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Review

The role of aquaporins in excretion in insects

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Summary

One of the aspects of insect osmoregulation that has most intrigued researchers is the ability of a simple tubular epithelium, such as the Malpighian tubule, to create both hypo- and hyperosmotic urine. Indeed, Ramsay's initial observation that isolated tubules could secrete a hypoosmotic urine led him to attribute the phenomenon to the active transport of water. In the ensuing decades several models for solute recycling have been proposed, but only in the last 15 years has it become clear that tubule water permeability is due to the presence of aquaporins (AQPs), the ubiquitous water transport proteins. There are 13 known human AQPs, and they are tissue and even membrane specific. It is now clear that the number and type of AQPs within a membrane are the major determinants of its water transport capacity. There are many gene homologs for the AQPs, so proof of function requires expression of the protein in a defined system. Within the insects, only seven AQPs have been functionally expressed and, of these, four directly or indirectly function in excretion. In this paper we review the basic structure and general function of AQPs and then examine the source, localization and functional attributes of those isolated from insects.

Key words: insect, aquaporin, water transport.

Background

On the occasion of the fiftieth anniversary of its publication, Maddrell (Maddrell, 2004) wrote a retrospective of Ramsay's ground-breaking paper (Ramsay, 1954). Ramsay's hypothesis was valid, his methods innovative and his conclusions almost certainly flawed, which should probably be a caveat to all of us in the field. Despite its thermodynamically untenable conclusion that water was actively transported, Ramsay's paper has remained a landmark in part because he developed methods to work with individual Malpighian tubules which with slight modifications are still in use today.

With this paper, Ramsay opened up the entire field of research in which fluid-secreting tubules can be studied individually. Certainly it expedited the discovery of stimulatory and inhibitory substances and made the study of secretion much more amenable to experimental manipulation.

We now know, of course, that Ramsay's discarded alternative explanation, that primary urine is formed by the secretion of ions to form an osmotic gradient, followed by selective reabsorption downstream, is in fact the correct mechanism. Water always follows the osmotic gradient established by the cell or the tissue. The ionic mechanisms and pumps that drive this fluid transport are discussed elsewhere in this volume.

The more difficult experimental question was to determine how solute and water movement could be de-coupled, so that water would freely follow a very small, essentially isosmotic gradient in one region of the tubule and not in another. Various forms of solute recycling (Wall, 1971) were proposed wherein the initial osmotic gradient required for fluid movement was established and then the solutes were recovered and pumped back into the same luminal space. At the time these studies were being done, the mechanisms by which water molecules moved across membranes were unknown. It was assumed that despite the intensely hydrophobic nature of the membrane interior, small molecules, even ones as polar as water, could somehow freely slip between the phospholipids and pass through the bilayer. The biggest problem with this explanation is that there is no way to regulate permeability and water should always follow the osmolytes. The best explanation at the time was that there were 'tight' and 'leaky' epithelia, and that the difference between the two was the degree to which water moved paracellularly.

Current theory holds that while membranes do indeed have a low but constant permeability to water, it is not nearly sufficient to account for the very rapid movement experienced under many physiological conditions. The discovery by Agre and co-workers that the 28 kDa channel-forming integral membrane protein (CHIP-28) was in fact the long sought-after water channel, now called Aquaporin-1 (AQP1), was sufficiently momentous to be recognized by the award of a Nobel prize (Preston et al., 1992; Agre et al., 1993). Currently there are 13 known human AQPs (AQP0 to 12), each of which is expressed in specific tissues and which differ in both transport specificity and regulation.

