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Investigation of the redox-dependent modulation of structure and dynamics in human cytochrome *c*

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Redox-dependent changes in the structure and dynamics of human cytochrome *c* (Cyt *c*) were investigated by solution NMR. We found significant structural changes in several regions, including residues 23–28 (loop 3), which were further corroborated by chemical shift differences between the reduced and oxidized states of Cyt *c*. These differences are essential for discriminating redox states in Cyt *c* by cytochrome *c* oxidase (C*c*O) during electron transfer reactions. Carr-Purcell-Meiboom-Gill (CPMG) relaxation dispersion experiments identified that the region around His33 undergoes conformational exchanges on the μs-ms timescale, indicating significant redox-dependent structural changes. Because His33 is not part of the interaction site for C*c*O, our data suggest that the dynamic properties of the region, which is far from the interaction site for C*c*O, contribute to conformational changes during electron transfer to C*c*O.

1. Introduction

Electron transfer (ET) processes are at the heart of numerous key physiological processes, such as aerobic and anaerobic respiration and fatty acid metabolism. Among them, ET from Cytochrome *c* (Cyt *c*) to Cytochrome *c* oxidase (C*c*O) in mitochondrial respiration is one of the best-studied processes [1,2]. Cyt *c* has one *c*-type heme, covalently attached to the two cysteine residues *via* thioether bonds, and mediates ET from the Cytochrome bc_1 complex to C*c*O, cycling between the reduced and oxidized forms. Although Cyt *c* can only carry one electron, the reduction of dioxygen to water molecules catalyzed in C*c*O requires four electrons, which implies that the regulation mechanism for the specific association of reduced Cyt *c* with C*c*O and the dissociation of oxidized Cyt *c* from C*c*O are efficient for the four electron reduction of dioxygen.

Although previous interaction analysis using NMR revealed that the interaction between Cyt *c* and C*c*O depends on the redox state of Cyt *c* [3,4], the structural factors for the redox dependent interactions are unclear because detailed structural comparisons between reduced and oxidized Cyt *c* are unavailable. Considering the concentrated protein milieu in the intermembrane space of mitochondria, discriminating between reduced and oxidized Cyt *c* is essential for successive ET reactions that reduce dioxygen. While structural similarities between reduced Cyt *c* and oxidized Cyt *c* have been reported for horse heart Cyt *c* [5–8], yeast iso-1-Cyt *c* [9–11], and Cyt c_{552} from *Paracoccus denitrificans* [12–14], some minor, though significant, redox-dependent structural differences were reported. For horse heart Cyt *c*, Qui et al. reported that the redox-dependent structure changes in residues 49–55 [5,6], while Banci et al. found structural changes in other regions, such as residues 32–38 [7,8]. No significant redox-dependent structural differences were observed for Cyt c_{552} from *P. denitrificans* [12,13] and *Saccharomyces cerevisiae* iso-1-Cyt *c* [9,10,15,16]. Conversely, the detailed analysis of the pseudocontact shifts (PCSs) on horse heart Cyt *c* and yeast iso-1-Cyt *c* indicated that there were redox-dependent structural changes in residues 28–31, 39–43, 52–60, and 79–82 [11].

These structural studies include conflicts attributed to methodological limitations. Although X-ray crystallography can provide

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Abbreviations: R_2, transverse relaxation rate; R_{ex}, contribution of relaxation caused by chemical exchange; $R_{2,eff}$, effective transverse relaxation rate; k_{ex}, chemical exchange rate constant.

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precise atomic models, structures of the crystalized proteins can be affected by crystal packing, which may obscure redox-dependent structural changes [15,16]. Protein structures determined by solution NMR are less affected by artifacts; however, due to the technical limitations of NMR measurements decades ago, such as the low sensitivity and limited isotope labeling, it was difficult to find minor, though significant, structural changes. In addition, protein dynamics are still controversial: NMR studies of yeast and horse Cyt *c* showed higher flexibility in the oxidized form compared with the reduced form [17], and the human protein showed more restricted backbone mobility in the oxidized form [3].

In this study, we report detailed study on the structure and dynamics of human Cyt *c* in oxidized and reduced states. The redox-dependent structural changes were detected in amino acid residues located in the interaction site for C*c*O, including loop 3 (residues 23–28) in the heme vicinity, and our ^{15}N NMR relaxation dispersion experiments highlighted novel conformational exchanges in both redox states around His33 located on the outside of the interaction site.

