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# Precise structural and functional analyses of cytochrome *c′* revealed by quantum beams and other multiple methods

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## Introduction

Cytochrome c' (cyt *c′*) are found in the periplasmic space of a number of photosynthetic, nitrogen-fixing and denitrifying bacteria. In denitrifiers, they have been proposed to have a role in mediating nitric oxide (NO) transfer and protecting the organism from the potentially toxic levels of NO that may otherwise accumulate.

The ligand binding property by this protein is anomalous when compared to other high spin hemeproteins except soluble guanlyate cyclease (sGC), which is activated by NO binding to the heme domain and enhances generating the cellular second messenger cGMP in mammal. Cyt *c′* and sGC form the stable complexes with NO and carbon moxide (CO), while they cannot bind oxygen ($O_2$). Interestingly, NO forms 5-coordinate heme adducts with both cyt *c′* and sGC.

Many spectroscopic studies have been conducted for these two proteins and it has been suggested that NO binding is restricted to the distal side of the heme. In 2000, the crystal structure of the NO-bound cyt *c′* was reported at 1.35 Å resolution. In the structure, NO disrupts His-Fe bond and binds to the proximal face of the heme, giving a 5-coordinate species. It was the first example that shows the 5-coordinate heme binding NO at the proximal side. However, we thought that this structure had some problems. The electron density map close to the heme iron had "Y" shape, which is likely to be corresponding to a molecule containing three atoms (non hydrogen). The authors interpreted that NO was binding in two alternative bent conformations, each with half occupancy. This model has been believed and accepted for last decade. However, there was no evidence that the electron density is from NO molecule.

We decided to determine the structure for NO-bound cyt*c′* in addition to the ferric, the ferrous and the CO-bound form at higher resolution. Furthermore, we would like to collect the single crystal absorption spectra to ensure what is the state of the iron center in these crystals. In addition to the crystal structural analyses, we planned to apply Resonance Raman spectra, EXAFS, and other spectroscopic techniques for the solution samples.

## Preparation of several states of cyt*c′* crystals

*Oxidized form*

Cyt*c′* is purified from the denytrifying bacterium *Alcaligenes xylosoxidans* NCMBI 11015. The crystal is obtained by batch method and vapor diffusion method using ammonium sulfate or polyethylene glycol (PEG) as precipitants. The crystals show bi-pyramidal shape. The crystals for oxidized form are prepared by flash freezing into the liquid nitrogen. The cryoprctectant is the reservoir solution containing ~ 25 % (v/v) glycerol.

*Reduced form*

The crystals for the reduced form were prepared by soaking the ferric crystals in an anaerobic solution containing an excess of sodium dithionite or sodium asocrobate. On reduction, the crystal color immediately became bright red. The crystals were frozen as described above in the ferric form.

*NO-bound form*

The NO-bound form was prepared by soaking the reduced crystals to an NO-saturated solution. The sodium ascorbate and NOC-12, an NO donor (Dojindo), were added in excess to the crystallization solution, the gas phase of which had been replaced with nitrogen ($N_2$) in advance by several cycles of alternate degassing and $N_2$ purge. In place of ascrobate to dithionite, we could not obtain the electron density map corresponding to the NO-bound form.

## Synchrotron radiation experiments

We conducted diffraction intensity data collection experiments at beamlines AR-NE3, AR-NW12, BL5A, and BL17A. The max resolution of obtained data for each type of crystal is listed in table 1.

It was not easy to obtain NO-bound form. Only using ascorbate, not dithionite, for a reducing agent, the electron density corresponding NO was obtained. The electron density is similar to the previously reported one. Thus, we must re-examine the structural analysis and must obtain the evidence for NO-binding by other methods.

Table 1: Max resolution for the cyt*c′* crystals

| | oxidized | reduced | NO-bound |
|---|---|---|---|
| Max resolution (Å) | 1.00 | 0.92 | 1.11 |

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