Structure and function of aquaporins

The overwhelming majority of published studies of AQP structure and function focus on vertebrates and even more specifically on humans, so it is to these we look for a basic understanding of AQP function. Such comparisons are valid inasmuch as AQP structure appears to be highly conserved even among kingdoms (Bansal and Sankararamakrishnan, 2007). The known AQPs consist of six membrane-spanning helices (TM1 to 6) separated by five loops (A–E; Fig. 1). Loops B and E are highly symmetrical and contain the canonical Asn–Pro–Ala (NPA) motifs. Both the N- and C-terminus of the protein are intracellular. The

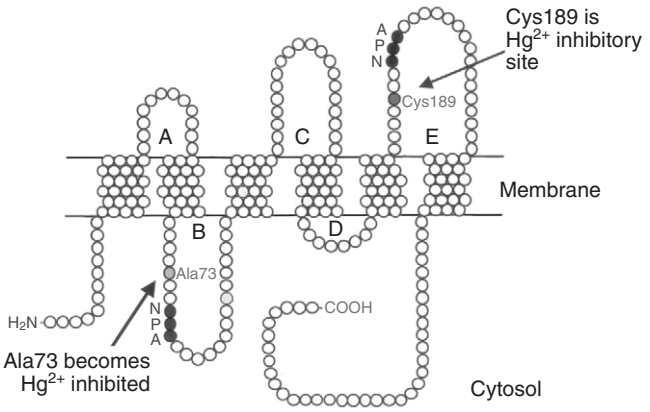

Fig. 1. Primary structure of human AQP1. The six transmembrane domains are shown as they are inserted in the membrane with the five connecting loops (A–E) linking them. The NPA (Asn–Pro–Ala) motifs in loops B and E are shown as well as cysteine 189 and alanine 73, which are the primary and secondary sites for mercury inhibition. After Agre et al. (Agre et al., 1993). For further details, see text.

classic 'hourglass' model (Jung et al., 1994) (Fig. 2) illustrates the folding of TM1–3 and TM4–6 to form a symmetrical pore. Loops B and E form two half-helices that fold into the pore from opposite sides, bringing their respective NPA regions into alignment. The prolines interact so that the two positively charged asparagines form one wall of the pore. This region is one of two major constrictions and gives the AQP its ion impermeability.

Protons are three times more mobile in aqueous solution than are water molecules themselves, so any aqueous pore should be a highly conductive proton channel. This is not the case for the AQPs, however, and the reason is the conserved NPA motifs. The two

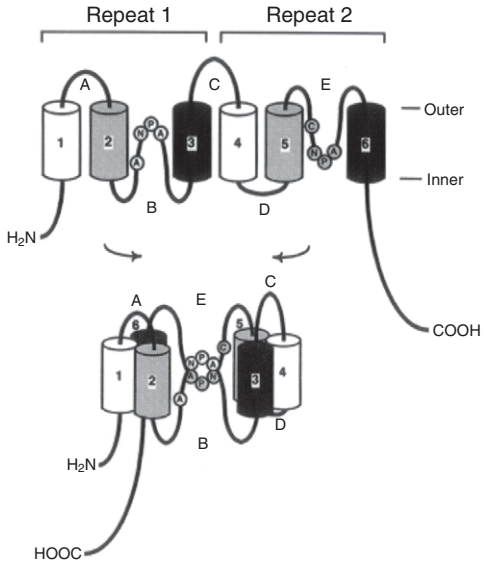

Fig. 2. The hourglass model for the folding of human AQP1. The six transmembrane domains consist of two tandem repeats and fold to form the central water-transporting pore. The cytoplasmic connecting loop B and the extracellular loop E, which contain the NPA motifs, fold inwards to meet in the center of the pore, forming the primary proton filter. C, Cys189; A, Ala73. After Jung et al. (Jung et al., 1994).

asparagines hydrogen bond with the oxygen in water, turning it at right angles to the pore and disrupting the hydrogen bonding of the water molecules filling the pore. Proton transport along water chains requires uniform orientation of the H-bonded water molecules which permits reorientation during proton transfer. This forced orientation of the water molecule filling the narrow region of the pore by the paired asparagines is sufficient to completely disrupt proton movement along the water chain, rendering the channel impervious to protons (Chakrabarti et al., 2004; Fu and Lu, 2007; Tajkhorshid et al., 2002). Most AQPs are also reversibly inhibited by Hg^{2+}, the inhibition being relieved by the reducing agent β-mercaptoethanol. The Hg^{2+} interacts primarily with the cysteine residue located near the NPA motif in the E loop and secondarily with the alanine of the B loop, both of which are brought into close proximity by the in-folding of the hemipore. This effectively blocks water from the pore.

