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Crystallization and preliminary X-ray analysis of dimeric and trimeric cytochromes *c* from horse heart

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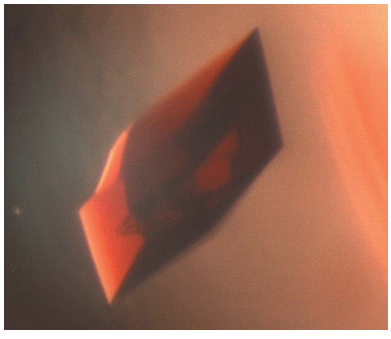


Cytochrome *c* (cyt *c*) is an electron-transfer protein in the respiratory chain of mitochondria. It is known to form polymers, but its polymerization mechanism is still unknown. Dimeric and trimeric cyt *c* from horse were successfully crystallized by the sitting-drop vapour-diffusion method using polyethylene glycol as a precipitating reagent. The crystal of dimeric cyt *c* belonged to space group $P1$, with unit-cell parameters $a = 41.8$, $b = 56.3$, $c = 60.8$ Å, $\alpha = 66.3$, $\beta = 89.9$, $\gamma = 73.7^\circ$, whereas that of trimeric cyt *c* belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 57.2$, $b = 95.7$, $c = 130.9$ Å. Initial structure models showed that the crystals of dimeric and trimeric cyt *c* contained two dimers and two trimers, respectively, in the asymmetric unit.

1. Introduction

Cytochrome *c* (cyt *c*) from horse consists of 104 amino acids and one haem group. Its main role is the transportation of electrons from the cytochrome bc_1 complex to cytochrome *c* oxidase in the respiratory chain in mitochondria. Cyt *c* is also related to the mitochondrial pathway of apoptosis in vertebrates. Many crystal structures of monomeric cyt *c* from eukaryotes have been reported, including those from bonito (Tanaka *et al.*, 1975), tuna (Takano & Dickerson, 1981), rice (Ochi *et al.*, 1983), yeast (Louie *et al.*, 1988) and horse (Bushnell *et al.*, 1990). The crystal structure of horse cyt *c* is essentially the same as that in solution (Banci *et al.*, 1997, 1999). The structure of horse cyt *c* is composed of five (three long and two short) α-helices and several loops which enclose the haem in the molecule. The haem is linked to the protein through two thioether bonds to the cysteine residues of the haem-binding motif Cys-X_1-X_2-Cys-His. The haem iron is coordinated by two axial histidine and methionine ligands. Cyt *c* is known to form amyloid fibrils when heated to 348 K for 12 h (de Groot & Ventura, 2005). In addition, the apo form can also form amyloid fibrils (Pertinhez *et al.*, 2001). Amyloid fibrils from other proteins cause neurodegenerative diseases and it has been suggested that they share a common structure and a common mechanism of toxicity regardless of their sequences (Kayed *et al.*, 2003). The formation of amyloid fibrils is accelerated by preformed amyloid seeds. However, the initial step of protein aggregation is not well understood and requires further study. In order to understand the mechanism of aggregation of the protein molecules, we have studied oligomeric cyt *c* using various physicochemical methods. In this paper, we report the purification, crystallization and preliminary X-ray structure analysis of dimeric and trimeric cyt *c* from horse.

2. Materials and methods

2.1. Purification

Soluble oligomeric cyt *c* was obtained by a modification of the method previously reported by Margoliash & Lustgarten (1962). Powdered horse heart cyt *c* (UniProt P00004) was purchased from Wako Pure Chemical Industries Ltd, Osaka, Japan. About 100 mg cyt *c* was dissolved in 10 ml 50 m*M* potassium phosphate buffer pH 7.0. Ethanol was added to the cyt *c* solution to 60%(*v*/*v*). The cyt *c*

solution was centrifuged at 8000*g* for 15 min and the precipitate obtained was freeze-dried. The freezed-dried precipitate was dissolved in 10 ml 50 m*M* potassium phosphate buffer pH 7.0, in which large cyt *c* oligomers were formed. The oligomeric cyt *c* obtained at this stage was larger than the exclusion limit of the gel-filtration column. After incubation of the large cyt *c* oligomers at 310 K for 40 min, smaller cyt *c* oligomers were produced. The obtained cyt *c* oligomer solution was filtrated and the cyt *c* oligomers were purified by gel chromatography (Superdex 75 26/60, GE Healthcare) several times in 50 m*M* potassium phosphate buffer pH 7.0.

2.2. Crystallization

Initial screening for the crystallization of dimeric and trimeric cyt *c* was carried out using the sitting-drop vapour-diffusion method with crystal screening kits from Emerald BioSystems, Washington, USA. The concentrations of dimeric and trimeric cyt *c* were 20 and 15 mg ml^{-1}, respectively. The drops for crystallization were prepared on ice by mixing 1 µl protein solution and 1 µl reservoir solution. The droplets were equilibrated against 80 µl reservoir solution at 277 K.

