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Crystallographic and solution studies of NAD^+- and NADH-bound alkylhydroperoxide reductase subunit F (AhpF) from *Escherichia coli* provide insight into sequential enzymatic steps

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ABSTRACT

Redox homeostasis is significant for the survival of pro- and eukaryotic cells and is crucial for defense against reactive oxygen species like superoxide and hydrogen peroxide. In *Escherichia coli*, the reduction of peroxides occurs *via* the redox active disulfide center of the alkyl hydroperoxide reductase C subunit (AhpC), whose reduced state becomes restored by AhpF. The 57 kDa *Ec*AhpF contains an N-terminal domain (NTD), which catalyzes the electron transfer from NADH *via* an FAD of the C-terminal domain into *Ec*AhpC. The NTD is connected to the C-terminal domain *via* a linker. Here, the first crystal structure of *E. coli* AhpF bound with NADH and NAD^+ has been determined at 2.5 Å and 2.4 Å resolution, respectively. The NADH-bound form of *Ec*AhpF reveals that the NADH-binding domain is required to alter its conformation to bring a bound NADH to the *re*-face of the isoalloxazine ring of the flavin, and thereby render the NADH-domain dithiol center accessible to the NTD disulfide center for electron transfer. The NAD^+-bound form of *Ec*AhpF shows conformational differences for the nicotinamide end moieties and its interacting residue M467, which is proposed to represent an intermediate product-release conformation. In addition, the structural alterations in *Ec*AhpF due to NADH- and NAD^+-binding in solution are shown by small angle X-ray scattering studies. The *Ec*AhpF is revealed to adopt many intermediate conformations in solution to facilitate the electron transfer from the substrate NADH to the C-terminal domain, and subsequently to the NTD of *Ec*AhpF for the final step of AhpC reduction.

1. Introduction

Reactive oxygen species (ROS) are inevitable byproducts during the course of normal aerobic metabolism. Biological cells have developed antioxidant systems to regulate the endogenous level of ROS, which damage the cells at higher concentration and can function as a cellular signaling molecule at lower concentration [1,2]. Peroxiredoxins (prx), a class of thiol-specific antioxidant proteins, together with peroxiredoxin reductases (prxR) are the predominant cellular defenses against oxidative stress and are also involved in cellular signaling pathways [3,4]. The coordinated interplay between prx and prxR is essential for enhanced peroxidative catalytic rates. Many bacteria possess an alkyl hydroperoxide reductase (AhpR) system, composed of the two most abundant enzymes called AhpF (prxR) and AhpC (prx), which together catalyze the NADH-dependent reduction of H_2O_2 [5–7]. AhpC undergoes oligomeric states, which are redox-sensitive. Reduced AhpC preferentially assembles five catalytic dimers into a doughnut-like decamer, whereby the oxidized form of the protein leads to a lower order of oligomers between decamer and dimer [8,9].

AhpF is a homo-dimeric protein and belongs to FAD-dependent NADH protein-disulfide reductases. The protein consists of an N-terminal domain (NTD), which catalyzes the reduction of redox-active disulfides in AhpC [10,11] (Fig. 1). The NTD is connected *via* a linker to the C-terminal catalytic core, containing an FAD- and NADH binding domain with the redox-active disulfide (C345/C348 according to *Escherichia coli* numbering). With regard to catalytic mechanism, AhpF has been proposed to alternate between two states known as flavin-oxidized (FO) and flavin-reduced (FR), in-order to accomplish the series of intra-molecular electron transfers from NADH to FAD and to the redox active disulfide, as well as the release of the oxidized NAD^+ [12–14]. The cascade of electron transfers from NADH *via* FAD, to the C-terminal redox-active center (C345/C348) followed by the disulfide center (C129/C132) of the NTD would provide the electrons for the redox-active center (C47/C166) of AhpC [11,15]. Apparently, conformational rearrangements of the NTD and C-terminal domain (CTD)

