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Short Conceptual Overview

Functional and evolutional implications of natural channel-enzyme fusion proteins

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Abstract

Channeling of ions or substrates across membranes and enzymatic activity are two highly distinct biochemical concepts. They are usually studied by different research groups, which focus on either subject. Nature has provided a challenge for specialized scientists by fusing genes coding for a transmembrane channel domain with an enzyme domain. There are examples of fusion proteins consisting of an N-terminal ion channel or sensor and a C-terminal, cytosolic kinase domain (or other enzymes involved in signaling) of which either domain may influence the functionality of the other. The physiological role of such fusions may reside in coupling ion flux or membrane potential sensing to cellular responses or vice-versa. Other examples can be found in metabolism. We have identified and characterized an arsenite-conducting aquaglyceroporin carrying a C-terminal arsenate reductase domain. Here, a function in the detoxification of arsenic is obvious, with the enzyme domain generating the substrate for the channel domain, which immediately shuttles the toxic metabolite out of the cell. We see two advantages in this latter concept: lowering of the cellular toxicity due to rapid release of the substrate and energetic coupling of the reaction enthalpy to extrusion due to high local substrate gradients. In this overview, we summarize and discuss the current view on functional and physiological aspects of channel/enzyme fusion proteins.

Keywords: arsenate reductase; arsenite channel; fusion protein; ion channel; kinase.

Introduction

According to Darwin's classical evolution theory, natural selection acts to preserve a functional advantage for a member of a species (1). An organism is in a particularly advantageous position if it is able to efficiently and economically capture and direct energy (2). It is consequent to apply the natural selection principle to the underlying cellular and molecular level as well.

All living cells are surrounded by an insulating lipid membrane, yet, it is essential that cells incessantly capture nutrients, exchange metabolites and transport ions across the membrane, e.g., for signaling purposes. Respective proteins that span the cellular membrane have evolved to facilitate the transport of organic compounds or inorganic ions in a highly selective fashion. Recently, various transmembrane channel fusion proteins have been identified, which carry functional enzyme domains at their C-termini (3–7). The combination of two such entities can be viewed as another evolutional step towards system optimization. Based on the selectivity properties of the channel domain, in context with the biochemical reaction that is catalyzed by the enzyme domain, one cannot only postulate a physiological role of such channel-enzyme fusion proteins, but also build and test hypotheses as to what the evolutionary advantage might be.

The first two channel-enzyme fusion proteins were reported from the family of transient receptor potential (TRP) channels (4, 5). These, and other proteins carrying an ion channel-like domain fused with a regulatory or a second messenger-generating enzyme domain, are most likely involved in cellular sensing or signaling processes. Another concept is represented by fusion proteins consisting of a metabolite channel and the respective metabolite-generating enzyme (7). This type may efficiently couple biochemical reaction energy and rapid metabolite diffusion processes due to high local concentration gradients. Here, we review recent progress made in the identification and biochemical characterization of channel-enzyme fusion proteins.

Fusions of ion channels or voltage sensors with enzymes

Ion channels facilitate the transport of inorganic ions across biological membranes along the prevailing electrochemical gradient. They are key in the primary physiological functions of any cells, for instance, by modulating the cellular membrane potential via sodium and potassium channels or by regulating the intracellular calcium and magnesium concentration for cellular signaling events.

Rapid switching between a non-conducting (closed) conformation and a conducting (open) conformation, i.e., ion channel gating, is essential for maintaining the cellular ion homeostasis. Most of the ion channels are modulated by phosphorylation and dephosphorylation events (8). In most cases, the required protein kinases and phosphatases associate transiently with the ion channel either by direct interaction (9) or via anchoring or scaffolding proteins (10). The

recent discovery of ion channels carrying covalently bound channel-regulating enzymes that are encoded by a single gene, brought up a new facet in the cellular signaling field. This topic has been reviewed before (11), and is hence discussed only briefly here.

TRP channels, a superfamily of cation-conducting membrane proteins, are involved in the sensory perception of mammals (12). Two members of the long TRP cation channel family, TRPM7 and TRPM2, have been identified combining both channel and enzyme activities within the same protein, whereby the enzyme domains can be direct modulators of the channel or of subsequent cellular signaling cascades.