2.3. Data collection and analysis

Diffraction data sets for dimeric and trimeric cyt *c* were collected on the BL26B2 beamline (λ = 0.8000 Å) using an MX-225 (MAR Research) detector at SPring-8, Japan. Both crystals were mounted on a cryoloop and flash-cooled at 100 K in a nitrogen cryosystem. The crystal-to-detector distance, oscillation angle, exposure time for each film and total number of frames were 220 mm, 1.0°, 20 s and 180, respectively, for dimeric cyt *c*, whereas those for trimeric cyt *c* were 230 mm, 0.6°, 20 s and 300, respectively. The diffraction data sets were processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Data-collection statistics are listed in Table 1.

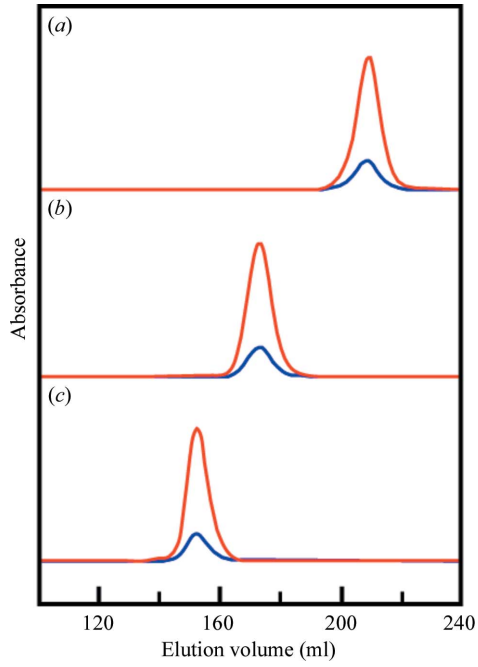


Figure 1
Elution curves of (*a*) monomeric, (*b*) dimeric and (*c*) trimeric cyt *c* from the HiLoad 26/60 Superdex 75 column monitored by absorbance at 409 nm (red) and 280 nm (blue). The flow rate was 0.8 ml min^{-1} and the running buffer was 50 m*M* potassium phosphate buffer pH 7.0.

Table 1
X-ray data-collection statistics.

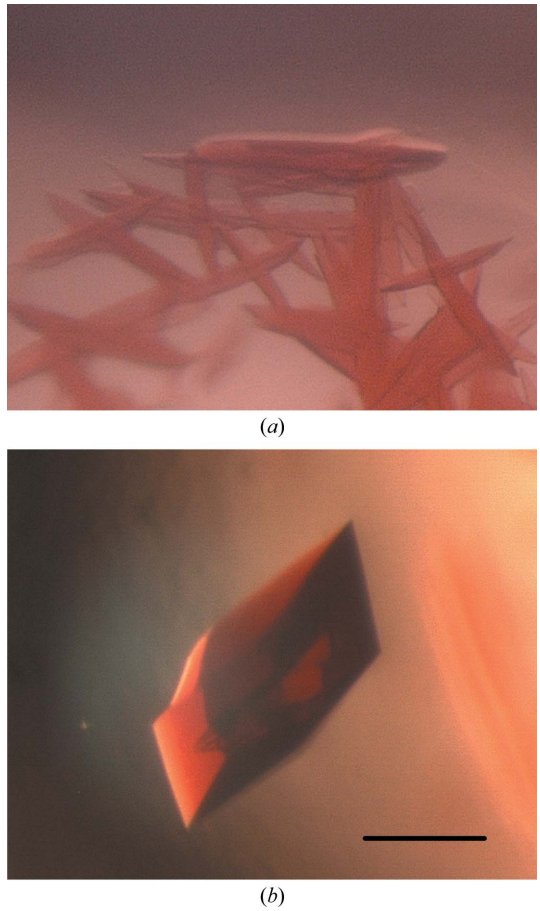
Values in parentheses are for the highest resolution shell.

	Dimer	Trimer
Source	SPring-8 (BL26B2)	SPring-8 (BL26B2)
Wavelength (Å)	0.8000	0.8000
Resolution limits (Å)	50–2.20 (2.28–2.20)	50–2.10 (2.18–2.10)
Space group	*P*1	$P2_12_12_1$
Unit-cell parameters		
a (Å)	41.8	57.2
b (Å)	56.3	95.7
c (Å)	60.8	130.9
α (°)	66.3	90
β (°)	89.9	90
γ (°)	73.7	90
No. of observed reflections	41227	312054
No. of unique reflections	22315	43002
$R_{\rm merge}$†	0.059 (0.141)	0.096 (0.380)
Completeness (%)	90.5 (70.9)	99.8 (100.0)
$I/\sigma(I)$	9.4 (5.16)	7.5 (5.59)
Redundancy	1.8 (1.6)	7.3 (7.4)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

3. Results

Dimeric and trimeric cyt *c* were successfully purified by gel chromatography (Fig. 1). Crystals of dimeric cyt *c* were obtained after one month in 0.1 *M* Tris–HCl buffer containing 30% PEG 200 and 200 m*M* $(NH_4)_2HPO_4$ pH 8.5 and those of trimeric cyt *c* grew in one week under the same conditions (Figs. 2*a* and 2*b*). The crystal of



(*a*)

(*b*)

Figure 2
Crystals of (*a*) dimeric and (*b*) trimeric cyt *c* from horse. The scale bar is 0.1 mm in length.

