Proteins that sense cellular environments – examples and implications

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Summary

The first step in the process of signal perception and transduction involves interaction between a stimulus and the specific protein that has the capacity to recognise the stimulus and to translate the interaction to the physical manifestation of a signal. The physical manifestation of the signal involves what is commonly referred to as a 'protein conformational change' that results in a change in a conformational equilibrium of the proteins that perceive the stimuli. Reflecting on our work I will describe two specific examples of stimuli perception and signal transduction mechanisms, one relating to protein AioX that is found in a prokaryotic organism adapted to living in the conditions of arsenic contamination and the second example involving neuropilins - transmembrane proteins of the significance for human health.

Keywords: signal transduction, AioX, neuropilins, VEGF.

Introduction

All cells need to respond to their environment. Bacterial organisms, in particular, rely on their capacity to sense varieties of stimuli that are of the essence for their survival, such as the presence of nutrients or deleterious chemicals. In a multicellular organism, activities of different cells and organs need to be coordinated in the range of timescales and distances (Williamson 2012). In both instances (single cell versus multicellular organisms) evolution has led to the development of the specific signalling pathways which enable cellular communication, coordination and responses. In addition to the simple, so-called one component signalling proteins, bacteria developed more complex two-component signalling systems, comprising a transmembrane sensory histidine kinase and an intracellular response regulator protein that frequently exhibits DNA-binding activity and regulates transcription (Stock et al. 2000). More complex organisms contain more complex signal transduction pathways with multitude of proteins involved and controlled by the range of regulatory mechanisms (Laub 2016). Interestingly, many of the proteins that are involved in signalling contain a common subset of structural domains whose topology is found in both prokaryotic and eukaryotic proteins (Ulrich et al. 2005; Borziak and Zhulin 2007; Mayer 2015). In part this is due to the need to provide the solution for the common problems associated with signalling such as overcoming membrane barrier via receptor dimerization (Lemmon and Schlessinger 2010) or transmembrane helix rotation (Fleishman et al. 2002). There is a plethora of other molecular mechanisms that signalling proteins and signalling pathways might use: co-localisation of proteins or combination of domains enable regulation and specificity (Mayer 2015); use of the common protein scaffolds with subtle modification or embellishments of the connecting loops also provides specificity; engagement of the protein hubs and adaptor proteins to bring multiple interacting proteins together (Ota et al. 2016;

Yablonski 2019); posttranslational modifications such as phosphorylation, lipidation and glycosylation (Campbell et al. 2019). The following examples will provide the context and insights into the ways by which some of these mechanistic and functional aspects are embedded in the specific signalling systems.

AioX and Rhizobium NT-26

Arsenic is a potent carcinogen that poses a significant threat to the ecological and public health globally (Ratnaike 2003). While immediate effects of acute arsenic poisoning include skin changes, vomiting, stomach cramps and diarrhoea, persistent exposure results in multisystem disease with malignancies as the most lethal consequence. Currently, there are no approved treatments for chronic arsenic poisoning and the management of the toxicity is focused on reducing arsenic ingestion from drinking water. Therefore there is a clear need for more detailed understanding of the carcinogenic mechanisms of arsenic as well as for the development of biological sensors that would facilitate rapid, selective and non-toxic detection of arsenic contamination in the environment and within the cells (Soleja et al. 2019).

Even though arsenic is toxic to most organisms, there are prokaryotic organisms that have been isolated from the arsenic-contaminated environments. Characterisation of these organisms have demonstrated that, to various degrees, they have adapted to living in these hostile environments by evolving molecular machineries that are engaged not only in detoxification and tolerance but also in exploitation of arsenic as an energy source (Santini et al. 2000; Oremland et al. 2002). Arsenite oxidase (Aio) is the best characterised arsenic-metabolising enzyme (Ellis et al. 2001; Warelow et al. 2013). Aio, isolated from aerobic

organisms, comprises two subunits, AioA - molibden-dependent, also containing 3Fe-4S cluster and AioB - Rieske [2Fe-2S]-containing subunit (Ellis et al. 2001).