The Ca^{2+} and Mg^{2+} conducting cation channel of TRPM7 is fused with a protein kinase (Figure 1A, left) (4, 13). As shown by knockout experiments in mice, TPRM7 is vital for cellular physiology (4). Yet, the role of the kinase domain in TRPM7 function is not fully understood. There is a controversy as to how the channel and the kinase domain interact on a functional basis. There is some evidence that the kinase is capable of autophosphorylating the channel domain and it was further suggested that ATP and the kinase domain are nessesary to open the channel (6). Others find that the activity of the kinase domain does not directly influence channel function, whereas intracellular Mg^{2+} and MgATP were seen to block channel permeability (4, 13, 14). In this case, binding of MgATP seemed to require the nucleotide binding site within the kinase domain (15).

Besides a proposed intramolecular action of the kinase domain, it may also be involved in signal transduction pathways if other substrates for the TRPM7 kinase exist. Recently, two such substrates have been reported, i.e., annexin A1, a central player in membrane trafficking and reorganization, and myosin IIA, the major motor protein driving cell contractility (16, 17). An additional functional connection of the TRPM7 kinase has been proposed with phosphorylation of the eukaryotic elongation factor EF2, which mediates ribosomal translocation. The kinase does not phosphorylate eEF2 directly, but the immediate upstream cognate kinase of eEF2 (18). Since the TRPM7 kinase responds to the free Mg^{2+} concentration (19), the channel function may be to link Mg^{2+} sensing to intracellular kinase activity.

Another member of the long TPR channel family, TRPM2, contains a calcium-permeable channel domain and a C-terminal ADP ribose pyrophosphatase-like domain (Figure 1A, right) (20). Accumulation of ADP ribose is a measure for oxidative stress in a cell. It has been shown that the enzyme domain acts as an intrinsic negative modulator of the channel function in the presence of ADP ribose. It is not clear, however, whether ADP ribose binding alone, or the actual hydrolysis of the substrate, is required for gating. Further, the question of whether an ion flux through the channel domain may modulate the enzymatic activity, is still open (3, 5, 11).

In certain protozoa, i.e., *Plasmodium*, *Paramecium* and *Tetrahymena*, an N-terminal ion channel domain with a C-terminal adenylyl cyclase domain has been reported (Figure 1B) (21). The ion channel domain harbors a sequence that is typical for voltage-sensors in the fourth of six transmembrane segments, and a predicted pore region consisting of a pore helix and a pore loop between the fifth and sixth transmembrane segments. This structure is indicative of a functional ion channel domain, however, ion permeability has not been shown yet. In terms of the physiological role, it can be speculated that this fusion protein may directly couple sensing of ion concentrations of the cell-surrounding medium to an intracellular second messenger system.

A variation of the theme has evolved by the fusion of a non-conducting voltage sensor domain with a C-terminal phosphoinositide phosphatase domain (Figure 1C) (22). The structure of the voltage sensor domain is similar to that of the first four transmembrane segments of voltage-gated ion channels, but it lacks an ion-conducting pore domain. With this type of a construct, the cell may be enabled to directly translate changes in the membrane potential into the turnover of phosphoinositides for signaling (23).

Very recently, fusions of high-affinity ammonium channels of the Amt/Rh family with various types of cytosolic enzyme domains have been identified by genome data analyses (Figure 1D). The physiological function of such fusions has not been investigated experimentally, but it is not farfetched to assume that they link an ammonium sensing or even transport function to intracellular metabolic processes that make use of nitrogen (24).

Metabolic channeling between enzymes and across compartment barriers

Metabolic channeling is the process in which biochemical reaction intermediates are directed to the active sites of consecutive enzymes by linking intramolecular tunnels (Figure 1E) (25). This layout prevents diffusion of the compounds away from the catalytic centers and, thus, increases the efficiency of the metabolic pathway (26).

The first mulitfunctional, multicomponent *Escherichia coli* enzyme, with a tunnel linking the α and β subunits, was reported in the 1950s (27). The complex catalyses the last two reactions of the tryptophan biosynthesis, i.e., the cleavage of indol 3-glycerol phosphate at the α subunit and synthesis of L-tryptophan from indol and L-serine at the β subunit (28). The intermediate product, indole, does not appear in the cytosol during this reaction, but diffuses within the enzyme complex from subunit α to subunit β (29, 30). Elucidation of the three-dimensional structure of tryptophan synthase from *Salmonella typhimurium* revealed the presence of a 25 Å long tunnel connecting the active sites of both enzyme domains (31). This setup strikingly enhances the conversion rate by up to two orders of magnitude compared to the rates obtained with separate subunits α and β (32).

