Crystal structure of the trypanosome cyanide-insensitive alternative oxidase

Alternative oxidase (AOX) is a non-protonmotive ubiquinol oxidase catalyzing the four-electron reduction of dioxygen to water and belongs to the membrane-bound diiron carboxylate protein family [1]. This protein has been found in many organisms from prokaryotes to eukaryotes, such as higher plants, algae, yeast, slime molds, free-living amoebae, subacteria, nematodes, and parasites including *Trypanosoma brucei* [2]. *T. brucei* causes human African sleeping sickness and nagana in livestock, which are called neglected tropical diseases (NTDs) [1]. These are endemic in low-income populations in developing countries of Africa. Since current treatments are poorly targeted and show serious side effects and poor efficacy, the development of chemotherapy and the continued search for new unique therapeutic targets for African trypanosomiasis are urgently required. *T. brucei* has two different stages within its life cycle; the procyclic form in the tsetse fly and the bloodstream form in the mammalian host. Once the parasites invade the mammalian host in the bloodstream, both the cytochrome respiratory pathway and oxidative phosphorylation disappear and are replaced by the trypanosome alternative oxidase (TAO), which functions as the sole terminal oxidase to re-oxidize NADH accumulated during glycolysis [2]. Since NADH re-oxidation is essential for parasite survival and mammalian hosts do not possess this protein, TAO is considered to be a unique target for anti-trypanosomal drugs. Indeed, we have previously reported that the antibiotic ascofuranone, isolated from pathogenic fungus *Ascochyta viciae*, specifically inhibits the quinol oxidase activity of TAO at sub-nanomolar concentrations (IC₅₀ = 0.13 nM) and rapidly kills the parasites. Furthermore, we have confirmed the chemotherapeutic efficacy of ascofuranone in vivo [3,4]. To develop a rational design of further potent and safer anti-trypanosomal drugs, we have determined the structures of TAO in the presence or absence of an ascofuranone derivative, AF2779OH, which also strongly inhibits TAO (IC₅₀ = 0.48 nM) and dissolves in water more easily than ascofuranone.

TAO was expressed in *Escherichia coli*, purified and crystallized using *n*-octyl β-D-glucopyranoside (OG) and tetraethylene glycol mono-octylether (C8E4) as detergents. Since TAO includes a diiron carboxylate center, phase angles were obtained by SAD using anomalous scattering effects of iron atoms. All X-ray diffraction experiments were performed at beamlines BL41XU and BL44XU of SPring-8 and BL-17A of Photon Factory. The crystal structure of the inhibitor-free TAO determined at 2.85 Å resolution reveals that there are two homodimers in an asymmetric unit [5]. As shown in Fig. 1, each monomer consists of a long N-terminal arm, six long α-helices (α1-α6) and four short helices (S1-S4). The α2, α3, α5, and α6 helices are arranged in an antiparallel fashion and form a four-helix bundle that accommodates a diiron center, as widely observed in other diiron carboxylate proteins [5]. A large hydrophobic region that is formed by α1, α4, the C-terminal region of α2, and the N-terminal region of α5 from both monomers (Fig. 2(a)) spreads out on one side of the dimer surface, but the opposite side of the dimer surface is relatively hydrophilic, suggesting that the dimer is bound to the mitochondrial inner membrane via this hydrophobic region in an interfacial fashion. In addition, basic residues (Arg106, Arg143, Arg180, Arg203, and Arg207) are distributed along a boundary between the hydrophobic and hydrophilic regions of the dimer surface (Fig. 2(b)). They are conserved across all amino acid sequences of the membrane bound AOXs and their locations make these residues ideal candidates to interact with the negatively charged phospholipid head groups of membranes [5]. The structure of the diiron active site was refined as an oxidized Fe(III)-Fe(III) form with a single hydroxo-bridge, as previously predicted.
from spectroscopic studies. The active site, which is located in a hydrophobic environment deep inside the TAO molecule, comprise the diiron center, conserved glutamate (Glu123, Glu162, Glu213, Glu266) and histidine (His165, His269) residues. In addition, the conserved hydrophobic residues (Lue122, Ala126, Leu212, Ala216, Tyr220, Ile262) are within 6 Å of the diiron center. The most striking feature of the diiron active site in the oxidized state is that, as predicted from earlier FTIR studies, His165 and His269 are too distant from both Fe1 and Fe2 to coordinate to the diiron center. Accordingly, TAO is the first detailed structure of an oxidized diiron active site that contains only carboxylate but no histidine ligands [5].

The structure of TAO in a complex with AF2779OH determined at 2.6 Å resolution reveals how the inhibitor, as well as ascofuranone, is recognized by TAO (Figs. 3(a), 3(b) and 3(c); [5]). The binding cavity of AF2779OH is located near the membrane surface between helices α1 and α4 and is lined by 16 residues (Val92, Arg96, Phe99, Arg118, Cys119, Phe121, Leu122, Glu123, Val125, Met190, Leu212, Glu213, Glu215, Ala216, Thr219, and Tyr220) plus Cys95 (Figs. 3(c) and 3(d)), which are highly conserved among the membrane-bound AOXs. It is also apparent that the aromatic head of AF2779OH is located close to the diiron active site and C2-OH forms hydrogen bonds with Arg118 and Thr219 (Fig. 3(d)). In the AF2779OH- TAO complex, the distance between His165 and Fe1 is shorter than that observed in the inhibitor-free structure, and hence, His165 can now coordinate with Fe1, unlike His269 [5].

In conclusion, we believe that the structures determined in this study will contribute to the more complete understanding of the structure-function relationship of all AOXs. In addition, the structure of the AF2779OH-TAO complex indicates that the interactions between substituents of the AF2779OH aromatic head and TAO play an important role in the inhibition of TAO. This information would help the development of ascofuranone derivatives with better properties as a drug, and will not only be beneficial for the control of trypanosomiasis and other human diseases, such as cryptosporidiosis and candidiasis, but also for the control of plant diseases caused by phytopathogenic fungi.