In the *Proteobacteria*, the expression of Aio is regulated by a three-gene cluster adjacent to the arsenite oxidase genes (Kashyap et al. 2006; Sardiwal et al 2010; Liu et al. 2012). Two of the genes from the cluster, *aioS* and *aioR,* comprise a cognate sensory histidine kinase/response regulator pair. We have characterised functional properties of *aioS* and *aioR* from the chemolithoautotrophic *Rhizobium* sp. str. NT-26 (NT-26 for short) (Sardiwal et al. 2010). The kinase protein AioS exhibits a typical histidine kinase topology with the periplasmic domain, flanked by two transmembrane helices and linked to the intracellular HAMP and kinase domains. The response regulator protein is a transcription regulator belonging to AAA+ family of σ^{54}-dependent regulators. The two proteins are required for arsenite oxidase expression and arsenite oxidation. However, functioning of this, apparently a canonical bacterial two-component signal transduction system, depends on a third protein component, which is encoded by the *aioX* gene – the third gene in the regulatory cluster. In *Rhizobium* sp. str. NT-26, *aioX* is positioned upstream of *aioS* and the three genes are co-transcribed but on a separate transcriptional unit from that of *aioAB* (Sardiwal et al. 2010).

Recently, we reported the three-dimensional crystal structures of AioX in an apo, phosphate and arsenite-bound state and we compared its ligand-binding properties with that of the homologues from other organisms (Badilla et al. 2018). Characterisation of these proteins allowed us to identify a new subfamily of arsenic oxyanion-binding proteins involved in regulation of bioenergetic arsenic metabolism. The periplasmic binding proteins in this structural family originate from phylogenetically distant bacteria, they exhibit exquisite substrate selectivity, and they could be developed into powerful sensors for toxic arsenic oxyanions.

Three-dimensional structure of AioX

AioX is a 304-residues long polypeptide that folds into two similar α/β domains organised in a typical bilobed structure that is characteristic of a class II periplasmic (substrate) binding proteins (Fukami-Kobayashi et al. 1999). Each subdomain comprises mixed beta-sheet with only one strand antiparallel to the rest in each of the subdomains (PDBID: 6ESK). The two structural halves of AioX are linked by two extended crossing-over peptide linkers. At the C-terminus, the canonical fold is extended by an additional (sixth) beta strand within the first beta-sheet and the terminal helix.The three-dimensional structure of AioX is highly similar to that of the *E. coli* phosphonate-binding protein PhnD (Alicea et al. 2011), with which AioX shares 25% amino acid sequence identity. In this class of proteins a ligand-binding pocket is characteristically located in the space between the two sub-domains and when the initial electron density maps for the crystals generated in a phosphate-containing buffer were examined, we were able to unambiguously identify electron density consistent with the presence of a phosphate ion (PDBID: ESV) at the inter-domain interface. Superposition of the AioXfrom NT-26 with the phosphonate-bound structure of *E. coli* PhnD further reinforced identification of a substrate-binding site within AioX as the coordinates of the AioX-bound phosphate and the PhnD-bound phosphonate partially overlapped (Badilla et al. 2018). The phosphate ion, chemical mimetic of arsenite, in the putative ligand-binding site was positioned near the AioX-specific residue Cys106, thereby suggesting that this unique cysteine might be involved in binding arsenite.

The crystal structure of an arsenite-bound AioX validated Cys106 as an essential residue (PDBID: 6EU7). Namely, the structure clearly showed covalent linkage between the arsenite ion and the sulphur atom of Cys106 side chain. The structures for the three forms of

AioX (apo, phosphate- and arsenite-bound) also allowed for examining the atomic-level features of the ligand-binding site and for identifying global conformational differences specifically associated with the presence of arsenite. The most pronounced differences in the conformation of the polypeptide backbone were detected in the structure of the arsenite-bound form, where the attachment of arsenite to Cys106 was associated with the hydrophobic collapse of the residues, forming the main loop (residues 53-61) at the edge of one of the subdomains and thus resulting in a subtle closure of the ligand-binding site (Figure 1). Importantly, the conformational rearrangement had direct impact on the molecular surface and the surface electrostatic potential of AioX. This direct manifestation of ligand-binding will inevitably change the nature of protein/protein interactions between AioX and the periplasmic domain of AioS sensory kinase leading to its activation and downstream regulation.