A similar layout is realized by the *E. coli* carbamoylphosphate synthetase enzyme. Structural analysis showed that there is a 96 Å long intramolecular tunnel between its three catalytic sites, which funnels three metabolic intermediates, i.e., ammonia, carbamate and carboxyphosphate, between the reaction centers (33).

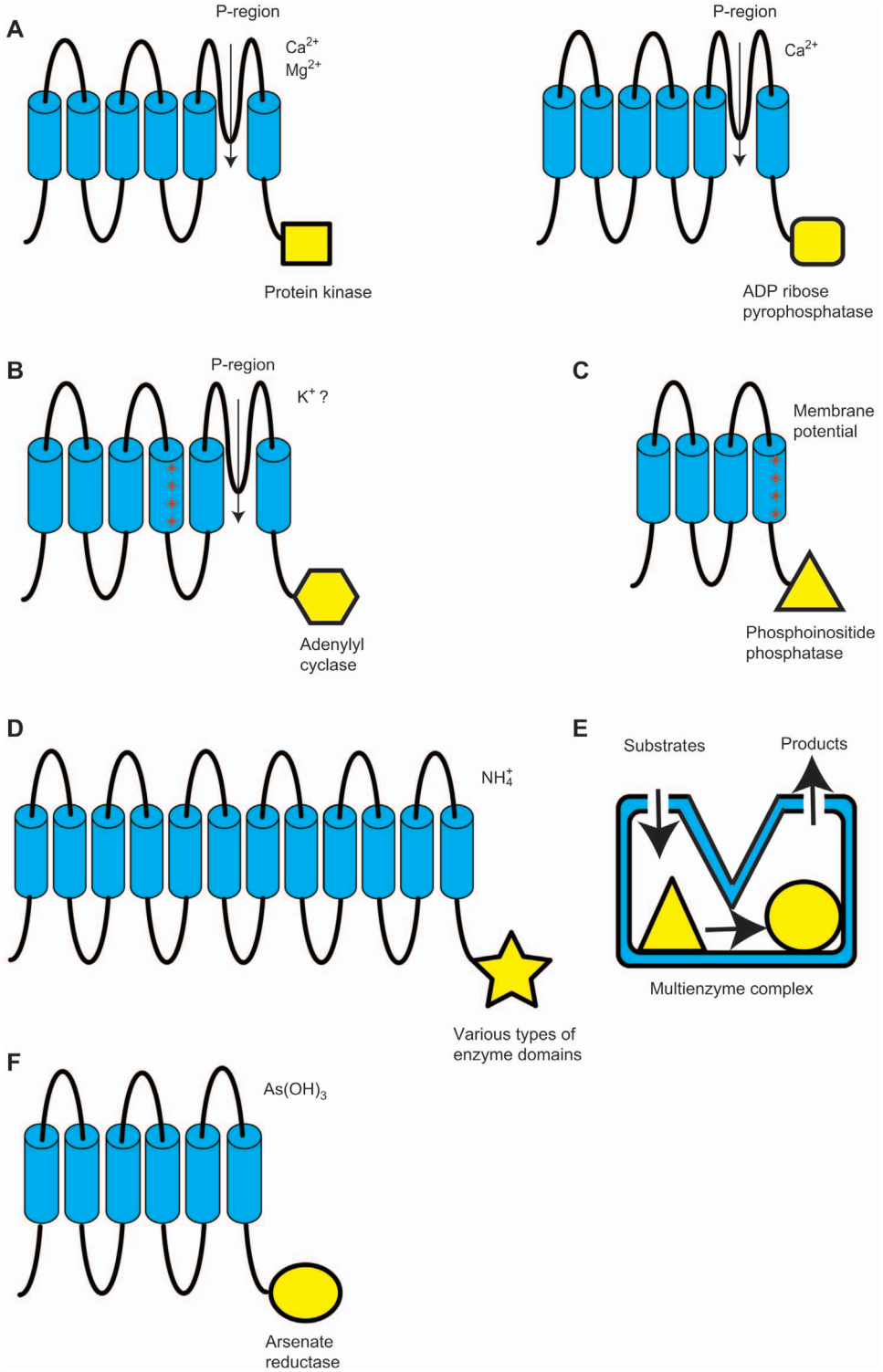

Figure 1 Overview of natural channel/enzyme fusion protein layouts.
(A) Proposed membrane topology and putative sites for ion transport (P-region) and enzyme domains of TRPM7 (left) carrying a protein kinase domain and TRPM2 (right) with an ADP ribose pyrophosphatase domain. (B) Predicted domain composition of some protozoan ion channel adenylyl cyclases. The voltage-sensor is marked by plus signs. The pore region (P-region) is located between the fifth and sixth transmembrane segments. (C) Proteins with a voltage sensor and a phosphoinositide phosphatase domain. The voltage-sensor is marked by plus signs. (D) Predicted ammonium channels of the AmtB family fused with different cytoplamatic domains such as regulators of nitrogen metabolism or Ser/Thr protein phosphatases. (E) Metabolic channeling between catalytic sites in multifunctional enzymes. The catalytic sites of an enzyme complex are connected via an intramolecular tunnel. (F) Topology prediction of Strop634, i.e., an arsenite-conducting aquaglyceroporin fused with an arsenate reductase domain.

Additional substrate channeling multienzyme complexes of this type are being discovered in plants, which enable a cell to synthesize specific natural products more effectively and minimize metabolic interference (34).

Certain metabolic reactions require that a metabolite is transported across a lipid bilayer, either into another compartment for further chemical modification, or out of the cell for detoxification purposes. A suitable construct to achieve this, by analogy to the above-discussed channel-linked enzymes, would consist of a metabolite generating enzyme fused to a metabolite-specific transmembrane channel. We have identified and characterized examples of this novel concept in metabolism, which has been realized for the detoxification of arsenic (Figure 1F). Today, the oxidized form of arsenic, i.e., arsenate, predominates in nature due to the oxidizing conditions of the atmosphere.

Arsenate detoxification in microorganisms consists of three steps. 1. Arsenate appears indistinguishable from phosphate by cellular transport systems and is hence taken up via phosphate transporters (35). 2. In the cytosol, arsenate is reduced by a cytosolic arsenate reductase enzyme (36). The resulting arsenite as a soft metal ion is chemically very active and even more toxic than arsenate. It can for instance form covalent bonds with thiols of cysteines and nitrogens of histidines and inhibit enzyme activity. 3. Arsenite is subsequently extruded from the cells by unidirectional arsenite transporters, such as ArsB in *E. coli* (37) or ACR3 in yeast (38). Certain bacteria lacking genes for ArsB or ACR3 transporters can release arsenite via an aquaglyceroporin (39). Aquaglyceroporins have recently have been implicated in bidirectional arsenite transport (40–42).

