Disruption of the dimerization interface of the sensing domain in the dimeric heme-based oxygen sensor AfGcHK abolishes bacterial signal transduction

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The heme-based oxygen sensor protein AfGcHK is a globin-coupled histidine kinase in the soil bacterium Anaeromyxobacter sp. Fw109-5. Its C-terminal functional domain exhibits autophosphorylation activity induced by oxygen binding to the heme-Fe(II) complex located in the oxygen-sensing N-terminal globin domain. A detailed understanding of the signal transduction mechanisms in heme-containing sensor proteins remains elusive. Here, we investigated the role of the globin domain’s dimerization interface in signal transduction in AfGcHK. We present a crystal structure of a monomeric imidazole-bound AfGcHK globin domain at 1.8 Å resolution, revealing that the helices of the WT globin dimer are under tension and suggesting that Tyr-15 plays a role in both this tension and the globin domain’s dimerization. Biophysical experiments revealed that whereas the isolated WT globin domain is dimeric in solution, the Y15A and Y15G variants in which Tyr-15 is replaced with Ala or Gly, respectively, are monomeric. Additionally, we found that although the dimerization of the full-length protein is preserved via the kinase domain dimerization interface in all variants, full-length AfGcHK variants bearing the Y15A or Y15G substitutions lack enzymatic activity. The combined structural and biophysical results presented here indicate that Tyr-15 plays a key role in the dimerization of the globin domain of AfGcHK and that globin domain dimerization is essential for internal signal transduction and autophosphorylation in this protein. These findings provide critical insights into the signal transduction mechanism of the histidine kinase AfGcHK from Anaeromyxobacter.

Heme complexes play essential roles in many important physiological processes, including oxygen transport and storage, electron transport, oxidative stress protection, and signaling. They are also vital components of heme-containing sensor proteins, a large group of biologically significant proteins that are represented in almost all living organisms and exhibits great functional diversity (1–6).

There is considerable interest in the structure-function relationships of heme-based oxygen sensors because the heme-iron complex serves as the signaling center (1, 7–14). Proteins of this type often act as one component of a two-component signal transduction system. These systems are associated with biofilm formation and virulence in certain pathogenic bacteria but also fulfill many other functions (15–18). The heme-based oxygen sensor AfGcHK4 is a globin-coupled histidine kinase from the soil bacterium Anaeromyxobacter sp. Fw109-5 (19). It has an N-terminal heme-bound globin domain and a C-terminal kinase domain. The binding of oxygen to a heme-Fe(II) complex in the oxygen-sensing globin domain induces autophosphorylation of its kinase domain via interdomain signal transduction (19). AfGcHK proteins containing a heme-Fe(II)-O2 complex or an Fe(III)-OH− complex formed by autooxidation are both enzymatically fully active (20). The values of the heme-Fe(II)-O2 and Fe(III)-OH−-bound AfGcHK forms were

The abbreviations used are: AfGcHK, globin-coupled histidine kinase from the soil bacterium Anaeromyxobacter sp. Fw109-5; AvGReg, Azotobacter vinelandii globin-coupled oxygen sensor; BpeGReg, globin-coupled oxygen sensor with diguanylate cyclase activity from Bordetella pertussis; CusS, E. coli copper and silver ions sensor; DevS (DoxS), heme-based oxygen sensor protein from Mycobacterium tuberculosis; EcDOs or EcdosP, heme-based oxygen sensor phosphodiesterase; HDX-MS, hydrogen deuterium exchange coupled with mass spectrometry; HemAT, globin-coupled oxygen sensor from B. subtilis; heme-Fe(II), heme-Fe(II)-protoporphyrin IX complex or hemin; Mpd, 2-methyl-2,4-pentanedion; PccGCS, globin-coupled oxygen sensor with diguanylate cyclase activity from P. carotovorum; PdB, Protein Data Bank; TEV, tobacco etch virus; YddV, globin-coupled oxygen sensor diguanylate cyclase from E. coli; RMSD, root mean square deviation.

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M. M. dedicates this paper to Professor Marie Stiborová of the Dept. of Biochemistry at Charles University on the occasion of her 70th birthday, in recognition of her devotion to science and people.

This article contains Figs. S1–S4 and Tables S1 and S2.

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1.0–1.1 min⁻¹, and their $K_m^{ATP}$ values were 18.9–23.0 μM (Table S1). However, the form with an Fe(II)-CO-heme complex had a $k_{cat}$ of 1.0 min⁻¹ but a very high $K_m^{ATP}$ of 357 μM. This high $K_m^{ATP}$ suggests that in addition to causing pronounced changes in the structure of the sensing site, CO coordination dramatically altered the structure of the kinase domain (20). The inactive AfGcHK forms either contain a heme-Fe(II) complex, lack the heme complex altogether (20), or lack the whole sensing domain (21).

