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# The key role of water in the dioxygenase function of *Escherichia coli* flavohemoglobin

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Flavohemoglobins (FHbs) are members of the globin superfamily, widely distributed among prokaryotes and eukaryotes that have been shown to carry out nitric oxide dioxygenase (NOD) activity. In prokaryotes, such as *Escherichia coli*, NOD activity is a defence mechanism against the NO release by the macrophages of the hosts' immune system during infection. Because of that, FHbs have been studied thoroughly and several drugs have been developed in an effort to fight infectious processes. Nevertheless, the protein's structural determinants involved in the NOD activity are still poorly understood. In this context, the aim of the present work is to unravel the molecular basis of FHbs structural dynamics-to-function relationship using *state of the art* computer simulation tools. In an effort to fulfill this goal, we studied three key processes that determine NOD activity, namely i) ligand migration into the active site ii) stabilization of the coordinated oxygen and iii) intra-protein electron transfer (ET). Our results allowed us to determine key factors related to all three processes like the presence of a long hydrophobic tunnel for ligand migration, the presence of a water mediated hydrogen bond to stabilize the coordinated oxygen and therefore achieve a high affinity, and the best possible ET paths between the FAD and the heme, where water molecules play an important role. Taken together the presented results close an important gap in our understanding of the wide and diverse globin structural-functional relationships.

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## 1. Introduction

Flavohemoglobins (FHbs) are members of the ubiquitous heme containing globin super family of proteins, which also includes the single domain globins and the truncated hemoglobins. Their characteristic fold combines an N-terminal globin domain (GD, residues 1 to ≈ 140), a FAD-binding domain (FBD, residues ≈ 140 to ≈ 250) and a NAD-binding domain (NBD, residues ≈ 250 to ≈ 400). These domains are organized in a “heart-shaped” structure, presenting a cleft in between the GD and the NBD (Fig. 1) [1,2]. Over the years, the discovery of novel globin genes among different types of organisms such as bacteria, algae, protozoa and fungi suggests that these proteins were not originally designed for oxygen transport and storage as the well known mammalian hemoglobin(Hb) and myoglobin(Mb) [3,4]. Instead, it has been proposed that they may perform a wide variety of tasks such as relief of nitro and oxidative stress; CO, NO or $O_2$ sensing; or even be involved in reductive and/or oxidative catalytic processes [3–5].

Particularly, FHbs have been suggested to play an important role in nitrosative stress resistance, due to their nitric oxide dioxygenase activity (NOD) [1,6]. This activity is also shared with other globins, such as some of the members of the truncated hemoglobins (also known as bacterial globins), like the *Mycobacterium tuberculosis* truncated hemoglobin N (MtTrHbN) [7–10]. Such function can make the difference between bacterial survival or death under stress conditions, as the attack of the hosts' immune system. Therefore, elucidating how FHbs carry out NOD activity is crucial for understanding bacterial resistance mechanisms. In this context, FHbs have been proposed as molecular targets for several antibacterial and antimycotic imidazole derived compounds, including the FDA approved azoles [11,12].

The NOD activity carried out by heme proteins consists in the conversion of nitric oxide (NO), which is toxic for the bacteria, into innocuous nitrate according to the NO detoxification reaction cycle, shown in Scheme 1 [9,13–16].

In order to perform NOD activity (see Scheme 1) the protein must be able to internalize $O_2$ into the GD and stabilize the iron bound oxygen, a process characterized by oxygen association and dissociation rate constants ($k_{on}$ and $k_{off}$). Afterwards, the NO molecule must also migrate into the globin domain and react with the oxy-heme ($Fe^{II}O_2$) to form ferric heme ($Fe^{III}$) and a nitrate ion ($NO_3^-$) [9,14], which is then released to the solvent [17]. The NO reaction with oxy heme is usually characterized by the rate constant $k_{ox}$NO which is extremely fast. Finally, the protein needs to reduce the heme back

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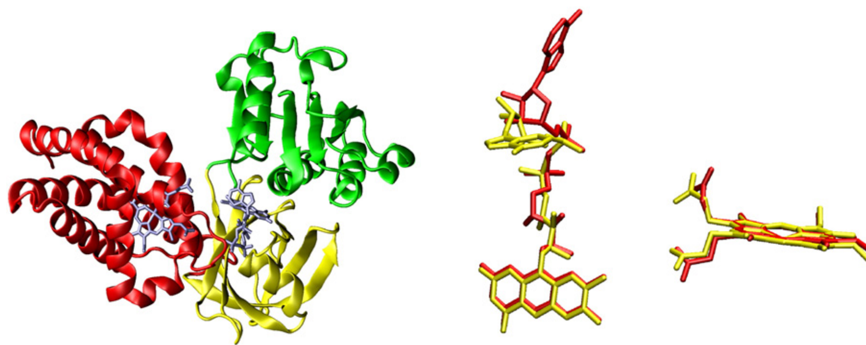


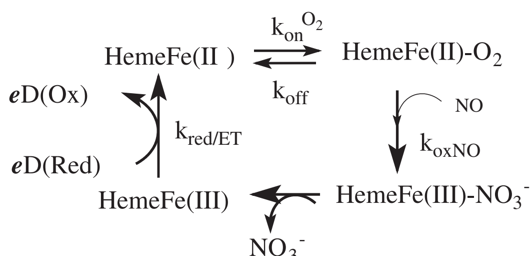
**Fig. 1.** Left panel: structure of *E. coli* flavohemoglobin. Red: globin domain. Yellow: FAD-binding domain. Green: NAD-binding domain. Right panel: FAD and heme crystal conformation. Red: A. eutrophus. Yellow: *E. coli*.

to the ferrous ($Fe^{II}$) state, usually through inter or intraprotein electron transfer (ET) from a suitable electron donor (***e***D) that is oxidized in one electron along the process, in order to start over the cycle [1].