The second major constriction and the primary selectivity filter lies 8 Å (1 Å≈0.1 nm) closer to the extracellular face than the proton filter. This is the aromatic/arginine (ar/R) region and is formed by one residue each from TM2 and TM5 and two residues from loop E. The aromatic side chains of the phenylalanine force the water molecules to hydrogen bond with arginine and histidine. These residues are conserved in both the AQPs and the aquaglyceroporins (GLPs), which allow the passage of both water and low molecular weight solutes such as glycerol. This filter is ~3.0 Å in diameter, slightly less (down to 2.8 Å) in strict AQPs, slightly more in GLPs. The hydrophobic interactions are essential for selectivity. The histidine residue (His180 in AQP1) is highly conserved in strict AQPs and is normally replaced by glycine in GLPs to relax the steric interaction with the alkyl chain of glycerol (Chen et al., 2006).

Within the membrane, AQPs form homotetramers, and in tissues with high densities of AQPs they may appear as orthogonal arrays (Verkman and Mitra, 2000). The reason for the functional tetramer formation is unclear as each subunit contains its own water pore and the central pore of the tetramer is water impermeable. The most plausible explanation is that AQPs serve multiple functions within the membrane. For example, AQP1 has been reported to be both a cyclic nucleotide-gated ion channel (Anthony et al., 2000) and the CO_2 permeability pathway in erythrocytes (Endeward et al., 2006). It is not unreasonable to suggest that these functions might be handled by the central pore while leaving the water transport function of the monomers intact, but definitive data are lacking at this point.

One other important feature of AQPs would be the distinction between constitutive and regulated components. In the vertebrate collecting duct, for example, AQP3 is a constitutive component of the basolateral membranes, providing a constant high H_2O permeability between the cell and the ECF. The apical membrane, of course, has a variable permeability regulated primarily by arginine vasopressin. Here AQP2 is retained in vesicles until the endocrine signal is received and then it is inserted into the apical membrane to promote H_2O reabsorption from the collecting duct. AQP2 also contains a consensus sequence that allows it to be phosphorylated by a cAMP-dependent protein kinase. Phosphorylation appears to promote the insertion of AQP2 into the membrane, rather than altering its intrinsic permeability (Lande et al., 1996; Valenti et al., 2005).

To demonstrate unequivocally that an AQP is more than a homologous gene sequence, of which there are many, it is necessary to demonstrate that it does indeed have a physiological role. This can be done either by injecting the purified protein into

liposomes or by injecting the appropriate mRNA into *Xenopus* oocytes. In either system, volume can easily be measured microscopically, and changes in volume following an osmotic challenge correlate directly with the degree of permeability conferred by the putative AQP. Different osmolytes can also be used to distinguish between AQPs, GLPs and ion channels.

Insect aquaporins

The tremendous amount of research being published on vertebrate systems brings into high relief the paucity of data on invertebrates, including the insects. Within the class Insecta, there are only seven members of the AQP family which have been functionally expressed (Table 1). Six of these appear to function as classic, water-transporting proteins. The exception is big brain (BIB) isolated from the fruit fly, *Drosophila* (Rao et al., 1990). Mutations in the BIB gene cause defects in cell fate determination during neurogenesis as it is required for endosome maturation and notch trafficking (Kanwar and Fortini, 2008). The BIB protein has sequence identity with the AQP family of proteins, but does not increase water permeability when expressed in *Xenopus* oocytes. Instead it acts as a voltage-insensitive, non-selective cation channel (Yanochko and Yool, 2002). Cation movement is *via* the central pore of the tetrad and the cation channel activity is regulated by an exogenous signaling pathway mediated by tyrosine kinase (Yool, 2007).