Several studies have investigated the structure-function relationships of AfGcHK (19–22). As a result, the X-ray crystal structure of the isolated AfGcHK globin domain dimer has been solved and the full-length protein has been studied by hydrogen-deuterium exchange coupled with MS (HDX-MS) (21). These studies have revealed that changes in the heme redox state and axial ligand binding to the heme-iron complex induce large changes in the protein’s structure in the vicinity of the heme, which in turn cause profound structural changes in the wider protein. It was suggested that these structural changes occur primarily within the dimerization interface of the globin domain and are linked to the regulation of the kinase domain’s catalytic activity (21). Here, we present X-ray crystallographic investigations and a thorough biophysical analysis including a kinase activity study that were conducted to determine how the oligomerization state of the globin (sensing) domain affects the autophosphorylation activity of AfGcHK. The crystal structure of the monomeric sensing domain co-crystallized with imidazole was solved, and inspection of this structure suggested that Tyr-15 is involved in the domain dimerization process and also forms important contacts with the neighboring heme group. Therefore, the oligomeric states and functions of Tyr-15 mutant proteins were investigated. Based on the results of these investigations, we suggest that the interaction of Tyr-15 with the heme-binding pocket of the neighboring globin domain in the AfGcHK dimer is critical for the sensing domain’s dimerization, and that the sensing domain’s dimerization is required to enable signal transduction between the sensing and functional domains and the functional domain’s autophosphorylation activity.

Results

Crystal structure of the monomeric imidazole-bound globin domain of AfGcHK

Overall crystal structure of the AfGcHK globin domain

A new crystal structure of the globin (sensing) domain of AfGcHK in the presence of imidazole was solved at a resolution of 1.8 Å (Fig. 1A and Table 1). The globin domain is monomeric in this structure. It has a globin-fold and consists of seven helices and seven loops. Additionally, it contains a heme molecule and three small ligands: two imidazole molecules and one molecule of 2-methyl-2,4-pentanediol (MPD). The MPD molecule (which originates from the crystallization solution) is bound at a crystal contact of two protein molecules, forming hydrogen bonds to Glu-62 and Arg-139 residues from neighboring protein chains; it has no biological significance. The binding of imidazole (also originating from the crystallization solution) is discussed in detail below. The data processing and structure refinement parameters are shown in Table 1. All residues of the protein chain fit well to the observed electron density. 98.1% of the residues lie in the favored regions of the Ramachandran plot and there are no outliers. The atomic coordinates and structure factors have been deposited in the Protein Data Bank (PDB) under the code 6OTD.

The heme-binding pocket contains two imidazole molecules

The heme is located in its standard position and interacts directly with His-99 on the proximal side (Fig. 1A). On the distal side, an imidazole molecule (IMD203) occupies a typical ligand-binding position, binding to the heme-iron via N3; the Fe-N3 distance is 2.2 Å. The imidazole nitrogen N1 forms a hydrogen bond with the main chain carbonyl group of Leu-68 (2.7 Å). The His-67 side chain projects toward the heme molecule and forms a hydrogen bond to its propionate group (ND1 of His-67 and O2D of the heme are within a distance of 2.7 Å), making the heme inaccessible to the bulk solvent. This propionate group also makes a crystal contact with Phe-110 of an adjacent protein molecule. The heme’s second propionate group makes a crystal contact with Arg-158, the last localized residue at the C terminus of the same crystal mate.

Another imidazole molecule (IMD204) is bound approximately in the plane of the heme (Fig. 1A), with a distance of 4.1 Å between the imidazole’s C2 carbon and the heme’s CMC carbon. This is the position occupied by Tyr-15 from the neighboring chain in the dimeric globin structure (Fig. 1B) (21). IMD204 is positioned in a small cavity with direct access to the solvent; there is no protein–protein crystal contact in this area of monomeric protein. Although the first imidazole molecule, IMD203, is well-localized in the electron density, IMD204 is relatively free within its cavity and forms no hydrogen bonds, so it is much less well-localized. Its position was therefore verified by generating a Polder map (Fig. S1) (23).

Structural differences between the monomeric imidazole-bound AfGcHK globin domain (6OTD) and the previously solved dimeric structures (5OHE and 5OHF)

Two crystal structures of the AfGcHK globin domain (PDB codes 5OHE and 5OHF) have been solved previously (21). Here we compare these structures to the new one (PDB code 6OTD). To this end, the globin domains (6OTD chain A, 5OHE chain G, and 5OHF chain G) were superimposed on the four nitrogen atoms of their heme groups. Structure 5OHE represents a dimeric CN-bound Fe(III) form of the AfGcHK globin domain, whereas structure 5OHF (in which chains G and H comprise the dimerization interface and exist in an alternative “B” configuration, denoted here as 5OHF-ligand-free) represents a dimeric form of the AfGcHK globin domain in the inactive state caused by treatment with sodium dithionite (21).