Although possibly all globins may carry out NOD activity in-vitro under optimal conditions [16], not all of them are equally efficient. The most studied and well understood efficient NODs are the FHbs from *Escherichia coli, Alcaligenes eutrophus* and *S. cerevisae* [1,18,19], and the truncated hemoglobin MtTrHbN [7–10,17]. The structural elements that determine an efficient NOD activity have been related to fast ligand migration into the active site ($O_2$ into the deoxygenated protein and NO into the oxygenated protein) [8], strong stabilization of the oxygen coordinated to the heme [9], and fast electron transfer to recover the ferrous state [16].

To ensure fast NO incorporation into the oxygenated protein, and also for oxygen entry to the free protein, MtTrHbN relies on the presence of a tunnel cavity system which is allosterically modulated by the presence of the oxygen ligand [8,20]. All globins structurally studied so far reveal the presence of internal cavities and tunnels [21,22] which have been related to both ligand migration [23] and multi-ligand chemistry processes [24]. Interestingly, while in the truncated hemoglobins the tunnel/cavity system connects the heme active site with the solvent [22,8,20,25,26] in other globins the cavities do not connect the heme with the solvent, which is accessible through the so called HisE7 gate [23,24], but allow the ligand to go deeper inside the protein core [27]. So far, the way in which the ligand enters and exits the GD and how the tunnel/cavities for ligand migration are related to the global protein structure is unknown for FHbs.

In MtTrHbN, as in many globins, strong oxygen stabilization is ensured due to the presence of distal tyrosine at position B10 (TyrB10) which forms a tight hydrogen bond (HB) with the bound oxygen ligand [7,9]. Strikingly, although in FHbs position B10 is indeed occupied by a tyrosine, in the crystal structures (*E. coli* pdbid 1GVH [28], and *A. eutrophus* pdbid 1CQX [29]) TyrB10 is too far from the iron to establish an HB with the bound ligand. Moreover, in *A. eutrophus* FHb the crystal structure displays a phospholipid molecule with the lipid side chain inside the distal pocket, on top of the iron [29].




**Scheme 1.** Complete nitric oxide dioxygenase reaction cycle performed by heme proteins.

Given that no other HB donor residues are present in the distal pocket of FHb, the mechanism by which high oxygen affinity is achieved is still unclear. To complicate the picture even further, resonant Raman studies of CO bound *E. coli* FHb suggested the presence of two different conformations, which have been assigned to open and closed conformations of the distal pocket [30]. The closed conformation presents similar Fe–C and C–O vibrational frequency values as those reported for MtTrHbN where a tight HB is established with the CO ligand by TyrB10 [31], while the open conformation has vibrational frequencies which are similar to the H64V/V68T Myoglobin mutant [32] or the *T. tencogensis* HNOX domain [33] displaying an oxygen lone pair pointing towards the bound CO ligand [34]. Both conformations are supposed to be in dynamic equilibrium and increasing the environment pH increases the relative population of the open state [30].

Finally, what makes FHbs special over other globins, is the presence of the FBD and NBD, which ensures a fast and efficient heme reduction during catalytic turnover [1,2]. Heme reduction has been suggested to occur by shooting electrons from the co-substrate NAD to the FAD and, from there, to the heme; although direct NAD to heme electron transfer cannot be discarded. It is important to note, that since NAD is a two electron donor, while for each NOD cycle the globin domain requires only one electron, the Flavin not only acts an electron bridge, but allows the storing of one electron, after one re-reduction cycle. In other words, if the protein is completely oxidized and without NAD, once the NAD enters the protein it will first perform a two electron reduction of the FAD. Then one electron from the reduced Flavin will reduce the heme, while remaining in the semi reduced radical state, until the heme is oxidized after an NOD cycle. FHbs are thus unique globins, since reduction involves an intra-protein ET between domains, and not inter-protein ET, as is the case for Mb and Hb reduction by Cytochrome-b5 [35–37]. Although understanding how structure, dynamics and domain–domain interactions in FHbs modulate ET could give insights into general reduction mechanisms of globins, the molecular details of the ET process in FHbs are still unknown. The crystal structures of the *E. coli* flavohemoglobin (HMP) and the *Alcaligenes eutrophus* flavohemoglobin (FHP) lack the NAD co-substrate, but interestingly, in both of them, the FAD molecule adopts significantly different conformations, (Fig. 1) suggesting a high mobility of the adenine moiety [1,2].

The aim of the present work is to unravel the molecular basis of FHb structural dynamics-to-function relationship using *state of the art* computer simulation tools. To achieve this goal, we studied the three key processes that determine NOD activity, namely i) ligand migration and ii) oxygen binding and iii) intra-protein electron transfer. Our results allowed us to determine key factors related to all three processes like the presence of a long tunnel for ligand migration, the presence of a water mediated hydrogen bond to achieve a string stabilization for the ligand and therefore a high oxygen affinity, and the best possible ET paths between the FAD and the heme.

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