Insect AQPs appear to be ubiquitous and affect cellular function in every tissue. Both desiccation and freeze tolerance in insects require the removal of water from the cells, either to suspend metabolic processes or to avoid damaging ice crystal formation. Additionally, freeze tolerance requires the accumulation of glycerol in the cells, a role admirably suited to the aquaglyceroporins. There are a number of interesting studies in rice stem borers (Izumi et al., 2006; Izumi et al., 2007) and gall flies (Philip et al., 2008) that point to the major role played by specific AQPs in these insects. Recently Kikawada and colleagues have begun an elegant study on the larvae of the sleeping chironomid, *Polypedilum vanderplanki* (Kikawada et al., 2008). They have functionally characterized two AQPs (*Pv*AQP1 and 2; Table 1) which play an important role in anhydrobiosis in this insect. These studies, however, lie beyond the scope of this paper.

The three areas relating to excretory water transport where the requisite combination of genetic and physiological studies have been performed are in the homopteran filter chamber, the tracheolar cells of the mosquito and of course the Malpighian tubules, primarily those of the fruit fly.

One of the first insect AQPs to be isolated and functionally characterized was AQP*cic*, isolated from the filter chamber of the homopteran *Cicadella viridis*. These homopterans are xylem feeders and so ingest large volumes of very nutrient-poor fluid. To cope with the fluid volume, the homopterans have a filter chamber. In essence the posterior midgut and ileum, with attached Malpighian tubules, have recurved to lie against the anterior midgut. The close association of the anterior midgut with the Malpighian tubules permits the shunting of water and ions directly to the excretory system thereby concentrating the nutrients 2.5- to 10-fold in the absorptive portion of the midgut (Hubert et al., 1989). The role of the midgut is passive: the osmotic driving force is provided by the Malpighian tubules.

To accomplish this extremely rapid flow of dilute fluid, it was presumed that the filter chamber membrane must be very rich in AQPs. An AQP (originally P25, now AQP*cic*) was isolated from the filter chamber and immunofluorescence showed that it was only present in the filter chamber membrane (Beuron et al., 1995). Expression in *Xenopus* oocytes demonstrated that AQP*cic* had a higher water permeability than human AQP1 and was reversibly inhibited by Hg^{2+} (Le Caherec et al., 1996). The *Cicadella* AQP forms orthogonal arrays, similar to those formed by AQP1, when expressed in *Xenopus.* In the leafhopper, AQP*cic* is preferentially expressed on the apical membrane of the filter chamber where it can constitute as much as 90% of the intrinsic membrane protein (Le Ceherec et al., 1997). Orthogonal arrays have been observed on both membranes, suggesting that there is a second AQP present in the filter chamber, but this has not been experimentally confirmed to date. Immunofluorescence studies using antibodies to AQP*cic* have shown similar staining of the filter chamber in other xylem feeders, but not Aphids, which are phloem feeders. Phloem is a relatively much more rich substrate than xylem, reinforcing the idea that water shunting by the filter chamber is an adaptation to an extremely nutrient-poor diet (Le Caherec et al., 1997).

Blood-feeding insects face a similar osmotic challenge to the xylem feeders. The rapid ingestion of a blood meal greatly impairs their mobility and so disposing of the relatively useless plasma becomes paramount. For flying insects, such as female mosquitoes, the situation is most critical in that they have to be able to leave the host and find cover quickly, so post-prandial diuresis begins within seconds of the initiation of feeding.