Comparison of monomeric imidazole-bound and dimeric CN-bound [Fe(III)]-CN forms

The monomeric imidazole-bound structure has some unique structural features including relaxation of the dimer-forming helices (due to its monomeric form), a more accessible heme, and differences in the architecture of the heme-binding site (Fig. 1B).
The monomer’s helices are relaxed—In the dimer (5OHE), Tyr-15 is immersed in the second chain’s heme-binding cavity. This enables the residue to form part of the heme pocket and to protect the heme’s equatorial region against interactions with the solvent, but requires the residue to adopt another conformation in which the torsion angle $\chi_1$ is $-165^\circ$. As a result, the position of the Tyr-15 $\mathrm{Ca}$ is shifted by 2.5 Å relative to that in the monomeric structure, creating tension in the helix (H1) containing this residue. Conversely, in the monomeric structure 6OTD, the Tyr-15 side chain adopts the most common rotamer ($\chi_1 = -65^\circ$) and helix H1 is straight and relaxed. Similar behavior is seen for the two long helices H6 and H7, which form the globin domain dimerization interface, are labeled. The chain termini are labeled N and C. The kinase domain of the full-length protein (not shown in the figure) would be located in front of the C terminus of helix H7.

The monomer has a new access tunnel—In the monomeric globin domain, the absence of Tyr-15 from a neighboring chain opens a new access tunnel to the heme pocket. This makes the heme more accessible to solvent molecules and oxygen, which is probably why the monomeric mutants Y15A and Y15G have high autoxidation rates (see below). In the monomeric crystal structure, this tunnel is occupied by the imidazole molecule IMD204, which is bound about 1 Å closer to the heme plane than the aromatic ring of Tyr-15 in the dimer.

Changes in the 6OTD heme-binding pocket—The Tyr-45 $\chi_1$ angles of the monomer and dimer differ by $113^\circ$ (Fig. 1B). In the monomer, the bulky imidazole molecule IMD203 binds to the heme distal side, pushing away the side chain of Tyr-45, which forms a hydrogen bond to the cyanide ligand in the dimeric structure (21). No such hydrogen bond exists between Tyr-45 and IMD203 in the monomeric structure, which is unsurprising because Tyr-45 interacts with the signal molecule O$_2$ (19) and other small heme ligands; imidazole is much bulkier than these species. The space occupied by the Tyr-45 side chain in the dimeric structure is in the monomeric structure occupied by water (HOH347) and partially by Leu-68, which relaxes into a

Figure 1. A, structure of the monomeric imidazole-bound AfGcHK globin domain (yellow chain, PDB code 6OTD) and B, a comparison of the imidazole-bound AfGcHK globin domain (yellow) to its dimeric cyanide-bound counterpart (cyan and dark blue; PDB code 5OHE, chains G and H). The rectangular boxes in the left-hand images enclose the heme-binding pockets, which are shown in expanded form in the right-hand images. The Tyr-15 residues (two in the dimeric complex and one in the monomer) and the two bound imidazole molecules (in the monomeric case only) are shown in ball-and-stick form. Helices H1, H6, and H7, which form the globin domain dimerization interface, are labeled. The chain termini are labeled N and C. The kinase domain of the full-length protein (not shown in the figure) would be located in front of the C terminus of helix H7.
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Table 1
Data collection statistics and structure refinement parameters for the isolated AfGcHK globin domain

| Data processing statistics | P6, 22 |
|---------------------------|--------|
| Space group               |         |
| Unit cell parameters a, b, c (Å) | 69.4, 69.4, 113.4 |
| Resolution range (Å)      | 41.25–1.80 (1.84–1.80) |
| No. of observations       | 213,525 (12,820) |
| No. of unique reflections | 15,414 (902) |
| Data completeness (%)     | 99.2 (99.9) |
| Average redundancy        | 13.9 (14.2) |
| Mosaicity (%)             | 0.2 |
| Average I/σ(I)            | 18.3 (2.0) |
| Solvent content (%)       | 43 |
| Matthews coefficient (Å³/Da) | 2.2 |
| Rmerge                    | 0.073 (1.320) |
| Rfree                     | 0.028 (0.506) |
| CC1/2                     | 0.999 (0.703) |

| Structure refinement parameters |
|----------------------------------|
| Rwork                             | 0.182 |
| Rfree                             | 0.231 |
| Rall                              | 0.186 |
| Average B-factor (Å²)             | 35 |
| RMSD bond lengths from ideal (Å)  | 0.017 |
| RMSD bond angles from ideal (°)   | 1.7 |
| Number of non-hydrogen atoms      | 1,404 |
| Number of water molecules         | 68 |
| Ramachandran statistics: residues in favored region (%) | 98.1 |

| PDB code | 6OTD |

† Rmerge = Σ||Fobs| − |Fcalc||/Σ|Fobs||
‡ Rfree = Σ||Fobs| − |Fcalc||/Σ|Fcalc||
§ R = Σ||Fobs| − |Fcalc||/Σ|Fcalc||
	where |Fobs| is the observed intensity, |Fcalc| is the mean intensity of multiple observations of symmetry-related reflections, and |Fobs| and |Fcalc| are the observed and calculated structure factor amplitudes.
=Rmerge calculated on 95% of reflections excluding a random subset of 5% of reflections marked as “free.” The final structure refinement was performed on all observed structure factors.

Overall protein structure—The conformations of the distal helices of the globin domain in 5OHF ligand-free, which lacks an axial heme ligand, differ from those in the ligand-bound (CN⁻ or imidazole) domains. The largest shift is observed for helix H2 (residues 20–50), which is located in similar positions in the CN⁻ and imidazole-bound structures but is shifted away from the heme in the inactive ligand-free protein.

