Crystal structure of O_2-tolerant [NiFe] hydrogenase reveals the mechanism of O_2-tolerance attributable to a redox-dependent conformational change of [4Fe-3S] cluster

Hydrogenases are metalloenzymes that catalyze the reversible oxidation of dihydrogen, and have been considered to be a potential catalyst for biofuel cells and biosensors or an attractive model for bio-mimetic chemical catalysts. [NiFe] hydrogenases have four metal centers: the Ni-Fe active site for catalytic reaction and three iron-sulfur clusters for electron transfer. The Ni-Fe active site is composed of two metals, Ni and Fe, which are supported by four cysteines in the protein molecule [1]. Fe has three additional intrinsic non-protein diatomic ligands [1]. One of the serious weak points in the application of hydrogenases to biocatalysts is their sensitivity to O_2. [NiFe] hydrogenases are reversibly inactivated by O_2, producing two inactive forms, Ni-A and Ni-B. Ni-A is a strong inactive form with a dioxygen species between two metals and requires a prolonged reactivation time, whereas Ni-B has a monoxygen ligand which can be immediately liberated to become an active form upon reduction with H_2 [1]. This kind of well-studied O_2-sensitive [NiFe] hydrogenase is referred to as the "standard" enzymes. Generally, standard [NiFe] hydrogenases do not display the catalytic activity even in the presence of small amounts of O_2. Some H_2-oxidizing bacteria, however, have O_2-tolerant [NiFe] hydrogenases that form only Ni-B after exposure to O_2 and are rapidly reactivated by H_2 to show catalytic activity even at ambient O_2 concentration. Therefore, it has been considered that the prevention of the active site from producing Ni-A upon oxidation is associated with the O_2-tolerance of [NiFe] hydrogenases [2]. In order to elucidate the mechanism underlying the O_2-tolerance of the enzyme, we have carried out diffraction experiments at **BL41XU** and **BL44XU** beamlines, and solved the crystal structures of membrane-bound O_2-tolerant [NiFe] hydrogenase (MBH) from *Hydrogenovibrio marinus* in the reduced and ferricyanide-oxidized forms at 1.18 and 1.32 Å resolution, respectively [3].

MBH is composed of two (large and small) subunits, and the complex with cytochrome *b* catalyses the oxidation of H_2 and the reduction of quinones in its energy metabolism. Standard enzymes are usually crystallized as a heterodimeric unit, whereas MBH is crystallized as a dimer of the heterodimer in the crystal. The overall structure of the heterodimeric unit of MBH and relative disposition of the four metal centers are similar to those of the standard enzymes (Figs. 1(a) and 1(b)). The notable difference is that the Fe-S cluster proximal to the active site of MBH is not a [4Fe-4S] type as in the standard enzyme, but a [4Fe-3S] type. In the H_2-reduced MBH, one of the corner sulfides (S4), which binds to Fe1, Fe2, and Fe4 in the usual [4Fe-4S] cubane cluster (Fig. 2(a)), is replaced by a nearby cysteinyl sulfur (Cys25) and another cysteinyl sulfur (Cys126) coordinate Fe4 (Fig. 2(b)). One bond, Fe4-S4, in the cubane [4Fe-4S] cluster is missing, but all four iron atoms are coordinated by four ligands. Despite the difference of the coordination features, the whole shape of the cluster including the coordinating cysteinyl sulfur atoms is very similar to that of the standard

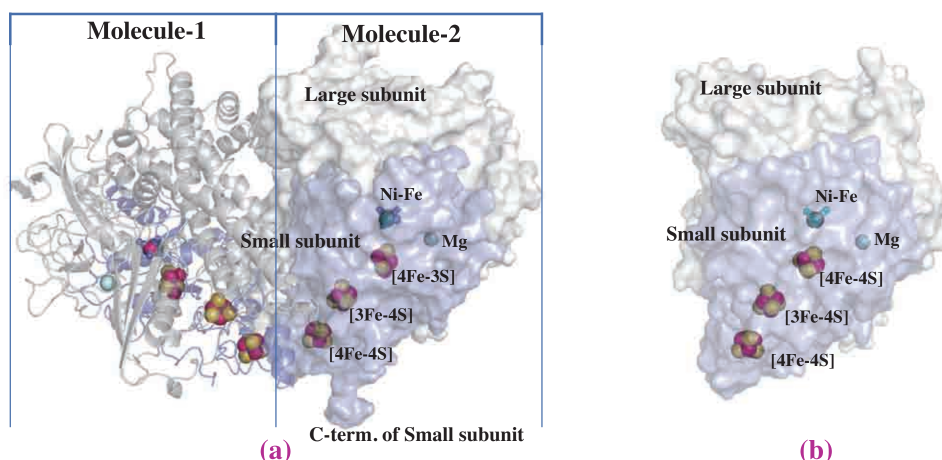

Fig. 1. Overall structures of [NiFe] hydrogenases. [NiFe] hydrogenases are composed of large (gray) and small (light blue) subunits. **(a)** Membrane-bound O_2-tolerant (MBH) enzyme from *H. marinus* in a dimeric form in this study. **(b)** Standard enzyme from *D. vulgaris* Miyazaki. Protein folding is schematically depicted in ribbon and/or surface models, whereas metal centers are shown in spheres. Ni, Fe, Mg, and S atoms are colored green, red, cyan, and yellow, respectively.