At least four AQPs are apparent in the genome of the yellow fever mosquito, *Aedes aegypti*; however, only one has been functionally identified. It is located not in the Malpighian tubule cells themselves but rather in the end cells of the tracheoles associated with the tubules. When expressed in *Xenopus* oocytes, *Aea*AQP increases water permeability to an even greater degree than AQP*cic*, and is reversibly inhibited by $HgCl_2$. Direct comparison with *E. coli* aquaglyceroporin (GlpF) shows that *Aea*AQP is a strict AQP and freeze fracture studies of the oocyte suggest that most, if not all, of the expressed *Aea*AQP forms orthogonal arrays within the membrane (Duchesne et al., 2003).

As unexpected as it was to find this AQP in the tracheolar end cells, rather than the tubule cells themselves, Pietroantonio and colleagues suggested that it may serve an important metabolic role (Pietroantonio et al., 2000). Rapid fluid transport by tubule cells is metabolically expensive. Malpighian tubule cells are all

Table 1. Functionally expressed insect aquaporins

Aquaporin	Species	Tissue expression	Transport	Reference
BIB	*Drosophila melanogaster*	Epidermal precursor regions of larvae	Monovalent cations (K^+ selective)	Yanochko and Yool, 2002
*Pv*AQP1	*Polypedilum vanderplanki*	Ubiquitous	H_2O	Kikawada et al., 2008
*Pv*AQP2	*Polypedilum vanderplanki*	Fat body	H_2O	Kikawada et al., 2008
AQP*cic*	*Cicadella viridis*	Filter chamber	H_2O	Le Caherec et al., 1996
*Aea*AQP	*Aedes aegypti*	Malpighian tubule tracheolar cells	H_2O	Duchesne et al., 2003
*Rp*MIP	*Rhodnius prolixus*	Malpighian tubules	H_2O	Echeverria et al., 2001
DRIP	*Drosophila melanogaster*	Adult Malpighian tubule stellate cells	H_2O	Kaufmann et al., 2005

heavily tracheated and we have anecdotal evidence that even a few minutes of oxygen deprivation will irreversibly harm *in vitro* Malpighian tubule preparations (Spring and Hazelton, 1987). As far back as some of Wigglesworth's earlier studies (Wigglesworth, 1972), it has been known that insect respiration is regulated internally by hydraulic valving. The tracheoles of inactive tissues fill with fluid, presumably H_2O from the hemolymph, and so shunt air to active tissues. In the Pietroantonio model, the *Aea*AQP is asymmetrically localized on the apical membrane of the Malpighian tubule tracheolar cells. In this way, as soon as the tubules begin rapid water and ion transport in response to diuretic factors, H_2O will immediately be withdrawn from the tracheoles providing the oxygen required to sustain rapid fluid transport. In effect this becomes a self-regulating mechanism, by which oxygen delivery to the tubules is controlled directly by the rate of fluid transport (and therefore oxygen consumption) of the tubules.

Another widely recognized hematophagous insect, and the one favored by Maddrell himself, is the assassin bug, *Rhodnius prolixus*. Eschevarria and colleagues isolated a single water-transporting protein belonging to the major intrinsic protein family (*Rp*MIP) from this insect (Eschevarria et al., 2001). *Rp*MIP appears to be relatively uniformly distributed throughout the proximal and distal tubules. When expressed in *Xenopus* oocytes, *Rp*MIP exhibits a relatively low H_2O permeability, perhaps 30% of that of human AQP3 expressed in the same system. Furthermore, *Rp*MIP is not inhibited by $HgCl_2$, although whole tubule preparations do exhibit some mercury sensitivity. To further confuse the issue, *Rp*MIP mRNA expression is significantly enhanced 6 h post-feeding, an effect that can be partially mimicked by both serotonin and cAMP (Martini et al., 2004).