Heme-binding pocket—The fine structure of the heme-binding pocket primarily depends on the volume of the heme distal ligand. Therefore, the architecture of the active CN-bound state represents something of an intermediate between those of the imidazole-bound monomer (6OTD) and the ligand-free dimer (5OHF-ligand-free).

The effect of Tyr-15 on the oligomeric state of full-length AfGcHK and the isolated globin domain

Because the crystal structure of the Fe(III)-imidazole complex of the isolated monomeric globin domain of AfGcHK showed that Tyr-15 plays an important role in the protein’s architecture, we prepared Tyr-15 mutants (Y15A, Y15G, Y15F, and Y15W) of both the full-length protein and the isolated globin domain, and performed detailed functional (biophysical) studies on them.

Oligomeric state of WT and mutant full-length AfGcHK

The WT full-length AfGcHK protein and the tested Tyr-15 mutants all had similar elution volumes (from 13.2 to 13.4 ml, respectively) during size exclusion chromatography on a Superdex 200 Increase 10/300 GL column (Fig. 3A). The theoretical molecular mass of full-length AfGcHK in monomeric, dimeric, and tetrameric forms is 43, 86, and 172 kDa, respectively. Calibration experiments indicated that the column would clearly separate protein complexes of these masses (Fig. S2). The experiments confirmed that the full-length AfGcHK WT exists as a dimer in solution (Table 2) and showed that all the tested Tyr-15 mutants of the full-length AfGcHK protein are also dimeric (Table 2).

Oligomeric state of WT and mutant isolated globin domains of AfGcHK

The size exclusion elution volumes of the isolated globin domains of WT AfGcHK and the tested Tyr-15 mutants varied significantly, ranging from 16.0 to 17.3 ml (Fig. 3B). The theoretical molecular mass of the isolated globin domain of AfGcHK in the monomeric, dimeric, and tetrameric forms is 19, 38, and 76 kDa, respectively. Calibration experiments indicated that the column would clearly separate protein complexes of these masses (Fig. S2). The results confirmed that the isolated WT globin domain of AfGcHK forms a dimer in solution (Table 2), and demonstrated that the Y15F and Y15W mutants behave in the same way. Conversely, the Y15A and Y15G mutants of the isolated globin domain exist as monomers in solution (Figs. 3B and Table 2).
The effect of Tyr-15 on the heme axial environment of full-length AfGcHK and its isolated globin domain

UV-visible spectroscopy was used to study the effect of Tyr-15 on the heme axial environment of full-length AfGcHK and its isolated globin domain. Both the full-length proteins and the isolated globin domains of the Y15A and Y15G mutants in the Fe(II)-O_2 form exhibited very high autoxidation rates (>10 min⁻¹), making it impossible to obtain clear spectra of these species. Remarkably, the autoxidation rates of the full-length Y15A and Y15G mutants were approximately twice those of the corresponding isolated globin domain mutants. However, the Y15A and Y15G mutations had little effect on the absorption bands in the spectra of the Fe(III)-OH⁻, Fe(II), and Fe(II)-CO complexes, causing minor changes that were most pronounced in the visible region (Table 3 and Fig. S3). The spectra of the Y15F and Y15W mutants were identical to those for the WT protein independently of the coordination and redox state of the heme-iron center, indicating that replacing Tyr-15 with an aromatic amino acid (Phe or Trp) does not greatly change the heme binding environment of the full-length protein or the isolated globin domain (Table 3 and Fig. S3).

The effect of Tyr-15 on the enzyme activity of full-length AfGcHK

A detailed kinetic analysis of the full-length AfGcHK protein in its WT and mutant variants of their Fe(III)-OH⁻ forms (Table 4 and Fig. S4) showed that the Y15A and Y15G mutations completely abolish its catalytic activity. However, the k_cat values of the Y15F and Y15W mutants are comparable with that of the WT. Additionally, the K_m value of Y15W is comparable with that of the WT but that of Y15F is approximately twice as high (Table 4 and Fig. S4).
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Table 3

| Protein                        | Fe(III)-OH | Fe(II)   | Fe(II)-O₂ | Fe(II)-CO |
|--------------------------------|------------|----------|-----------|-----------|
| **AfGcHK full-length**         |            |          |           |           |
| WT                             | 411, 538   | 431, 559 | 413, 545, 580 | 420, 541, 565 |
| WT                             | 410, 539   | 430, 556 | 415, 545, 580 | 420, 544, 566 |
| Y15A                           | 410, 536   | 423, 557 | 415, 455, 580 | 420, 533, 565 |
| Y15F                           | 410, 541   | 425, 550 | 415, 545, 580 | 420, 545, 572 |
| Y15G                           | 410, 536   | 428, 556 | 415, 545, 580 | 420, 535, 566 |
| Y15W                           | 410, 537   | 425, 553 | 415, 545, 580 | 420, 544, 570 |
| **AfGcHK-isolated globin domain** |          |          |           |           |
| WT                             | 410, 539   | 430, 554 | 415, 545, 580 | 420, 543, 565 |
| Y15A                           | 410, 535   | 430, 559 | 415, 545, 580 | 420, 544, 565 |
| Y15F                           | 410, 540   | 430, 551 | 415, 545, 580 | 420, 545, 566 |
| Y15G                           | 410, 535   | 430, 560 | 415, 545, 580 | 420, 535, 564 |
| Y15W                           | 410, 539   | 430, 550 | 415, 545, 580 | 420, 541, 570 |

*As described in Ref. 19.

