ($\Delta\delta_{ij}/\Delta\delta_{t}$, where $\Delta\delta_{ij}$ is the induced chemical shift variation and $\Delta\delta_{it}$ is the maximal induced chemical shift variation) as a function of the cytochrome c_{553}/ferredoxin ratio. Chemical shift variations obtained from 1D experiments were deduced from two different sets of data with 0.15 mM ferredoxin and 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2 and 3 equivalents of cytochrome. Chemical shift variations observed in 2D heteronuclear experiments were obtained from a single set of experiments at 0.1 mM cytochrome c_{553} concentration and 0.5, 1 and 2 equivalents of ferredoxin.

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Abbreviations: NMR, nuclear magnetic resonance; HSQC, heteronuclear single quantum coherence; *Dd*N, *Desulfovibrio desulfuricans* Norway; *Dv*H, *Desulfovibrio vulgaris* Hildenborough

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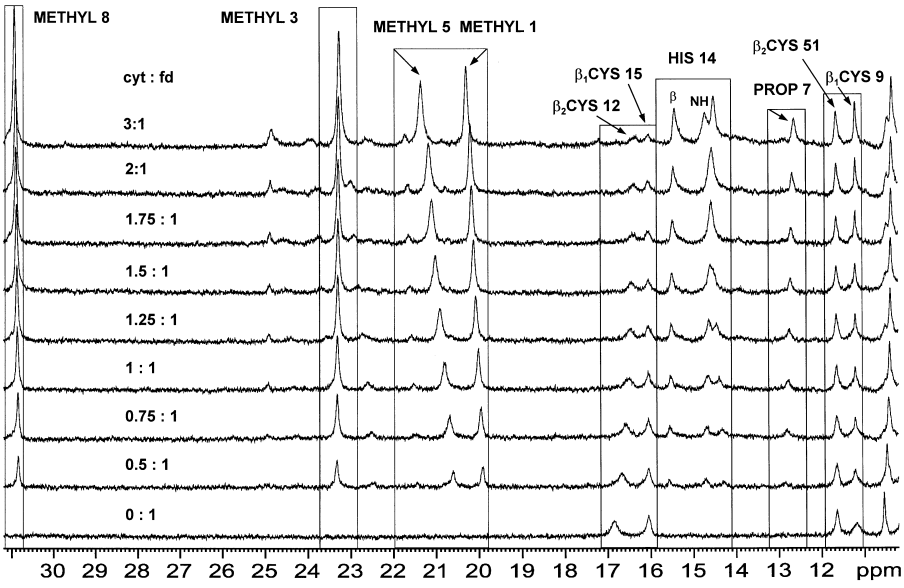

Fig. 1. 1D NMR titration of ferredoxin-cytochrome c_{553} complex formation. The experiment was performed on a Bruker DRX 500 spectrometer using a ferredoxin solution at 0.15 mM concentration and various concentrations of cytochrome in a 30 mM phosphate buffer pH 5.9 at 296 K. Only the 31–10 ppm part of the spectra, containing the protons of the cytochrome c_{553} heme (Me1, 3, 5 and 8 and propionate 7), the protons of the fifth heme axial ligand H14 and the β protons of the four cysteine ligands of the ferredoxin (4Fe-4S) cluster, are shown.

3. Results

3.1. 1D NMR analysis of the complex formation

The heme methyl lines of *Dv*H cytochrome c_{553} are particularly well resolved and were previously assigned at 296 K (Me1, 20.69 ppm; Me3, 23.40 ppm; Me5, 22.11 ppm and Me8, 30.70 ppm) [9]. The resonances of the cysteines bonded to the [4Fe-4S] cluster of *Dd*N ferredoxin I were also previously assigned (C9β1, 11.25 ppm; C12β2, 16.87 ppm; C15β1, 16.08 ppm and C51β2, 11.69 ppm) [10]. Any changes to these proton environments induce chemical shift variations in the 1D NMR spectra.

Fig. 1 shows the 1D NMR titration of the complex formation. Addition of cytochrome c_{553} to ferredoxin I provides proton chemical shift variations. The βCH_2 of C12 is particularly affected by complex formation ($\Delta\delta$ 0.55 ppm). The resonance corresponding to Me5 of the cytochrome is highly affected ($\Delta\delta$ 1.51 ppm). Calculating the titration curve plotted from the chemical shift variations of the ferredoxin C12β, one can obtain the stoichiometry (1:1) and the dissociation constant ($K_d \sim 3\times10^{-6}$ M) of the complex. It is necessary to keep in mind that the concentrations used in these experiments (0.15 mM) are too high to determine lower values.

3.2. 2D NMR titration of the complex formation

In the ^{1}H-^{15}N HSQC experiment the correlation peaks of

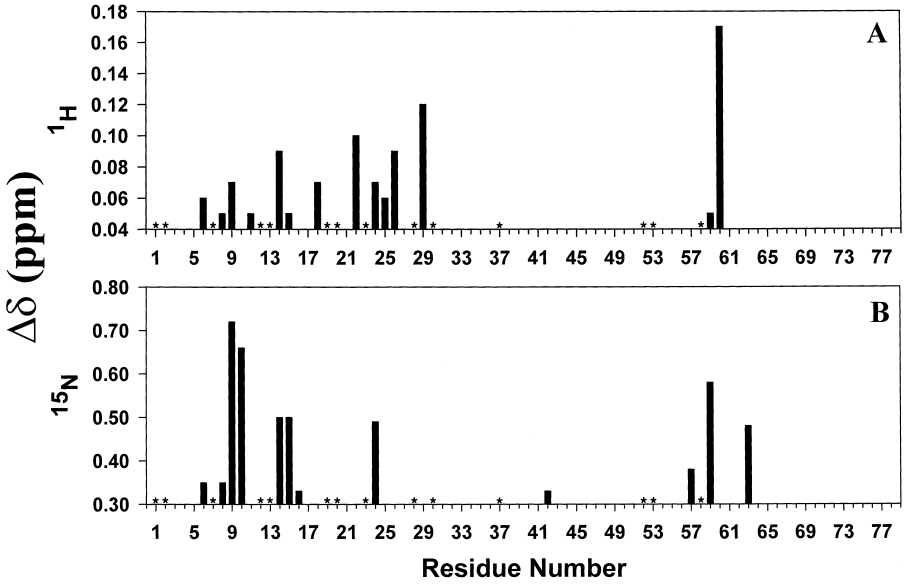

Fig. 2. ^{1}H and ^{15}N chemical shift variations observed on the NH groups of cytochrome c_{553} in 2D NMR experiments in the presence of two excesses of ferredoxin. Stars label non-assigned residues (1, 2, 12, 13, 19, 20, 23, 28, 52 and 58) and non-observable residues in the complex (7, 30, 37 and 53).

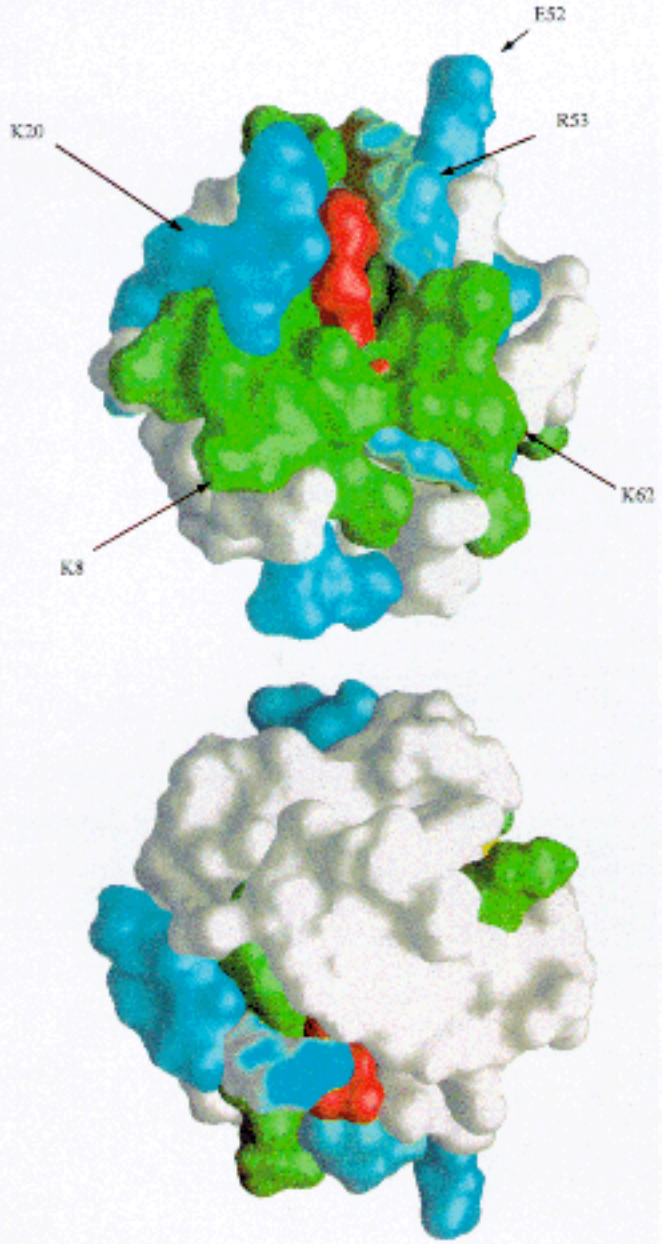

Fig. 3. Structural representation of the cytochrome c_{555} interacting site. In the top figure, the residues found to be involved in the interaction by heteronuclear experiments are represented in green. The amino acids colored in blue were either not assigned in the heteronuclear spectra or underwent proton resonance overlaps, which prevents analysis. However, these amino acids might be involved in the interacting site. The heme is represented in red and the amino acids which are not involved in the complex formation are in white. The bottom figure is a 180° rotation along the z axis and represents the 'back side' of the molecule.

NH groups are observable and have been previously assigned [7]. Only the NH groups of the ferricytochrome are observed, and no signal of the ferredoxin is present. Fig. 2 summarizes the measured chemical shift variations. The ^{1}H chemical shift variations do not exceed 0.18 ppm (Fig. 2A) while ^{15}N chemical shift variations are up to 0.75 ppm (Fig. 2B). However, for a residue the ^{15}N resonance frequency might be affected while the ^{1}H is not (V29) and vice versa (K63). Four parts of the molecule are modified by the presence of ferredoxin. They include amino acids 6–10, 13–16, 22–30 and 56–63. The amide proton resonance of the fifth heme axial ligand (H14) is particularly shifted. This result is in agreement with the involvement of H14, which was shown in the 1D experiments (Fig. 1).

3.3. Structural representation of the cytochrome c_{555} interacting site

Fig. 3 (top) represents the mapping of the interacting site obtained from chemical shift variation analysis. The 1D NMR experiments provide information concerning the extent of complex formation around the metallic center (see the heme in red), and thus give the orientation of the molecule in the complex. The 2D heteronuclear NMR experiments (HSQC) allow us to identify 85% of the NH of the backbone of the molecule. In the 3D structure the four zones are close together and form the 'front side' of the molecule (in green). Fig. 3 (bottom) reveals that the 'back side' of the molecule is not affected by complex formation (in white).

4. Discussion

Even though numerous X-ray or NMR structures of metalloproteins are now available in the Protein Data Bank, only a few electron transfer complex structures have been solved. One of the limitations is certainly the protein co-crystallization. NMR is helpful for studying protein interactions, but the factor that limits the method is the molecular weight. Molecular expression of structural domains is currently being developed to decrease the size of the complexes. In the present work, ferredoxin I was used as a model for the 'ferredoxin-like' domain of hydrogenase and formate dehydrogenase.

Another problem with electron transfer systems is the short lifetime of the productive complexes. Therefore structural studies can only be carried out on an unproductive complex where both partners are in the oxidized form. However, in most of the structural studies which concern electron transfer complexes, it is assumed that the interacting site is the same in the productive and unproductive complexes. For 20 years, 1D homonuclear NMR methods have been applied for studying complex formation involving cytochromes such as cytochrome c_3 [11], plastocyanin [12] and cytochrome b_5 [13]. 2D homonuclear NMR experiments have been developed in the last decade for structural analysis of these electron transfer complexes [12,13]. If the proton NMR spectra of cytochrome c are generally well resolved, complex formation with oxidoreduction partners induces considerable peak overlaps. ^{15}N labeling of cytochrome c_{555} gave us the opportunity to develop a classical heteronuclear NMR approach. In these experiments only the cytochrome is observable, removing the problem of the ferredoxin line superimposition and preserving the resolution of the spectrum. Moreover, the sensitivity of the experiments permits titration at a very low protein concentration (10^{-5} M).

Structural comparison [14] of cytochrome c_{555} with other cytochromes c underlined that despite the lower sequence homology, the overall structure of cytochrome c_{555} is similar to the rest of the cytochrome c family. Notably, the heme pocket architecture, the electrostatic surface distribution and the accessibility of the encounter surface at the system edge are conserved. The present data clearly demonstrate that the interacting site for cytochrome c_{555} is that which was previously predicted from cytochrome c structure comparison [14]. In this low oxidoreduction potential cytochrome, the main struc-

tural variation observed when compared with other *c*-type cytochromes concerns the propionate 6 solvent accessibility. In cytochrome c_{553}, this group is directly exposed to the solvent, a feature unique among cytochromes *c*. Moreover, the homonuclear experiment (data not shown) evidenced that the lateral chain (CαH) of R53 as well as the loop between residues 20 and 30 are found in the interaction site. The involvement of these two arms of the heme crevice is probably related to the structural characteristics found for this cytochrome [1].

The encounter surface described for the cytochrome c_{553}-ferredoxin I complex is in complete agreement with the characteristics of the formate dehydrogenase-cytochrome c_{553} complex [15]. We have previously reported that the electrostatic interactions are probably the driving force in the complex formation and that K62 and K63 substitution gives a drastic decrease in the affinity constant. In the present mapping both residues are found in the interacting site. Formate dehydrogenase (M_r 120 kDa) and Fe-hydrogenase (M_r 60 kDa) are large multimeric enzymes, and new insights into structural NMR give the opportunity to study large proteins involved in complexes. Therefore, transverse relaxation-optimized spectroscopy experiments [16,17] on these high molecular weight electron transfer complexes should confirm the accuracy of the present model. This approach may be largely developed for other electron transfer complexes such as cytochrome *c*/cytochrome *c* oxidase, cytochrome *c*/complex bc_1, etc. However, few cytochromes *c* have been successfully labelled [18–21]. Cytochrome *c* overexpression and labelling is a special challenge, mainly for mammalian cytochromes. This is a serious limitation for the study of such oxidoreduction complexes which have not yet been cocrystallized and for which thermodynamic parameters can only be interpreted through molecular models.

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