We have identified and biochemically characterized two variants of bacterial arsenate detoxifying fusion proteins in *Mycobacterium tuberculosis* (Rv2643) and in marine *Salinispora tropica* (Strop634) consisting of an N-terminal arsenite channel domain of the ACR3 or aquaglyceroporin type, respectively, and a C-terminal thioredoxin-coupled arsenate reductase domain (7). Both channel/enzyme proteins mediate arsenate resistance in a highly sensitive yeast strain, by coupling arsenate reduction with immediate arsenite efflux. We have also shown in phenotypic, as well as in direct assays, that both domains are functional when expressed separately (7). Is there an advantage connected with the physical fusion of an arsenite reductase enzyme with an arsenite channel? Mainly two positive aspects come to mind in this scenario: 1. reduction of cellular arsenite toxicity and 2. energetic coupling of the reaction enthalpy with the extrusion process. Regarding the effect on toxicity, it seems likely that the close proximity of the arsenite generating enzyme and the arsenite export channel at the cell periphery, will reduce the overall cellular concentration of arsenite similar to the situation in metabolic channeling described above. In terms of energetic coupling, the enzymatic reduction reaction will generate a high local concentration of arsenite at the plasma membrane, which will drive the export by diffusion more efficiently than equally distributed, i.e., diluted, arsenite molecules throughout the cytosol would do (Figure 2). In addition to the dilution effect, diffusion in the cytosol is strongly hampered by the high protein concentration of 200–300 mg/ml, also referred to as molecular crowding (43). According to Fick's first law of diffusion, the concentration gradient (Δc) in conjunction with the permeability coefficient of the conducting channel (P) directly determines the solute flux (J): $J = -P \cdot \Delta c$. Without an export channel nearby, any excess in reaction energy would go unused and the product molecules would disperse. In fact, some bacteria express an accessory ATPase domain protein, i.e., ArsA, to the ArsB arsenite channel. It is thought that the function of ArsA is in propelling arsenite molecules towards the ArsB channel for higher export flux rates at the cost of ATP hydrolysis. The newly discovered channel/enzyme fusion proteins may achieve a similar effect at a lower cost by not letting slip away the arsenite molecules in the first place. It will be interesting to see whether these proposed effects are quantifiable and whether the extent suffices to generate a distinct phenotype.