**Discussion**

**The structural role of Tyr-15 in AfGcHK**

In the previously reported structure (21) of the CN-bound Fe(III) form of the dimeric AfGcHK globin domain (PDB code 5OHE), the side chain of Tyr-15 from one chain of the globin dimer forms part of the neighboring chain’s heme-binding pocket: the distance between the heme CMC carbon and the O of the Tyr-15 OH group is between 3.8 and 4.2 Å (21). The imidazole-bound globin domain in this work was monomeric (PDB code 6OTD). Because Tyr-15 has no neighboring chain to interact with in the monomer, it adopts a different side chain conformation and its main chain position is relaxed, leading to the relaxation of helices H1, H6, and H7 (Fig. 2). This drew our attention to Tyr-15’s possible role in the globin domain dimerization interface. We therefore performed a detailed functional analysis of selected Tyr-15 mutants to determine how Tyr-15 affects the full-length protein’s oligomerization, oxygen-sensing ability, and enzymatic activity.

**Biophysical characterization of Tyr-15’s role in AfGcHK**

The isolated globin domain of WT AfGcHK is dimeric in solution. If Tyr-15 is replaced with an aromatic residue (phenylalanine or tryptophan), the globin domain still forms a dimer, although the size exclusion chromatography results suggest that these dimers are somewhat more bulky than the WT dimer (Table 2 and Fig. 3). Conversely, if Tyr-15 is replaced with a nonaromatic residue, as in the Y15A and Y15G mutants, the globin domain becomes monomeric in solution (Table 2 and Fig. 3B).

The globin domain variants that are monomeric in solution (Y15A and Y15G) exhibit much higher rates of autoxidation than those that form dimers (Table 3, Fig. S3). This is probably because the inability to form a dimer causes profound changes in protein structure near the heme group, destabilizing the Fe(II)-O₂ state. Additionally, as noted above, the absence of the aromatic amino acid from the neighboring chain in the monomers opens a new tunnel allowing water and oxygen to access the heme pocket. Note that both the Fe(II)-O₂ form of the full-length AfGcHK enzyme and its autoxidation product, the Fe(III)-OH⁻ form, are catalytically fully active (20) (Table S1).

Therefore, the increased autoxidation rates of the nonaromatic Tyr-15 mutants cannot explain their loss of enzyme activity.

The biophysical characteristics of the full-length mutants are similar to those of the corresponding isolated globin domains other than with respect to their oligomeric states (Tables 2 and 3 and Fig. 3A). All the full-length Tyr-15 mutants form dimers, but it is reasonable to speculate that the globin domains of the full-length Y15A and Y15G mutants are dissociated in the same way as in the cases of the isolated globin domains. The preservation of the oligomeric state of the full-length mutants is understandable because the kinase domain’s dimerization interface is probably larger than that of the globin domain (21, 22).

Finally, it should be noted that UV-visible spectroscopy indicated that the protein structural changes caused by replacing Tyr-15 with another aromatic residue did not significantly alter the heme environment (including the neighboring chain and the tethering protein structure linking the globin and functional domains), and that kinetic studies showed that the Y15F and Y15W mutations did not significantly affect the enzyme’s full-length activity (Table 4, Fig. S4).

**Dimerization interfaces in the structures of other heme-containing oxygen sensors**

There are four heme-containing oxygen sensor proteins with heme-bound globin-folds (7, 10, 11, 13, 14) for which the structure of the sensing domain has been solved: a globin-coupled sensor from *Geobacter sulfurreducens* (PDB code 2W31) (24), the HemAT protein from *Bacillus subtilis* (PDB codes 1OR4...
and 1OR6) (25), the *Azotobacter vinelandii* globin-coupled oxygen sensor *AvGReg* (PDB code 4UII), and the *Escherichia coli* protein YddV or DosC (PDB codes 4ZVA–4ZVH) (26). All these globin domains are dimeric but none has an amino acid from one subunit that interacts as strongly with its partner subunit as does Tyr-15 in the *AfGcHK* globin domain dimer. Therefore, the results of the mutation experiments presented here are unlikely to be directly applicable to these proteins, and their dimerization interfaces may behave differently to that of *AfGcHK*.

**Effect of imidazole binding in structures of similar proteins with globin-folds**

Three-dimensional structural changes in globin domains and other globins induced by the binding of imidazole to the heme have been reported previously. For example, imidazole binding significantly changed the heme environment of the Fe(III) form of sperm whale myoglobin (PDB code 1MBI) because it caused the His-64 side chain (which otherwise acts as the distal site ligand) to swing outwards (27). This is similar to the changes observed for Tyr-45 in *AfGcHK*. Structural changes induced by imidazole binding have also been identified by comparing the structure of the imidazole-bound globin-coupled sensor from *Bordetella pertussis* (PDB code 4U1Q) to its imidazole-free counterpart (4UII). Unfortunately, because these structures have not yet been discussed in any publication, it is difficult to say anything further about them. Conversely, the protoglobin from *Methanosarcina acetivorans* (PDB code 3ZJP) exhibited only small changes in the conformations of two heme-distal site residues (Tyr-61 and Phe-93) following imidazole binding (28).

