Structural Basis of Inter-protein Electron Transfer for Nitrite Reduction in Denitrification
Paper in journals : this is the first page of a paper published in ***Nature***.
[*Nature*] 462, 117-120 (2009)

Vol 462|5 November 2009|doi:10.1038/nature08507 nature

LETTERS

Structural basis of inter-protein electron transfer for nitrite reduction in denitrification

Masaki Nojiri[1], Hiroyasu Koteishi[1], Takuya Nakagami[1], Kazuo Kobayashi[2], Tsuyoshi Inoue[3], Kazuya Yamaguchi[1] & Shinnichiro Suzuki[1]

Recent earth science studies have pointed out that massive acceleration of the global nitrogen cycle by anthropogenic addition of bio-available nitrogen has led to a host of environmental problems[1]. Nitrous oxide (N_2O) is a greenhouse gas that is an intermediate during the biological process known as denitrification[2]. Copper-containing nitrite reductase (CuNIR) is a key enzyme in the process; it produces a precursor for N_2O by catalysing the one-electron reduction of nitrite (NO_2^-) to nitric oxide (NO)[3]. The reduction step is performed by an efficient electron-transfer reaction with a redox-partner protein[4–6]. However, details of the mechanism during the electron-transfer reaction are still unknown. Here we show the high-resolution crystal structure of the electron-transfer complex for CuNIR with its cognate cytochrome *c* as the electron donor. The hydrophobic electron-transfer path is formed at the docking interface by desolvation owing to close contact between the two proteins. Structural analysis of the interface highlights an essential role for the loop region with a hydrophobic patch for protein–protein recognition; it also shows how interface construction allows the variation in atomic components to achieve diverse biological electron transfers.

The biological reduction of nitrite ions to gaseous nitrogen monoxide ($NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O$) is catalysed by two very different enzymes called haem cd_1-containing nitrite reductase (cd_1NIR) and CuNIR, which are less commonly known by their product gene names, NirS and NirK, respectively[3]. They are mutually exclusive at the species level in the denitrifying bacteria distributed widely on Earth[7]. For the one-electron reduction of NO_2^-, the enzyme is linked to respiratory electron-transfer chains by cytochrome *c* (Cyt *c*) and/or cupredoxin in the cell[8,9]. Although the electrons destined for the enzyme pass through the cytochrome bc_1 complex in the chain, how they pass on from this complex to the enzyme is still a matter of uncertainty. In general, CuNIR folds a homotrimeric structure with two distinct Cu-binding sites through an approximately 37-kilodalton (kDa) monomer unit[10]. The type 1 Cu site (T1Cu) buried within each monomer relays an electron from the redox-partner protein to the catalytic type 2 Cu site (T2Cu), where NO_2^- is reduced to NO. This enzyme is further classified into two subgroups based on the spectroscopic properties of the T1Cu site, which are blue and green[11]. Whereas the physiological redox-partner protein for green CuNIR is pseudoazurin (PAz), the blue CuNIR has been suggested to accept an electron from azurin (Az) or Cyt *c*[11–13].

In a ubiquitous Gram-negative non-fermenting rod, *Achromobacter xylosoxidans* GIFU 1051 (the organism formerly known as *Alcaligenes xylosoxidans*)[14], it has been found that two structural genes (*nirK* and *nirM*) of the blue CuNIR (AxgNIR) and Cyt c_{551} constitute an operon presumed to be a redox-partner pair (Supplementary Fig. 1). The *nirM* gene is also part of the operon that harbours the *nirS* gene coding for cd_1NIR in other denitrifying bacteria, and the product Cyt c_{551} protein acts as a physiological redox partner for cd_1NIR[15]. Recent gene disruption studies on the *nirK* gene-harbouring bacterium *Bradyrhizobium japonicum* have shown that Cyt c_{550} is required for growth under denitrifying conditions[16]. Moreover, earlier work by Zumft and colleagues has demonstrated that CuNIR can function instead of cd_1NIR; this is most likely caused by the use of Cyt *c* as a redox partner in *Pseudomonas stutzeri*[17]. Thus, it is reasonable to assume that not only A2 but also Cyt c_{551} plays a role as an *in vivo* redox partner for AxgNIR.