But how did arsenite binding 'cause' this conformational change? Close inspection of the residues surrounding bound arsenite shows that residue Tyr88 plays unique function such that it adopts two different rotamer conformations depending on the presence of a ligand. Most likely the two distinct Tyr88 rotamer conformations and the two different AioX global conformations represent the hallmarks of the two extreme states in the conformational equilibrium with one of the states stabilised by arsenite binding. Indeed, the crystal structures show that Tyr88 rotamer observed in the apo form of AioX and the specific conformation of the ligand-binding site loop, as observed in the arsenite-bound AioX, are mutually sterically exclusive. In other words, loop closure by rearrangement of hydrophobic residues (hydrophobic collapse) can only occur if Tyr88 adopts a rotamer orientation that is consistent with arsenite-binding. Interestingly, even though we were able to detect a phosphate ion in one of the determined AioX crystal structures, suggesting that the phosphate ion mimics arsenite-binding, phosphate-binding did not sufficiently stabilise the ligand-binding loop and

the corresponding electron density was disordered (Badilla et al. 2018). Apart from the Tyr88 rotamer, overall the phosphate-bound structure had more similarity to the apo rather than the arsenite-bound form of AioX.

Conserved and unique features of AioX-binding pocket have implications for signalling mechanism of AioX

Alignment of AioX amino acid sequence with that of the close homologues ArrX, ArxX and PhnD, identified conserved residues in the AioX subfamily of periplasmic binding proteins (for details see Badilla et al. 2018). These residues, corresponding to Tyr88, Cys106, Tyr131, Ser161, Ser163, His192 and Asp210 in AioX, indicate evolutionary and functional relationship linked to similar substrate-binding capabilities. Differences among the polypeptides, such as the absence of Cys106 in PhnD, and the presence of the specific hydrophobic loop residues in AioX, point towards the structural requirements for arsenite binding. Isothermal titration calorimetry (ITC) experiments demonstrated distinct substrate specificity of these proteins, confirming AioX as a protein with the highest affinity for arsenite (Kd=170 nM) while ArrX selectively bound arsenate (As(V)) with a micromolar affinity. Structural studies and the ligand-binding experiments jointly suggested that the conserved binding residues alone were not sufficient to confer full ligand specificity and that additional region in the polypeptide, affecting overall conformation of the protein, plays a profound role in selectivity. With consideration of the significant conformational changes that impact the shape and electrostatic potential of the molecular surface of AioX upon arsenite binding we could postulate signalling mechanism analogous to the mechanisms seen previously for proteins LuxP and TorT (Neiditch et al. 2006; Baraquet et al. 2006; Moore and Hendrickson 2012). The two proteins form complexes with the sensory domains from their

specific histidine kinases, LuxQ and TorS respectively, and kinase activation/repression is linked to the symmetrical/asymmetrical state of the kinase complexes, regulated by the specific ligand binding to LuxP and TorT. In AioX, similarly, the new molecular surface generated by arsenite-binding would be a trigger of a putative symmetry/asymmetry switch associated with AioS activation. High affinity and selectivity of AioX, together with its molecular dynamic characteristics, enable rapid response to arsenite and these molecular properties could provide a unique platform for the development of a new type of a biosensor.

Neuropilins

As a second example of signalling proteins I will comment on neuropilins. Neuropilins (NRP1/NRP2) are transmembrane proteins with the pivotal role in key physiological processes including: vasculogenesis, angiogenesis, lymphangiogenesis, axonal guidance, and immunomodulation (Parker et al. 2012; Djordjevic and Driscoll 2013; Roy et al. 2017). They are essential in development but they are also implicated in pathologies such as various cancers and proliferative neuropathies. Role of neuropilins have been demonstrated in many cancers; autocrine $VEGF_{165}$-dependent signalling mechanism promotes breast cancer (Bachelder et al. 2001); preclinical studies support a role for NRP1 in lung and renal cancer cells migration, proliferation and invasion (Castro-Rivera et al. 2004; Hong et al. 2007; Cao et al. 2008); NRP1 is essential in skin tumorigenesis – NRP1 deletion abrogates the response of cancer stem cells to autocrine VEGF (Beck et al. 2011). NRP2 is implicated in colon cancer, prostate cancer and a triple negative breast cancer (Elaimy et al. 2019); NRP1 supports proliferation of human glioma stem-like cells in glioblastoma multiforme (Hamerlik et al. 2012). All of these functions result from neuropilins' interactions with a plethora of ligands. Neuropilin-1 (NRP1) was initially discovered as a neuronal

