Structures of dimeric and trimeric cytochrome *c* and its polymerization mechanism

Cytochrome *c* (cyt *c*) is a well-known globular heme protein. It receives electrons from the cytochrome bc_1 complex and provides them to cytochrome *c* oxidase in the respiratory chain in mitochondria. It also plays a key role in apoptosis, where it is released to the cytosol when permeabilization of the mitochondrial outer membrane occurs. Cyt *c* contains three long α-helices, and the heme of cyt *c* forms covalent bonds with two cysteine residues through their sulfur atoms and is coordinated in native form by His18 and Met80. Met80 dissociation from heme iron induces peroxidase activity and leads to cardiolipin oxidation, which in turn leads to the release of proapoptotic factors.

Protein polymerization has gained interest owing to its relationship with neurodegeneration. The crystal structure of a stable dimer of serpin, a family of proteins that forms large stable multimers leading to intracellular accretion and disease, revealed a domain-swapped structure of two long antiparallel β-strands inserting into the center of the neighboring monomer [1]. Domain swapping is a phenomenon where the secondary or tertiary structural unit of a protein molecule is replaced by the corresponding unit of another molecule of the same protein [2]. It has also been shown that β_2-microglobin can oligomerize and form amyloid fibils via runaway domain swapping [3]. It has been known for nearly half a century that cyt *c* forms polymers, but the polymerization mechanism remains unknown [4]. Recently, we have solved the X-ray crystal structures of dimeric and trimeric cyt *c* (Figs. 1(d) and 1(f), respectively) [5]. Diffraction experiments were carried out at **BL26B2** beamline. From the dimeric and trimeric structures and the physicochemical properties of the oligmers of cyt *c*, we found that it forms polymers by successive domain swapping (runaway domain swapping), where the C-terminal helix is displaced from its original position in the monomer and Met-heme coordination is perturbed significantly (Fig. 2) [5].

Ferric horse cyt *c* oligomers were prepared by the treatment of monomeric ferric horse cyt *c* with ethanol. Both dimeric and trimeric cyt *c* exhibit domain-swapped structures, where the C-terminal regions beyond Thr78 of the dimer and Lys79 of the trimer are relocated from their original position observed in the monomer owing to Met80 dissociation from the heme (Fig. 2). The relocated C-terminal region includes one α-helix, and the vacant area produced is occupied by the corresponding region of another cyt *c* molecule (Figs. 1(a), 1(d), and 1(f)). Polyethylene glycol (PEG) molecules, added for crystallization, were identified in the crystal structures. Diethylene glycol lies at the interface between two protomers in dimeric cyt *c*, whereas it interacts with the hinge loop (Thr79–Ala83) in trimeric cyt *c*. These interactions contribute to the stabilization of the dimer and trimer structures, resulting in closed-ended mutual swapping and cyclic structures, respectively. Tetraethylene glycol interacts with Lys55 and sits at the interface between the oligomers in the crystal structures of both dimeric and trimeric cyt *c*. This interaction reduces the positive

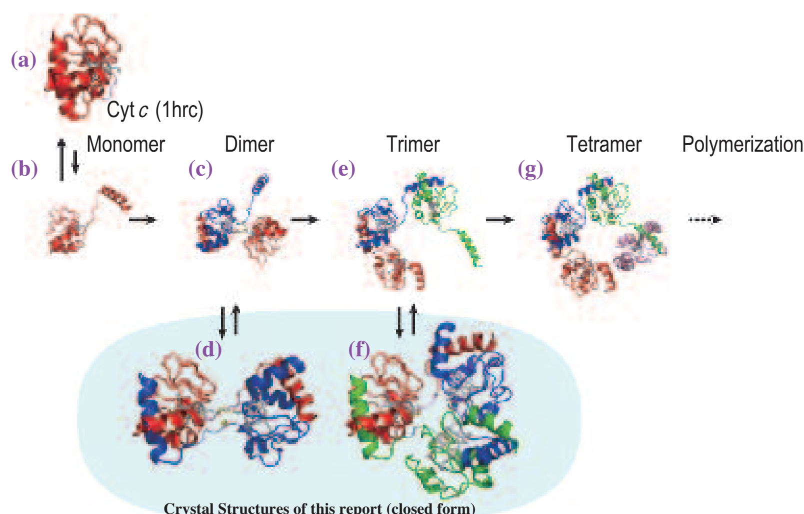

Fig. 1. Structures of monomeric and oligomeric cyt *c*. Crystal structures of monomeric (**a**, 1 hrc), dimeric (**d**) and trimeric (**f**) cyt *c* from horse heart. Model structures of monomeric (**b**), dimeric (**c**), trimeric (**e**), and tetrameric (**g**) cyt *c* in solution. The cyclic forms of dimeric and trimeric cyt *c* were produced owing to interaction with PEG and $(NH_4)_2HPO_4$ molecules, which were added as crystallization precipitants. Oligomeric cyt *c* is formed by the displacement of the C-terminal helix of one monomer from its original position and the vacant area produced is occupied by the corresponding helix of another molecule. Each protomer is shown in red, blue, and green.

charge of the protein surface and the repulsion between the oligomers in the crystals. However, the linker loop is not long enough to cause domain swapping without steric hindrance, consequently, perturbation in the protein structure around the heme is induced. The heme–heme distance is shorter in the dimer (18.4 Å) than in the trimer (19.8–20.0 Å). As a result, the Thr78 residual position moves toward the solvent in the dimer, whereas it remains the same as that in the monomer in the trimer. However, the solution structures of dimeric, trimeric, and tetrameric cyt *c* were linear on the basis of small-angle X-ray scattering measurement (Figs. 1(b), 1(c), 1(e) and 1(g)), where the trimeric linear structure shifted toward the cyclic structure with the addition of PEG and $(NH_4)_2HPO_4$ (Fig. 1(f)).

Met80 was dissociated from the heme in cyt *c* oligomers allowing the C-terminal α-helix to swap (Fig. 2). For dimeric, trimeric, and tetrameric cyt *c*, the ΔH of the oligomer dissociation to monomers was estimated to be about -20 kcal/mol per protomer unit, where Met-heme coordination appears to contribute largely to ΔH. This suggests that Met–heme coordination stabilizes the interaction of the C-terminal helical region with the N-terminal region, resulting in a native monomeric structure (Fig. 1(a)). In fact, residues 83-86 are reported to be among the highest disordered regions in the solution structure of horse ferric cyt *c* according to NMR investigation. The side chain of Met80 is exposed to the solvent and Phe82 occupies the Met80 position in the dimer (Figs. 2(a) and 2(b)), while the side chains of Met80 and Phe82 in the trimer move but remain in the vicinity of their corresponding positions in the monomer (Figs. 2(a) and 2(c)). The dissociation of Met80 from heme iron allows a water molecule or a hydroxide ion to bind in its place. According to the absorption spectra of the dimer and trimer and the previous reports on Met-depleted cyt *c*, a hydroxide ion appears to be coordinated to the heme in the crystal (pH 8.5). The peroxidase activity of cyt *c* is thought to play an important role in apoptosis, where it depends on the perturbation of the Met-heme coordination. In fact, the peroxidase activity of dimeric cyt *c* was higher than that of monomeric cyt *c*.

Although the dimer and trimer have different heme pocket environments their optical absorption spectra were similar, which can be due to coordination of the hydroxide ion in both oligomers. The absorption and CD spectra of high-order oligomers (~40mer) were similar to those of dimeric and trimeric cyt *c* but different from those of monomeric cyt *c*. The present results suggest that cyt *c* polymerization occurs by the successive domain swapping of the C-terminal helix. We believe that the present new structural and thermodynamic work on cyt *c* polymerization provides a new and important concept, which may lead to a common mechanism of protein polymerization.

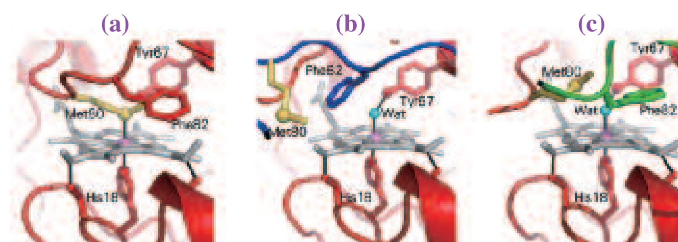

Fig. 2. Enlarged view around the heme pocket of monomeric, dimeric and trimeric cyt *c*. Heme is supported by two thioether bonds with two cysteine residues and Fe is coordinated by His18 and Met80 in the native monomeric form **(a)**. Met80 is shifted away from the heme and Phe82 occupies the Met80 position in the dimer **(b)**, whereas the side chains of Met80 and Phe82 in the trimer move but remain in the vicinity of their corresponding positions in the monomer **(c)**. In the dimer and trimer, additional species (probably a hydroxide ion) are coordinated to Fe at the Met80 ligand position in the monomer. The heme and side chain atoms of His18, Tyr67, Met80, and Phe82 are depicted as stick models. Fe of heme (pink), Nε2 of His18, Oη of Tyr67, Sδ of Mat80 and O of the water molecule (cyan) are shown as spheres. Each protomer is shown in red, blue and green except for the heme (gray) and the side chain of Met80 (yellow).

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