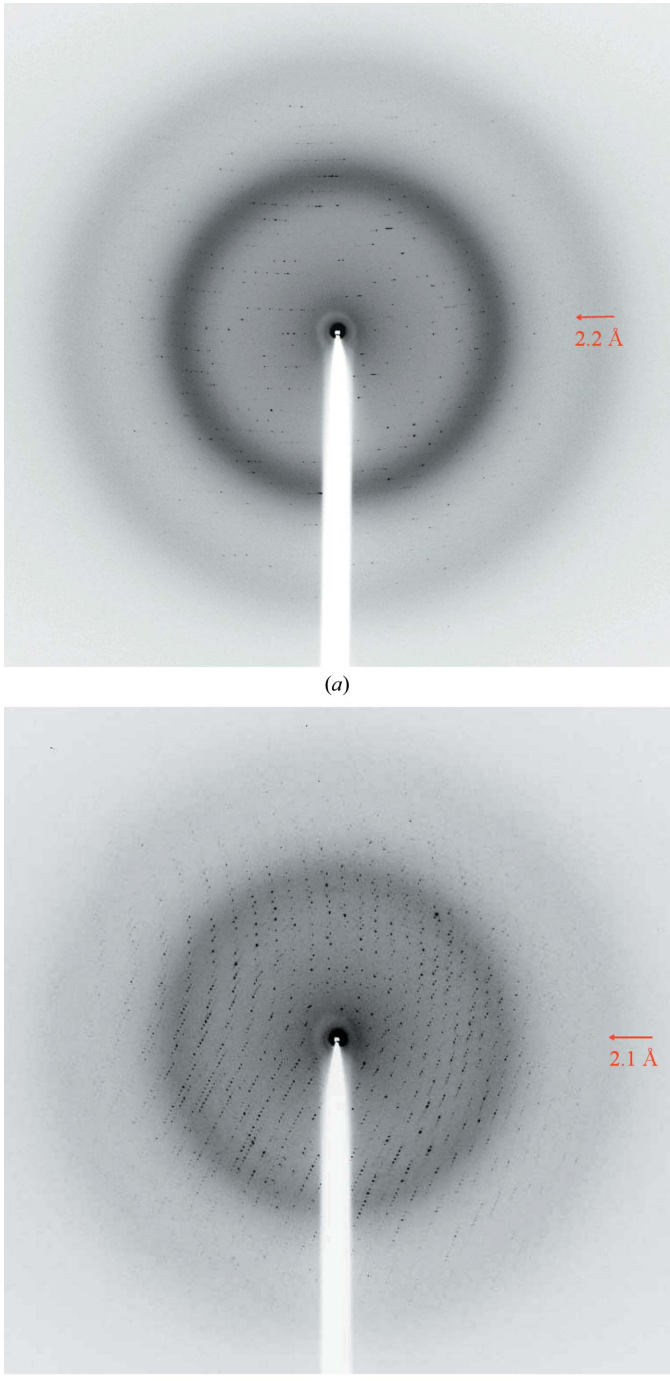


Figure 3
Diffraction images of (*a*) dimeric and (*b*) trimeric cyt *c* (BL26B2, SPring-8). The crystal-to-detector distance, oscillation angle and exposure time for dimeric cyt *c* were 220 mm, 1.0° and 20 s, respectively, whereas those for trimeric cyt *c* were 230 mm, 0.6° and 20 s.

dimeric cyt *c* diffracted to 2.2 Å resolution and belonged to space group $P1$, with unit-cell parameters a = 41.8, b = 56.3, c = 60.8 Å, α = 66.3, β = 89.9, γ = 73.7°. Assuming the presence of two cyt *c* dimers in the asymmetric unit, the Matthews coefficient was calculated to be 2.52 Å^3 Da^{-1}. The crystal of trimeric cyt *c* diffracted to 2.1 Å resolution and belonged to space group $P2_12_12_1$, with unit-cell parameters a = 57.2, b = 95.7, c = 130.9 Å. The Matthews coefficient was calculated to be 2.41 Å^3 Da^{-1} assuming the presence of two cyt *c* trimers in the asymmetric unit. Typical diffraction images of dimeric and trimeric cyt *c* crystals are presented in Fig. 3.

Initial models of the structures of dimeric and trimeric cyt *c* were obtained by the molecular-replacement method using the program *MOLREP* (Vagin & Teplyakov, 2010). The coordinates of the monomeric structure of horse cyt *c* (PDB code 1crc; Sanishvili *et al.*, 1995) were used as the search model. The result showed that dimeric cyt *c* contains two dimeric units in the asymmetric unit, whereas trimeric cyt *c* contains two trimeric units in the asymmetric unit. Structure refinement of both dimeric and trimeric cyt *c* is now in progress.

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