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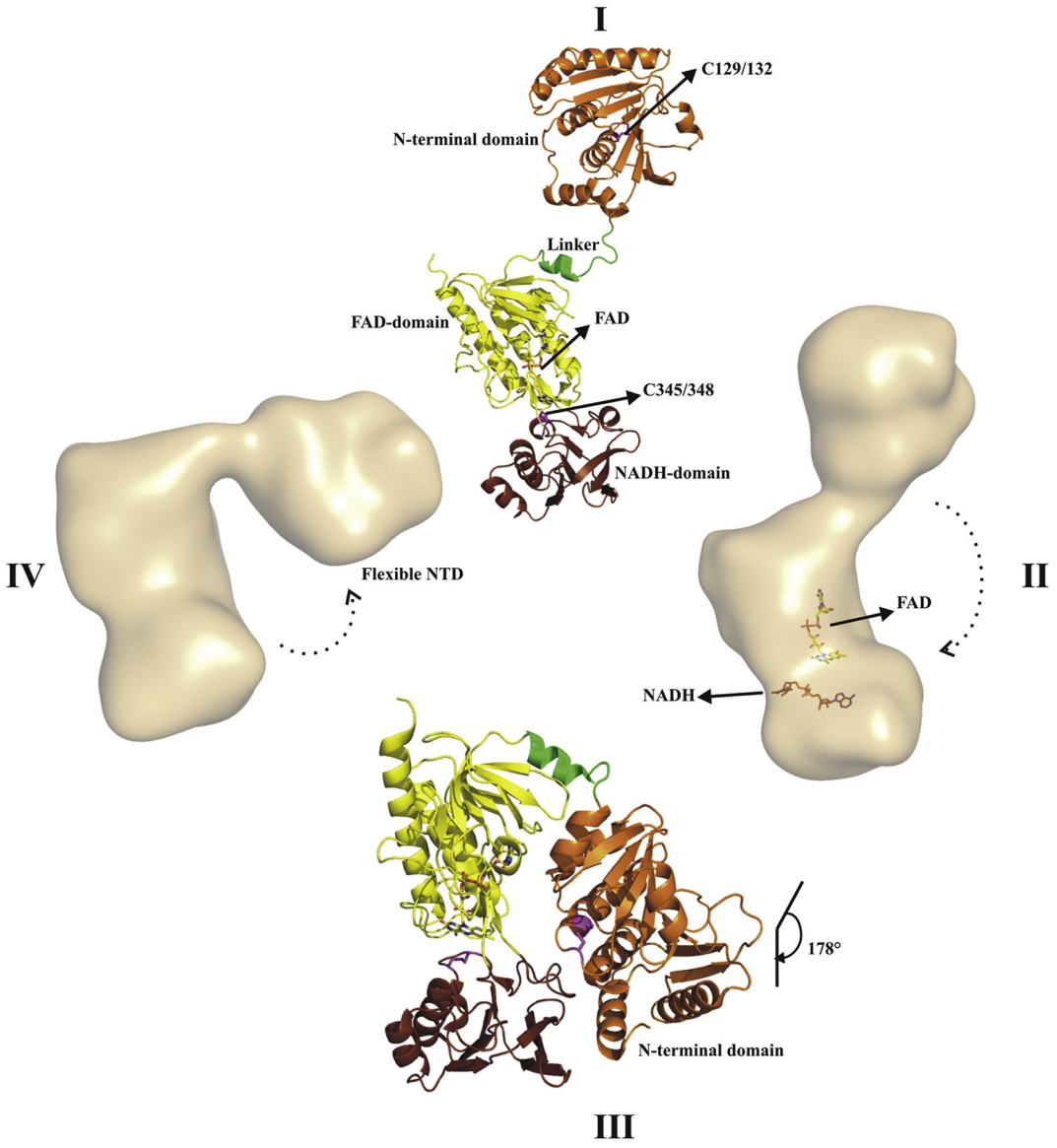

Fig. 1. *Structural details of AhpF:* cartoon representation of the structures available for full-length AhpF show the extended (I) and compact (III) conformations. (II) The model represents the lack of structural details for substrate binding and the alternating conformation of the NADH-domain. (IV) The plausible model, showing the flexibility of the N-terminal domain during electron transfer. The arrows in panels II and IV indicate the flexibility of the N-terminal domain of AhpF. For clarity, the AhpF molecule is shown as a monomer.

are essential to mediate the electron transfer during catalysis. The recent crystallographic structures of the *E. coli* [11] (PDB ID: 4O5Q) and *Salmonella typhimurium* [16] (*St*AhpF, PDB ID: 1HYU) AhpF revealed an elongated and a compact conformation, respectively (Fig. 1). The elongated form of *Ec*AhpF is proposed to have a prominent role in AhpC reduction, in which the dimeric *Ec*AhpF binds to the AhpC decamer [11]. In comparison, the compact conformation shown in the *St*AhpF structure potentially mimics the disulfide–dithiole exchange taking place between the NTD and CTD redox centers, even though they are not in a proximity for electron transfer to occur [16] (Fig. 1III).

So far, the structural studies on the apo-form of AhpF reveal only the FO-state of the NADH-domain (Fig. 1I and III), where the NADH-redox active disulfide is adjacent to the flavin. However, no detailed structural insight is available with regard to substrate binding, specificity and the intra-molecular electron transfer mechanism (Fig. 1II) [11,16,17]. In addition, the degree of conformational flexibility and possible relevant intermediate conformations of the NTD in solution is not yet characterized (Fig. 1IV). Here, an attempt has been made to solve the crystal structure of *E. coli* AhpF with NADH or NAD^+ bound to the enzyme. The first crystal structure of *Ec*AhpF with bound substrate implies that the NADH-domain is required to rotate relative to the FAD-domain by 65°. The underlying intra-molecular electron transfer mechanism is discussed. The existence and the extent of conformational flexibility of the NTD are demonstrated using SAXS, studying the NADH- and NAD^+-bound forms of *Ec*AhpF in solution.

2. Materials and methods

2.1. Crystallization of the NADH- and NAD^+-bound forms of EcAhpF

The purification of *Ec*AhpF was carried out using a recently published method [11]. The purified protein was concentrated to 10 mg/ml in a buffer composed of 50 mM Tris/HCl (pH 8) and 200 mM NaCl. Plate-shaped crystals with the dimensions of 0.25 × 0.12 × 0.05 mm were obtained by the micro-seeding method with the precipitant solution of 0.1 M Na-HEPES (pH 7.5), 2.5% (v/v) PEG 400, 2 M ammonium sulfate