**Mechanism of signal transduction in full-length AfGcHK**

It has been challenging to determine the signal transduction mechanisms of various histidine kinases and globin-coupled oxygen sensors because the conformational flexibility of their WT (full-length) forms makes it difficult to resolve their structures.

Here, we show for the first time that Tyr-15 is necessary for the dimerization of the *AfGcHK* globin domain. When this residue is replaced with a nonaromatic amino acid, the isolated globin domain dimer dissociates into monomers. As discussed above, it is reasonable to assume that although the full-length aliphatic Tyr-15 mutants exist as dimers, their sensing domains would be dissociated into monomers in the same way as the isolated sensing domains. It thus seems that a dimerization process involving intimate contact between the heme group and the side chain of a residue from the neighboring side chain of the dimer is essential for the dynamic properties and/or conformation of the system that permit signal transduction between the sensing and functional domains. The conformational changes of helix H1 upon dimer formation and the development of tension within the globin dimerization interface probably contribute to the fine-tuning of the dimer’s dynamic properties and signal transduction. We therefore suggest the following model of signal transduction in *AfGcHK*: the receipt of a signal in the heme-sensing site of the globin domain (i.e. the binding of a suitable ligand to the heme) influences the properties of the globin domain dimerization interface. Changes in the dimerization interface (or changes that act indirectly via the interface) in turn affect the functional domain, switching its catalytic activity on or off. Therefore, when globin domain dimerization is hampered, the protein becomes incapable of signal transduction and is enzymatically inactive, as also occurs when the heme or the entire sensor domain is missing (20–22). A schematic representation of this model is shown in Fig. 4.

As discussed above, the loss of enzyme activity observed for the aliphatic Tyr-15 mutants of the full-length *AfGcHK* protein is not due to increased autoxidation of these mutants. Both the Fe(II)-O2 form of the WT full-length enzyme and its autoxidation product, the Fe(III)-OH form, are catalytically fully active (20) (Table S1). Moreover, the enzyme activities of all mutants were determined using their...
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Fe(III)-OH\textsuperscript{−} forms to avoid complications resulting from the presence of mixtures of Fe(II)-O\textsubscript{2} and Fe(III)-OH\textsuperscript{−} species formed by autoxidation. Therefore, it is reasonable to conclude that the observed changes in AfGcHK kinase activity were due to changes in the monomeric/dimeric status of the sensing domain caused by the Tyr-15 mutations.

Signal transduction in the full-length AfGcHK compared with other histidine kinases or other heme-containing sensor proteins

Several mechanisms have been proposed to explain how the functional domains of various histidine kinases might be altered in response to signaling from the sensing domain (1, 9, 29–32). The results presented here together with some previously reported findings (21) strongly suggest that in AfGcHK, signal transduction is regulated without direct contact between the sensing and functional domains, probably via a short peptide linker between the two domains. This signal transduction mechanism (see paragraph above) resembles that proposed for transmembrane signal propagation in membrane-bound two-component systems (15–18), although AfGcHK is not a membrane-bound protein. In particular, a similar mechanism involving sensing domain dimerization that is subsequently propagated to a histidine kinase functional domain has been suggested for the E. coli copper and silver ion sensor CusS (33). Moreover, oligomerization plays an important role in signal transduction in the heme-based oxygen sensor protein DevS (DosS) from Mycobacterium tuberculosis. In this protein, the ability to rapidly change the oligomerization state based on the status of the heme in the sensing domain is directly connected to the catalytic activity of the functional domain (9). In addition, the equilibrium between oligomeric states in globin-coupled oxygen sensor proteins from B. pertussis (BpeGReg) and Pectobacterium carotovorum (PccGCS) was shown to depend on both O\textsubscript{2} binding to the sensing domain heme and c-di-GMP binding to the functional domain. These shifts in the position of equilibrium in turn affected the sensors’ catalytic activity (10, 13). The catalytic activity of these two proteins was also sensitive to heme distortion modes and heme-protein interactions (8). In keeping with the results obtained for AfGcHK, it has been suggested that the globin dimerization interface may be involved in regulating the properties and catalytic behavior of the full-length BpeGReg and PccGCS proteins (11). A similar set of global interactions within the N-terminal dimeric heme-binding domain was proposed to explain the signal transduction process in the heme-based oxygen sensor phosphodiesterase EcDOS (also known as EcDosP), which has a heme-bound PAS (34).

Summary

Several artificial inactive variants of AfGcHK have been prepared, including monomeric isolated heme-bound oxygen-sensing globin domains and dimeric full-length variants whose heme-bound globin domains are probably monomeric. Analyses of these variants suggested that monomerization of the heme-bound globin domain entirely abolishes the protein’s catalytic activity by impairing internal signal transduction, indicating that the dimerization of the heme-bound globin domain is vital for signal transduction to the functional domain. This is consistent with our previously reported crystallographic and HDX-MS studies on the molecular mechanism of AfGcHK (21), which suggested that the globin domain’s dimerization interface is crucial for inter-domain signal transduction between the globin and functional domains.

Experimental procedures

Materials

Ampicillin was obtained from P-lab (Prague, Czech Republic). Isopropyl β-D-thiogalactopyranoside, hemin, and acrylamide were obtained from Sigma-Aldrich. Water, doubly distilled over quartz, was purified using a Milli-Q Plus system (EMD Millipore, Billerica, MA). All glassware used for sample preparation was conditioned in advance by standing for 24 h in 10% HCl suprapur (Merck, Darmstadt, Germany). Phos-tag was from the Phos-tag consortium Wako Pure Chemical Industries (Osaka, Japan). All chemicals used were of the highest purity grade available from commercial sources and used without further purification.

Protein production and purification

Tyr-15 mutants of AfGcHK were prepared using a site-directed mutagenesis kit (Agilent Technologies, CA) (Table S2). Cloning, overexpression in E. coli, and purification of the full-length WT AfGcHK proteins and its Tyr-15 mutant proteins were performed as previously described (19). Briefly, His-tagged AfGcHK was expressed in BL21(DE3) (Novagen, Madison, WI) harboring the pET-21c(+) plasmid. Cell lysates containing His-tagged AfGcHK were isolated by affinity chromatography on TALON\textsuperscript{®} Metal Affinity Resin (Clontech Laboratories) followed by size exclusion chromatography on a Superdex 200 GL 10/300 column (GE Healthcare, Amersham Biosciences, UK). Protein and heme concentrations were determined using the BCA assay (Sunrise Absorbance Reader, TECAN, Männedorf, Switzerland) and the pyridine hemochromogen assay, respectively. Purified proteins were >90% homogenous, as confirmed by SDS-PAGE.

Tyr-15 mutants of the isolated AfGcHK globin domain were prepared by In-Fusion cloning (Clontech Laboratories). For this purpose, Tyr-15 mutants of full-length AfGcHK were shortened by PCR using the primers 5′-GAAGGAGATATCACATATGCAATCATCAATCATGATACCGGTGTCCACGAAAACGTGTG3′ and 5′-GTGGTGATGCTGACAGTACAGTACTTCTACATAGGTTGCAG-3′. The sequence obtained by PCR was then inserted into the pET-21c(+) vector using standard cloning techniques. The isolated globin domain of AfGcHK and its Tyr-15 mutants were prepared by inactivating 500 ml of TB media containing 100 μg/ml of ampicillin with 50 μl of overnight BL21(DE3) cells containing the appropriate pET-21c(+) plasmid. The cells were grown at 37°C for 4 h (170 rpm), then the temperature was lowered to 18°C (140 rpm) and protein expression was initiated by adding 0.1 mm isopropyl 1-thio-β-D-galactopyranoside followed by further shaking for 20 h. Cells were harvested by centrifugation at 4°C, 3,000 × g, and stored at −80°C. Pellets were resuspended in 50 mM Tris, 100 mM NaCl, pH 8, containing...
1 mM PMSF, 1 mM EDTA, and 0.2 mg/ml of lysozyme. The desired proteins were isolated and purified by sonication 6×1 min, reconstitution with 300 μM hemin for 20 min, centrifugation for 70 min at 50,000 × g, incubation of the supernatant with TALON® Metal Affinity Resin (Clontech Laboratories) for 1 h at 4°C, and elution of the protein using 200 mM imidazole. Finally, the protein was concentrated to a volume of 1 ml and desalted on a Superdex 200 GL 10/300 column equilibrated with 20 mM Tris, 150 mM NaCl, pH 8. In the case of the WT globin domain of AfGcHK, the protein was diluted to 0.5 mg/ml and tobacco etch virus (TEV) protease was added to a final concentration of 0.05 mg/ml. Cleavage was performed at 10°C for 48 h. TEV protease and uncleaved protein were removed using a TALON® Metal Affinity Resin column and the isolated protein was collected in the flow-through fraction. Finally, size exclusion chromatography on a Superdex 200 GL 10/300 column (GE Healthcare, Amersham Biosciences, UK) was applied and the final protein preparation was frozen in liquid nitrogen for further use. Tyr-15 mutants were analyzed without the TEV cleavage procedure. The purified isolated globin domain of AfGcHK was >99% homogenous, as confirmed by SDS-PAGE.

Determination of the oligomeric state of the full-length and isolated globin domains of WT and Tyr-15 mutant proteins of AfGcHK

The oligomerization states of the prepared proteins were determined by size exclusion chromatography on a Superdex 200 Increase 10/300 GL column (GE Healthcare). The mobile phase consisted of 20 mM Tris-HCl, pH 8.0, and 150 mM NaCl, and the flow rate was 0.5 ml/min with an injection volume of 100 μl. Eluted proteins were detected by monitoring the absorbance at 280 nm. The globular proteins thyroglobulin (669 kDa), apoferritin (481 kDa), BSA (66 kDa), ovalbumin (43 kDa), and myoglobin (17 kDa) were used as molecular mass standards and analyzed on the same column. The resulting calibration curve is shown in Fig. S2B. The theoretical molecular masses (calculated based on amino acid sequences) of the monomeric and dimeric forms of AfGcHK were 43 and 86 kDa for the full-length protein, and 19 and 38 kDa for the isolated globin domain, respectively. Samples for analysis were prepared by dissolving the appropriate AfGcHK protein in 50 mM Tris-HCl, 150 mM NaCl, pH 8.0, to a final concentration of 10 μM.

Optical absorption spectroscopy

Optical absorption spectral data were obtained at 24°C using a Cary 60 UV-visible spectrophotometer (Agilent Technologies, CA). To ensure that the solution was maintained at an appropriate temperature, the reaction mixture was incubated for 5 min prior to spectroscopic measurement. After purification, spectra of the full-length and the isolated globin domain proteins with the Fe(III)-OH− heme of AfGcHK were obtained. The corresponding heme-Fe(II) species were prepared by placing a 5 μM solution of the Fe(III)-OH− protein in a quartz cuvette and reducing it using 1 mM sodium dithionite. Fe(II)-CO complexes were then prepared by gently bubbling gaseous carbon monoxide (Linde, Wiesbaden, Germany) through the cuvette for ~2 min. The Fe(II)-O2 complex was prepared by removing sodium dithionite using PD Minitrap™ G-25 Desalting columns (GE Healthcare) and exposing the Fe(II) species to air for 5 min.

Enzyme activity of AfGcHK

The reaction mixture contained the appropriate full-length AfGcHK protein at a concentration of 10 μM, 50 mM Tris-HCl, pH 8.0, 50 mM KCl, and 5 mM MgCl2. The reaction mixture was preincubated for 5 min at 20°C, and the reaction was initiated by adding ATP at a concentration between 0 and 1 mM. All experiments were performed at 20°C. At predetermined times, the AfGcHK autophosphorylation assay reaction was terminated by adding a 100-μl aliquot of termination buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromphenol blue). Samples of the quenched reaction mixtures were then loaded on a 10% SDS-polyacrylamide gel containing 75 μM Phos-tag acrylamide and 0.2 mM MnCl2. Each lane was loaded with a quantity of quenched reaction mixture containing 0.5 μg of the AfGcHK proteins. Phosphorylated proteins in the sample interacted with the Phos-tag manganese complex in the gel, reducing their mobility relative to phosphate-free proteins (19, 35, 36). After electrophoresis, proteins were visualized by staining with Coomassie Brilliant Blue R-350 and the stained gels were imaged using a Scanjet G3010 (HP, USA) scanner. The protein loadings were then quantified by analyzing the scanned images using ImageJ.

Crystallization of the AfGcHK globin domain

The crystallization solution contained the AfGcHK globin domain at a concentration of 5.4 mg/ml in 150 mM NaCl, 20 mM Tris, pH 8.0. Droplets (100 nl of reservoir solution and 100 nl of crystallization solution) were set up using a Cartesian Honeybee 961 robot (Genomic Solutions) at 21°C. The crystal grew under C4 Morpheus conditions without any optimization. The C4 Morpheus condition contained 0.09M ligand stock (NaNO3, Na2HPO4, (NH4)2SO4), 0.1M buffer system, pH 6.5 (imidazole, 2-ethanesulfonic acid), and 37.5% precipitant stock MPD_P1K_P3350 (MPD, PEG 1000 and PEG 3350).

Diffraction data collection

Data were collected at the Diamond Light Source using the I02 beamline and a Pilatus 6 M-F detector. The wavelength was 0.97 Å, the crystal-detector distance was 350 mm, the exposure time per image was 0.05 s, and the oscillation width was 0.1°. The diffraction images were processed using XDS (37) and scaled using AIMLESS from the CCP4 program package (38). Finally, the best images (numbers 1100–1800) were selected based on their batch Rmerge values and overall statistics. Final data processing statistics are shown in Table 1.

Structure solution and refinement

The phase problem was solved by molecular replacement with the program MOLREP (39) based on the previously reported structure of the protein, one monomer of the dimer was used as a model (PDB code 5OHF). The asymmetric unit of the structure presented here contains a monomer, and inspection of the interfaces within the crystal lattice confirmed the
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monomeric form of the protein in the crystal. The structure was refined using REFMAC5 (40) with manual editing using COOT (41) and using 5% of reflections as a test set ($R_{	ext{free}}$ set). The last refinement cycle was performed using all reflections. Structure quality was checked using the validation tools implemented in MOLPROBITY (42) and REFMAC5 (40).

Author contributions—A. L., J. D., and P. M. investigation; P. K. methodology; M. M. conceptualization; M. M. writing-original draft; T. Skalova, J. D., P. K., M. S., and J. B. designed, performed and interpreted the X-ray crystallographic investigations; A. L. performed the biophysical analysis including a kinase activity study; K. H. helped with the X-ray crystallographic data collection; T. Shimizu participated in the results discussion; M. M. designed and interpreted the biophysical analysis including a kinase activity study.

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