adhesion molecule in the retinotectal projection of Xenopus tadpoles (Takagi et al. 1987). NRP1 was specifically identified as a receptor for the class III semaphorin SEMA3A, and this interaction was associated with a collapse of neuronal growth cones (Kolodkin et al. 1997). Since then, neuropilins have been shown to also interact with class IV semaphorins, HGF, PDGF, EGF, integrins, cell-cell adhesion receptors, galectins, TGF-beta (reviewed in Parker et al. 2012). But, the best characterised interaction is that with various forms of VEGF with the direct implications for angiogenesis and immunomodulation.

Three-dimensional structure of neuropilins – conserved features

Neuropilins are type one transmembrane proteins. They contain ectodomain region which consists of five distinct structural domains, a single transmembrane helix and a short 44-residues long intracellular region. Extracellular domains are named **a1**, **a2**, **b1**, **b2** and **c**. Domains **a1** and **a2** are structurally homologous to one another and they have the beta-sandwich topology of the so-called CUB structural domains (Nakamura et al. 1998; Appleton et al., 2007). The CUB domain structural motif is utilised to carry out wide range of functions but primarily in a context of extracellular or plasma membrane proteins. In neuropilins, these CUB domains are required for interaction with semaphorins. Neuropilin domains **b1** and **b2** also have a beta-strand based fold and they belong to the family of coagulation factor V/ VIII type C domains or discoidin structural family. In neuropilins, while both **b1** and **b2** domains are required for maintaining receptor integrity, series of functional and structural studies have demonstrated that it is the specific site on the **b1** domain that engages with VEGFs C-terminal end (Vander Kooi et al. 2007; Tsai et al. 2016). Membrane proximal **c** domain is also referred to as MAM domain owing to its similarity to the domains found in meprin, A5, and receptor protein tyrosine phosphatase mu (Takagi et al. 1991; Beckmann and Bork 1993).

MAM domains also form a stable beta structure known as a jellyroll topology. Several research groups, including ours, investigated the detailed atomic structure of the first four extracellular domains, and their complexes with biological or small molecule ligands (Lee et al. 2002; Vander Kooi et al. 2007; Appleton et al. 2007; Jarvis et al. 2010; Parker et al. 2012b, Tsai et al. 2016; Mota et al. 2018). More recently, we reported that a crystal structure of the MAM domain of NRP1 comprises a variable N-terminal region with short alpha helix connected through an extensive linker to a single beta strand, and an adjacent 8-stranded β-barrel, followed by the short α-helical turn at the C-terminus (Yelland and Djordjevic 2016). The structure was stabilised by the specific Ca^{2+} binding site. Thus, all of the neuropilins' extracellular domains adopt some type of the stable beta-structures that have been found in other extracellular-domain containing proteins. The presence of these domains provides neuropilins with the multiple surfaces for interactions with various ligands and protein partners which is why neuropilins are frequently referred to as pluripotent receptors.

A single transmembrane helix connects this large ectopic domain with the intracellular domain made of the terminal 44 residues. Our knowledge with respect to the structure of the intracellular region is limited and all of the current experimental evidence, including NMR spectroscopy, indicates that this part of the polypeptide can be described as intrinsically disordered, at least when not engaged in interactions with other partner proteins (unpublished data).