The secretion data make *Rp*MIP an unlikely candidate for the AQP that enables a *Rhodnius* Malpighian tubule to increase fluid secretion 1000-fold within seconds of being stimulated, and the time course for mRNA expression is far to slow to affect post-prandial diuresis. RNA expression seems even odder, given that *Rhodnius* normally feed only once per instar. Likewise, given that the distal tubule is secretory and the proximal tubule reabsorbs salts to produce the hypoosmotic urine, one would not expect a uniform distribution of an AQP. The obvious conclusion is that *Rp*MIP is more of a housekeeping protein, associated with the slow absorption of the blood meal, rather than one involved in the extremely rapid diuresis that is initiated by feeding. Nevertheless, this is the only AQP that has been found in this system to date, even though other members of the AQP family have been actively sought (A. Gutierriez, personal communication).

In more recent years, particularly in the hands of Dow's research group (see Dow and Davies, 2003), the fruit fly, *Drosophila melanogaster*, has become the standard for tubule research. The four Malpighian tubules have a secretory capacity that equals or exceeds that of *Rhodnius* and the preponderance of *Drosophila*-oriented genetic research makes it easier to address the fundamental questions of fluid transport. Although a number of putative AQPs have been isolated from *Drosophila*, only one, whimsically named *Drosophila* Integral Protein, or DRIP, has been functionally expressed. DRIP exhibits the characteristics of a pure AQP; proteoliposomes loaded with DRIP are water permeable, but impermeable to glycerol or urea. DRIP is also reversibly inhibited by $HgCl_2$. DRIP is expressed solely in the Malpighian tubule stellate cells in adult *Drosphila*. This corresponds to the location of the low-resistance Cl^- pathway (O'Donnell et al., 1998), and suggests that rapid H_2O transport in *Drosophila* may be localized primarily to these cells. Certainly, the spatial separation of primary cation transport and secondary anion and water transport would represent an elegant solution to the difficulty of moving the equivalent of one cell volume of fluid every 10 s across the metabolically active principal cell.

Several other putative AQPs have been identified and each localizes to a specific cell type and in some cases a cell type within a specific region of the tubule (e.g. AQP17864 is present only in the principal cells of the main tubule segment) (Kaufmann et al., 2005). With all of these AQPs present, assigning a specific role to DRIP becomes problematic. It will most likely require expression of the other AQPs to determine the specific function (at least one is a GLP; N. Kaufmann, personal communication), and then selectively knocking them out, possibly with antisense RNA. These experiments are very time consuming and doubtless lie some distance in the future.

Using antibody to DRIP, we have shown the presence of a DRIP-like protein in the Malpighian tubules of the house cricket, *Acheta domesticus* (Spring et al., 2007). The immunoreactivity is present in both the distal and mid-regions of the tubule. Cyclic-AMP, which mimics the effect of the corticotropin releasing factor-like diuretic peptide in *Acheta* (Spring and Kim, 1995), has little effect on the distribution or staining intensity of DRIP, and the apparent increase in staining is confined to internal membranes. Achetakin-2 (AK-2) is a member of the myokinin family of peptides and acts through the Ca^{2+}-mediated second-messenger pathway. AK-2 acts synergystically with cAMP, affecting the low-resistance Cl^- pathway in the mid-tubules, but inhibiting fluid transport by the distal regions. In response to AK-2, both the apical and basal membranes of the mid-tubule stained intensely for DRIP, suggesting that the presumed *Acheta* AQP is quickly inserted into the membranes to facilitate rapid H_2O transport by the tubule. Fluid transport by *Acheta* tubules can be reversibly inhibited with $HgCl_2$, although again this does not prove that it is the DRIP-like protein that is being inhibited (Spring et al., 2007).

Clearly we have barely scratched the surface in our understanding of the roles of AQPs in excretion in insects. The handful of AQPs that have been functionally characterized provide tantalizing clues to the mechanisms of the transmembrane movement of water, and at the same time shed little light on the cellular means for coping with a veritable torrent flooding through the cytoplasm on its way to the lumen. As far as we have come in the more than half a century since Ramsay first isolated single Malpighian tubules, it is clear that there are enormous gaps in our knowledge of the underlying mechanisms. We trust that the next half-century will be just as exciting.

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