To elucidate the physiological relationship between AxgNIR and Cyt c_{551}, the formation of a functional electron-transfer complex between these proteins was analysed by stopped-flow kinetics and a crystallographic approach (Fig. 1). The kinetics of electron transfer from the reduced Cyt c_{551} to AxgNIR was monitored at a wavelength of 420 nm originating from a Soret band of the haem *c* group under anaerobic conditions. The rapid decay of the absorbance is due to the oxidation of Cyt c_{551} and concomitant reduction of AxgNIR (Fig. 1a). The second-order electron-transfer rate constant between the two proteins, $(4.8 \pm 0.2) \times 10^6\ M^{-1}\ s^{-1}$ (s.d.) (Fig. 1a, inset), strongly supports the idea that Cyt c_{551} interacts functionally with AxgNIR as an electron donor. To understand the detailed mechanism further, the crystal structure of the binary complex of AxgNIR with Cyt c_{551} was determined at a resolution of 1.7 ångströms (Å). A model of the complex, which consists of one Cyt c_{551} and one trimeric AxgNIR molecule within an asymmetric unit, contains 8,242 protein atoms, one haem *c* group, and six copper atoms (Fig. 1b; see also Supplementary Figs 2 and 3a). The AxgNIR molecules contribute to major contacts in the crystal lattice and provide only one space for Cyt c_{551}; this is bound to its docking site on a subunit (Sub-I) of AxgNIR without steric hindrance (Supplementary Fig. 3b).

Within the docking interface, the change in solvent-accessible surface area upon complex formation is estimated to be $-529\ Å^2$ for Sub-I and $-553\ Å^2$ for Cyt c_{551}; the gap index (the ratio of gap volume to buried interface solvent-accessible surface area) is 3.55 Å. The haem *c* group partly protrudes from the protein interior of the Cyt c_{551} molecule at two parts: the thioether-bonded substituent linked to pyrrole ring C and the propionate groups on pyrrole rings A and D (Fig. 1c). The Cyt c_{551}-docking site near the T1Cu site of Sub-I and the haem *c* group are in close contact at a 3.5-Å distance between the Cε atom of Met 87 in Sub-I and the edge CBC methyl carbon of haem *c* (Fig. 2a). At least ten amino-acid residues of AxgNIR are associated with at least 11 residues and the haem *c* group of Cyt c_{551} at the interface (Supplementary Table 2). There is no salt bridge at the interface, and only three direct hydrogen bonds were observed. In general, direct hydrogen bonds between the docking

[1]Department of Chemistry, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan. [2]The Institute of Scientific and Industrial Research, Osaka University, Ibaraki, Osaka 567-0047, Japan. [3]Department of Materials Chemistry, Graduate School of Engineering, Osaka University, Suita, Osaka 565-0871, Japan.

The following is a comment on the published paper shown on the preceding page.

Atomic Description of Inter-protein Electron Transfer for Biological Nitrite Reduction in the Global Nitrogen Cycle

NOJIRI Masaki and SUZUKI Shinnichiro
(Graduate School of Science)

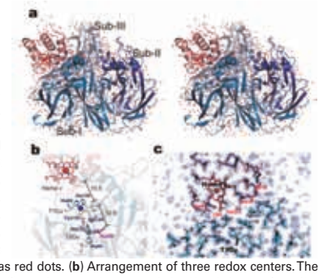

Fig. 1 Overall structure of the interprotein ET complex of AxgNIR with Cyt c_{551}. (**a**) Stereo side view. The Cyt c_{551} molecule is represented as a pink-colored ribbon, and the Cyt c_{551}-docked subunit (Sub-I) of AxgNIR is shown in sky blue, the undocked subunit (Sub-II) in navy, and the other one (Sub-III) in light blue. The heme group (red), T1Cu (dark blue), and T2Cu (gray) are depicted as balls and sticks. Water molecules are shown as red dots. (**b**) Arrangement of three redox centers. The heme group and ligand residues of both Cu centers are shown as sticks. The distances from the CBC methyl group on heme *c* to T1Cu and from T1Cu to T2Cu are given in angstroms. Marks A to D of the heme are indicated the four pyrrole rings. (**c**) Electron density map of the docking interface between AxgNIR (blue) and Cyt c_{551} (pink).