VEGF receptor complexes and the role of neuropilins in mediating response to VEGFs

Best characterised interactions of neuropilins are those with vascular endothelial growth factor VEGF. However, even describing interactions with this 'single' ligand is intrinsically complex – namely in humans there are several VEGF genes: VEGFA, VEGFB,

VEGFC, VEGFD, which either exist as splice variants or are subject to posttranslational proteolytic processing (Eichmann and Simons 2012). Physiologically, perhaps most significant is VEGFA with $VEGFA_{165}$ (here 165 refers to the number of amino acids) being its most abundant splice variant and comprising regions encoded by exons 2-5, exon 7 and a short 6-residues long sequence at the C-terminus encoded by exon 8.

VEGFs mediate their function primarily via family of VEGF receptors (VEGF-Rs) which belong to classical tyrosine kinases whose activation mechanism involves ligand-induced dimerization thereby enabling tyrosine phosphorylation. However, neuropilins play a profound role in stabilizing this complex and enhancing tyrosine kinase activity. Figure 2 shows a putative organisation of the ternary complex formed by $VEGF_{165}$, VEGF-R2 and NRP1. While the structures of nearly all of the components of this complex have now been determined, the actual three-dimensional structure of the full receptors-ligand assembly is still not known. For the illustrative purposes, the image of the complex was constructed with consideration of restrains imposed by knowing the partial structures, the size of the individual components, and by the knowledge of the relative orientations of the individual domains, within the proteins and with respect to the cellular membrane. Core region of a dimeric, disulphide bridged VEGF molecule is bound to a dimer of the VEGF receptor, via interactions with immunoglobulin homology domains 2 and 3 (Leppanen et al. 2010). Terminal 55 residues, provide a positively charged, heparin-binding (HBD domain) (Parker et al. 2012) link to an adjacent neuropilin molecule with the C-terminus of HBD specifically interacting with the ligand-binding grove on the neuropilin's **b1**-domain. In the illustration, for simplicity, only a single neuropilin molecule was included although it is known, that neuropilins can dimerise, particularly in the presence of heparin. Previously, molecular biology data were suggesting that the function of the membrane proximal **c**/MAM domain of neuropilins might be in mediating dimerization (Nakamura et al. 1998). However, our work

have shown that the MAM domain from NRP1 is monomeric in solution and insufficient to drive receptor dimerization (Yelland and Djordjevic 2016). We postulated that the MAM domain in the context of neuropilins, shields the ectodomain from the membrane surface and positions other domains (**a1a2b1b2**) for their interactions with partner co-receptors. This domain might also modulate multimerization and organisation of the higher order receptor clusters which would have implication for signalling by supporting cooperativity and modulating signal to noise ratios of the signalling complexes. Additionally, as the N-terminal region of the **c**/MAM domain is subject to O-linked glycosylation (Windwarder et al. 2015), this domain might mediate interactions with other binding partners, such as galectins and modifying enzymes.

So, how do neuropilins 'signal' and how is their activity regulated? As already mentioned, neuropilins do not have catalytic activity and they are perceived to function as 'co-receptors' together with VEGF-Rs in order to mediate response to VEGFs. The name 'neuropilins' primarily refers to two proteins: NRP1 and NRP2, and while they share 44 % sequence identity they are characterised by differences in expression patterns and ligand preferences and thus they play different physiological role. NRP1 is for example localized preferentially in arteries. In contrast, NRP2 is expressed preferentially in veins and lymphatic endothelial cells (Hercog et al. 2001). This differential expression parallels differences in physiological responses to the main ligands such that NRP1 is considered to be a primary receptor for VEGFA while NRP2 mediates responses to VEGFC. The intracellular C-terminal region of neuropilins is also required for the full signalling response, in particular the terminal –SEA sequence motif, which interacts with the PDZ domain of the scaffolding protein GIPC/Synectin and which is indispensable for the full signalling function of neuropilins as well as for the neuropilins' coupling with integrins and of relevance for cell migration (Prahts et al. 2008; Robinson et al. 2009). It should also be recognized that

neropilins are subject to extensive modifications by glycans – NRP1 is a substrate for chondroitin sulphate addition, NRP2 is a subject to polysialilation (Shintani et al. 3006; Frankel et al. 2008; Bhide et al. 2016). While addition of these large polar molecules will inevitably have a dramatic effect on neuropilins' capacity to form protein/protein interactions, the full extent by which these modifications control signalling outputs is poorly understood.