enzyme. The proximal Fe-S cluster revealed redox-dependent structural changes when the enzyme was exposed to an oxidant. In the ferricyanide-oxidized condition, deprotonated amide nitrogen of Cys26 replaced the S3 ligand of Fe2 (Fig. 2(c)). This ferricyanide-oxidized form of the cluster was recovered upon re-reduction with H_2 or titanium(III) citrate, indicating that this redox-dependent structural change is reversible.

EPR redox titration of MBHs from *R. eutropha* H16 and *A. aeolicus* showed four distinct one-electron transitions attributable to three iron-sulfur clusters [4,5]. Three of four mid-potentials were consistently assigned to three clusters. The metal center with the remaining highest potential had some interactions on both Ni in the active site and the medial [3Fe-4S], suggesting that this high-potential species should be assigned to the proximal cluster. Namely, the proximal cluster was proposed to have two midpoint potentials (dual redox character). These results are astonishingly consistent with our findings described above. The net charge of the [4Fe-3S]-6Cys in MBH established in this study is equal to that of [4Fe-4S]-4Cys in the standard enzymes, assuming that the oxidation states of the iron atoms are identical. The redox transition of 4+/3+ (corresponding to 2+/1+ for cubane [4Fe-4S] cluster) is most probable for the proximal [4Fe-3S] cluster during the H_2-oxidation catalytic cycle. The superoxidation of the proximal cluster to $[4Fe\text{-}3S]^{5+}$ should require the conformational change and concomitant donation of an additional negative charge to the Ni-Fe active site. In this superoxidized structure, an additional negative charge of the deprotonated amide nitrogen, which coordinates Fe2, should stabilize a higher oxidation state of the iron atoms. Two previously proposed single-electron transitions of the proximal cluster in MBH (that is, $[4Fe\text{-}3S]^{5+/4+/3+}$) can thus be reasonably explained by the redox-dependent conformational change observed in this study. In standard enzymes, Ni-A is considered to be produced by the oxidation of the active site with O_2 under electron-deficient conditions, whereas Ni-B is formed under electron-rich conditions [2]. Therefore, having a higher redox potential and a two-electron donation property in the proximal cluster are advantageous in preventing the formation of Ni-A. Our results provide a structural basis that underlies the unprecedented function of the [4Fe-3S] cluster in the O_2 tolerance of MBH, which acquires a superoxidized state to supply two electrons and one proton for the reduction of O_2 through a redox-dependent conformational change.

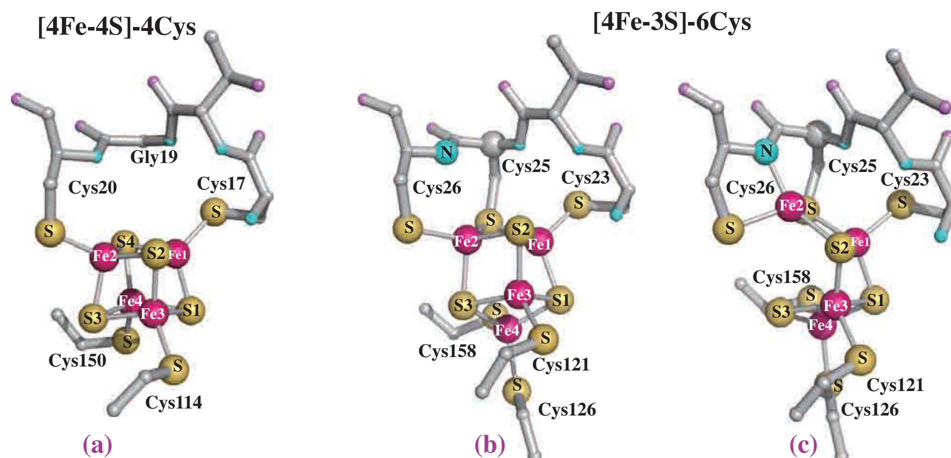

Fig. 2. [4Fe-4S] and [4Fe-3S] clusters. Cubane-type [4Fe-4S] cluster **(a)** in standard enzymes is coordinated by four cysteines, whereas [4Fe-3S] cluster in H_2-reduced **(b)** and ferricyanide-oxidized **(c)** forms found in this study are coordinated by six cysteines. Fe and S atoms in the inorganic part are labeled by element symbols with numbers, whereas S atoms belong to cysteine residues, and the amide N atom participating in the coordination to Fe2 are without numbers. Fe, S, N, O, and C atoms are colored red, yellow, cyan, pink, and gray, respectively.

Yasuhito Shomura and Yoshiki Higuchi*

Department of Life Science, University of Hyogo

*E-mail: hig@sci.u-hyogo.ac.jp

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