2. Materials and methods

2.1. NMR spectroscopy and calculations of solution structure

^{13}C/^{15}N- or ^{15}N-labeled human Cyt *c* was expressed and purified according to previously reported methods [3]. NMR samples were prepared in 50 mM sodium phosphate buffer (pH 6.8) containing 5% ^2H_2O. The protein concentration was adjusted to 0.5–1.0 mM. Backbone and side chain resonances of reduced and oxidized Cyt *c* were assigned using standard triple resonance pulse sequences. NMR experiments were recorded on Agilent UNITY Inova 800 and 600 MHz NMR spectrometers. The NMR spectra for the assignment and structure determination were recorded at 298 K. To assign the resonances of heme in reduced Cyt *c*, ^{13}C- and ^{15}N-filtered NOESY spectra were measured with mixing times of 75, 100 and 150 ms [18] (Table S1). To assign the resonances of the heme group in oxidized Cyt *c*, two-dimensional homonuclear NOESY in 100% ^2H_2O buffer was measured. To determine the distance restraints, ^{13}C-edited NOESY-HSQC and ^{15}N-edited NOESY-HSQC were measured with a mixing time of 75 ms. Backbone dihedral angle restraints were predicted by TALOS+ [19]. For the TALOS + prediction for oxidized Cyt *c*, the ‘diamagnetic’ chemical shifts were calculated by subtracting the pseudocontact shifts (PCSs) from the observed chemical shifts for oxidized Cyt *c*. The structures of reduced and oxidized Cyt *c* were calculated using CYANA [20]. The atomic coordinates and structural restraints for reduced and oxidized Cyt *c* were deposited in the Protein Data Bank, www.pdb.org (PDB ID code 2n9i and 2n9j).

2.2. $\Delta\chi$ -tensor fitting and PCS calculation

Oxidized Cyt *c* is paramagnetic due to the electronic spin of 1/2 in Fe^{3+}. The paramagnetic center contributes to the chemical shift *via* hyperfine interactions, leading to contact and pseudocontact shifts. For C′, C^α, C^β, H^α, H^N, and N atoms, the observed redox-dependent changes in the chemical shift δ_{obs} can be described as

$$\delta_{obs} = \delta_{ox} - \delta_{red} = \delta_{CS} + \delta_{PCS} + \delta_D \tag{1}$$

where δ_{ox} and δ_{red} are the observed chemical shifts in the oxidized and reduced proteins, respectively, and δ_{CS} and δ_{PCS} correspond to the chemical shift changes caused by Fermi contact and pseudocontact interactions, respectively [7]. The chemical shift changes attributed to the diamagnetic term, δ_D, arise from structural changes associated with the redox state [7].

A magnetic susceptibility tensor ($\Delta\chi$-tensor) was first calculated using chemical shift differences between the oxidized and reduced states, assuming that δ_D is the major component of δ_{obs} for most nuclei. Cys14 and Cys17, which are covalently bound to the heme, and the two axial ligand residues, His 18 and Met 80, were excluded because of the possible contribution from contact shift. During the tensor refinement, atoms showing large deviations were further excluded. The tensor parameters were calculated using the Numbat program [21] with the following equation:

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

where δ_{PCS} is the pseudocontact shift, *r*, θ and ϕ are polar coordinates of the nucleus with respect to the principal axes of the magnetic susceptibility tensor, and $\Delta\chi_{ax}$ and $\Delta\chi_{rh}$ are the axial and rhombic components of the anisotropic magnetic susceptibility tensor, respectively. The tensor was further refined by excluding the PCSs leading to large deviations due to large $\Delta\delta_D$ values.

2.3. ^{15}N relaxation dispersion experiment

An ^{15}N-Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence [22] was used to examine the conformational exchanges in oxidized and reduced Cyt *c*. Two-dimensional data sets were acquired as 128 × 1024 complex points in the t1 × t2 time-domain dimensions with a constant relaxation delay of 40 ms. The experiments were performed using ^{15}N labeled Cyt *c* on Agilent UNITY Inova 800 MHz NMR spectrometers at 279 K. The exchange rate constant for the two conformers, k_{ex}, was fit using NESSY [23] with the Meiboom equation [24], which was applied to the fast exchange processes between the two states. The exchange rates were extracted independently for each residue with the appreciable exchange contribution (>5 Hz).

3. Results

3.1. Structure of reduced Cyt c

The ^{1}H–^{15}N HSQC spectrum of reduced Cyt *c* with backbone resonance assignments is shown in Supplemental Fig. S1a. Resonances corresponding to four non-proline residues, Glu21, Thr28, Gly45, and Gly84, were missing in the ^{1}H–^{15}N HSQC spectrum. All of the backbone amide proton resonances were assigned based on the NOESY spectra, except for Thr28, and all of the side-chain resonances were assigned, except for the δ-proton of Tyr67. The missing assignments were possibly due to the fast exchange of amide protons with solvent, overlap with the water signal, or local exchange broadening. The structure of reduced Cyt *c* was calculated based on 1890 distance restraints from NOEs, and 58 dihedral angle restraints from TALOS+ [19]. The ribbon model of the lowest energy structure of reduced Cyt *c* is shown in Fig. 1a. An overlay of the 20 structures with the lowest CYANA energy is shown in Fig. 1c. The RMSD values for the 20 models in the refined structure are 0.49 and 0.91 Å for the backbone and all heavy atoms, respectively (Table 1).

3.2. Structure of oxidized Cyt c

Supplemental Fig. S1b illustrates the ^{1}H–^{15}N HSQC spectrum of oxidized Cyt *c* with backbone resonance assignments. The assignments of the four non-proline residues Glu21, Thr28, Gly45, and Gly84 were missing in the ^{1}H–^{15}N HSQC spectrum. Based on the NOESY spectra, all of the backbone amide proton resonances were assigned except for Thr28 and Gly45. All of the side-chain resonances were assigned except for the δ- and ε-protons of Tyr67 and