Introduction

Protein electron transfer (ET) reactions play a critical role in biologically vital processes in living cells, most notably respiration and photosynthesis. The reactions occur between protein-bound prosthetic groups separated by long distances, often greater than 10 Å and display high efficiency and specificity. In addition to a dependence on factors inherent to the long-range ET processes, numerous studies have revealed that intermolecular ET reactions requiring a balance of specific binding and fast dissociation are highly sensitive to protein association modes and their protein-protein interfaces. Hence, the conformational changes of the amino acid residues and the behavior of solvent molecules at the interface formed by redox-partner proteins have the potential to regulate inter-protein ET[1].

Recent earth science and geochemical studies have pointed out that the massive acceleration of the global nitrogen cycle as a result of the production and industrial use of artificial nitrogen fertilizers worldwide has led to a host of environmental problems, ranging from eutrophication of terrestrial and aquatic systems to global acidification[2]. Denitrification is one of the biological processes contributing to the maintenance of the nitrogen balance on the earth. Dissimilatory copper-containing nitrite reductase (CuNIR) is a key enzyme in denitrification, catalyzing one-electron reduction of nitrite (NO_2^-) to nitrogen monoxide (NO)[3]. The reaction is specifically regulated by the efficient ET reaction with a redox-partner protein. CuNIRs fold a trimeric structure with two distinct Cu sites per a ca. 37-kDa monomer unit. The type 1 Cu site (T1Cu) buried within each monomer relays an electron from the redox-partner protein to the catalytic type 2 Cu site (T2Cu), where NO_2^- is reduced to NO. Despite much effort by several groups, a crystal structure of the protein–protein complex between CuNIR and its redox-partner has not yet been determined.

Crystal structure of a transient ET complex of CuNIR with Cyt *c*

The crystal structure of a binary complex of CuNIR (AxgNIR) with its redox partner cytochrome c_{551} (Cyt c_{551}) from a ubiquitous gram-negative non-fermenting rod, *Achromobacter xylosoxidans* GIFU1051 (the organism formerly known as *Alcaligenes xylosoxidans*), was determined at a resolution of 1.7 Å. A model of the binary AxgNIR:Cyt c_{551} complex, which consists of one Cyt c_{551} and one AxgNIR molecule within an asymmetric unit, contains 8,242 protein atoms, one heme *c* group, six copper atoms, and 1,073 solvent molecules (Fig.1). Only one Cyt c_{551} molecule is bound to one subunit (Sub-I) of trimeric AxgNIR. The high-resolution crystal structure allows an accurate description of the complex interface between Sub-I and Cyt c_{551}. Within the interface, the change in solvent-accessible surface area upon complex formation is estimated to be -529 Å^2 for Sub-I and -553 Å^2 for Cyt c_{551}. In the isolated Cyt c_{551} structure, the heme c group is partially exposed to solvent at the two parts: the thioether-bonded substituent linked to pyrrole ring C and the propionate groups on pyrrole rings A and D (Fig.1b). At the center of the interface, the Cyt c_{551} docking site near the T1Cu site of Sub-I and the heme *c* group are in close contact at a 3.5-Å distance between the Cε atom of Met87 in Sub-I and the edge CBC methyl carbon of the thioether-bonded substituent on heme *c*. At least 10 amino acid residues of AxgNIR are associated with at least 11 amino acid residues and the heme *c* group of Cyt c_{551} at the interface. The atoms of these residues and heme *c* interact with each other at a distance of ≤3.5 Å. There is no salt bridge at the interface, and only three direct hydrogen bonds were observed: Thr192 Oγ1/Gly67 O (2.8 Å), Glu195 Oε1/Ala69 N (2.8 Å), and Gly198 N/Ser18 O (2.9 Å) of Sub-I/Cyt c_{551}. In general, direct hydrogen bonds between the docking proteins seem to be unfavorable for a transient ET complex because of energetically disadvantageous desolvation[4]; namely, interface of the reaction center (RC):Cyt c_2 complex also has three intermolecular hydrogen bonds[5] and that of the yeast cytochrome *c* peroxidase (CcP):Cyt c complex one hydrogen bond[6], but that of the cytochrome bc_1 (Cyt bc_1):Cyt *c* complex has no hydrogen bond[7].

Interface between CuNIR and Cyt *c*

The building blocks of the interface between AxgNIR and Cyt c_{551} are shown in Fig. 2. The interacting residues of AxgNIR are localized both at the hydrophobic patch near the T1Cu site and at the "tower loop" region extending toward the T1Cu site. These primarily non-polar and neutral residues indicate that hydrophobic and van der Waals interactions strongly contribute to complex for-