Apparently, Strop634 is not the only example of an aquaglyceroporin channel fused with an arsenate reductase. Related *Frankia alni* soil bacteria also encode a Strop634-like channel and enzyme domain in a fusion gene, *Fraal3366*. Strikingly, however, another *Frankia* species, CcI3, encodes highly similar proteins in two separate, consecutive genes

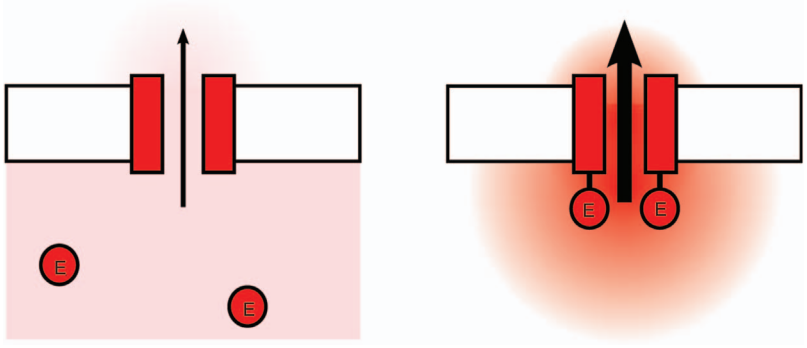

Figure 2 Depiction of the dependency of facilitated transmembrane solute flux from the chemical gradient.
Separation of the enzyme (E) from the channel proteins results in cytosolically dispersed metabolite molecules (pink shading) and a flat outward gradient (left panel), whereas channel-enzyme fusion proteins create a high local metabolite concentration (red shading) and, subsequently, a steep outward gradient (right panel).

(*Francci3_2325* and *_2326*) (7). The coding regions of both genes overlap in a GTGA sequence (44), which is able to serve as both a terminator codon (TGA) for the upstream aquaglyceroporin gene and an initiator codon (GTG) for the downstream arsenate reductase gene. Such an overlapping sequence is prone to initiate gene fusions, which will derive from a single frameshift mutation. The fact that bacterial species from the same genus display a different genetic makeup with regard to separate or fused aquaglyeroporin and arsenite reductase proteins, strongly hints at very recent gene fusion events in terms of the evolutional time scale.

Outlook

Gene clusters are widespread in nature. In 1959, Demerec and Hartman postulated that 'the existence of gene clusters shows that they must be beneficial, conferring an evolutionary advantage on individuals and populations which exhibit them' (45). Clusters imply functional interaction of the resulting gene products. With a cluster structure, the expression of genes can be coordinated in a concerted fashion (46). The next level of coordination is the formation of fusion genes and the production of multifunctional proteins. The presence of complex multidomain proteins typically involves vital enzymes in energy metabolism and is indicative of a high level of evolution of the organisms. Fusions of channel proteins with enzyme domains are just being picked up from the growing number of sequenced genomes. Each newly identified channel/enzyme fusion and its genetic and biochemical characterization will help elucidate their role in driving evolution.

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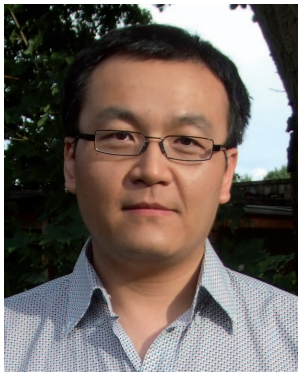

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