Targeting neuropilins

In light of neuropilins' role in angiogenesis, cell migration and maintenance of cancer cell stemness, as well as in immunomodulation, there is substantial merit in producing therapeutics to this pleiotropic target. Importantly, studies have shown that targeting these molecules would provide effective means for tumour growth inhibition while decreasing resistance to other therapies (Goel et al. 2013; Mercurio 2019). There are several areas of neuropilin molecules and the receptor assembly that could be targeted but the most explored and most effective approach so far involved development of the small-molecule antagonists that target well-described VEGF C-terminus binding-site on the **b1** domain. Using a range of structural studies, in vitro experiments, functional assays and the proof-of-concept studies it was shown that the main tool compounds, EG00229 and EG01377 bind to the **b1** domain of NRP1 selectively, that they inhibit VEGF receptor tyrosine kinase phosphorylation and associated VEGF-mediated functions such as angiogenesis and cell-migration (Jarvis et al. 2010; Miyauchi et al. 2016; Powell et al. 2018). Crucially, the compounds were shown to be effective in mice glioma models where they suppressed glioma progression and their activity was linked to the function of microglia, macrophages and Treg cells.

Final comments and general conclusions

Proteins involved in receiving and responding to environmental signals exhibit structural features that are shared across the domains of life. Commonality of structural domains is the evolutionary consequence of the requirements for the molecular plasticity and molecular dynamics of the proteins and molecular mechanisms employed to carry out these functions. The presence of these common structural threads and many of the global functional and mechanistic features allow us to transpose the knowledge gained from one signalling system to another. However, unique functional and structural properties of each of the molecular interactions necessitate detailed characterisation of each of the protein, and protein complexes involved, in order to fully understand the signalling processes and their regulatory outcomes. Ultimately, this detailed understanding could be exploited for the development of new reagents, devices and therapies.

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Figure legends

Figure 1. Comparison of the apo form of AioX (yellow) and the arsenite-bound (pink). In the crystal, arsenite is covalently linked to cysteine C106. Arsenite-binding causes Y88 side chain rotamer change in concert with a large backbone conformational change spanning the residues 53-61 (circled in the figure). While apo and the arsenite-bound form exhibited distinct conformations of this loop, the electron density for the corresponding region in the phosphate-bound AioX crystal structure was very disordered, suggesting higher flexibility of the loop for the phosphate-bound form.

Figure 2. Putative model of a ternary assembly between the ectodomains of neuropilin (dark gold) and VEGF receptor (grey) with the dimeric VEGF molecule (salmon red). The illustration is showing molecular surfaces generated from the crystal structures and combined as described in the text. A: The ternary complex. B: Molecular surface of the NRP1-b1 domain in a complex with antagonist EG01377 shown as sticks and coloured based on the atoms. C: The same as in B but rotated by 90 degrees providing the view from the top of the complex showing how EG01377 competes for binding with the C-terminus of VEGF.

Figures

Figure1:

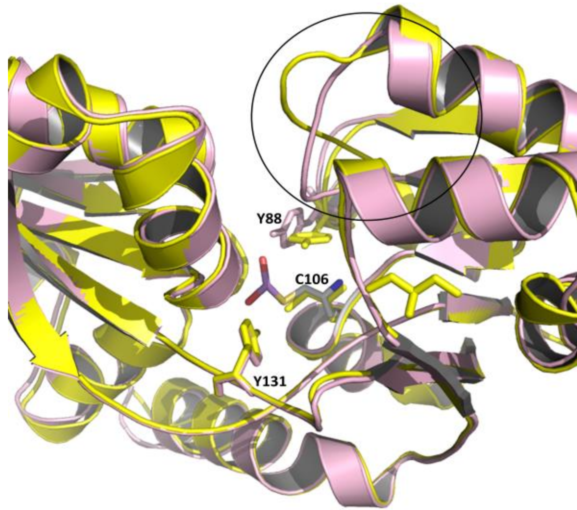

Figure 2: