The essential roles of metal ions in insect homeostasis and physiology

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Abstract

Metal ions play distinct roles in living organisms, including insects. Some, like sodium and potassium, are central players in osmoregulation and 'blood and guts' transport physiology, and have been implicated in cold adaptation. Calcium is a key player as a second messenger, and as a structural element. Other metals, particularly those with multiple redox states, can be cofactors in many metalloenzymes, but can contribute to toxic oxidative stress on the organism in excess. This short review selects some examples where classical knowledge has been supplemented with recent advances, in order to emphasise the importance of metals as essential nutrients for insect survival.

Metals and life

Every living organism must regulate its internal milieu, and metals are key players in the transport processes, and many enzymatic activities, that underpin life. Given the broad scope of the short review, only a few examples of recent progress will be selected; some of these will feature the best-studied epithelium (the Malpighian tubules) or the best-studied insect (*Drosophila melanogaster*), or both.

The critical role of the insect Malpighian tubules

A critical advantage of multicellular organisms is specialization. Different cells, organized into tissues, allow the separation of different compartments, each optimized for particular sets of functions.

In terms of homeostasis, an insect can be seen as a bag of haemolymph, surrounded by a relatively impermeable cuticle, and penetrated by a highly permeable alimentary canal, with distinct regions specialized for distinct transport roles. To a first approximation, homeostasis can be seen as the sum of the activities of four key epithelia, the salivary glands, midgut, Malpighian (renal) tubules, and hindgut (Fig. 1A). Although these tissues are too complex to be ascribed single roles, it is a convenient shorthand to consider the salivary glands as secretory, the midgut as absorptive, the tubules as excretory, and the hindgut as selectively reabsorptive [1]. As most insect guts process in a linear fashion from mouth to anus, it follows that the ultimate gatekeeper for what stays in, or leaves, the insect is the hindgut. The organization of the alimentary canal is well-covered in textbooks [2]. The Malpighian tubules are well-described by recent reviews [3-7]; their transport physiology is summarized below (Figs 1B-C).

The tubules achieve their key roles (of supporting ionic homeostasis and excreting waste solutes) by actively excreting the cation present in excess (usually potassium, but sometimes sodium in blood-feeding insects [8]). Rather than use a primary pump for the ion, tubules pump out protons across

their apical membranes [9], then use this gradient to drive alkali metal cation exchangers to lift the desired ion up its electrochemical gradient (Fig. 1B-C). Ion resupply is guaranteed by a collection of ATPases, channels and cotransporters on the basolateral surface. The resulting net charge transfer is balanced by a chloride flux, and the net movement of salt allows osmotic flux of water to produce a primary urine. Transcriptome analysis has suggested that this basic model can be extended to all *Drosophila* transporting epithelia [10], and to other species [11]. There is experimental evidence of ion gradients to support the model, for example by X-ray microanalysis in *Drosophila hydei* Malpighian tubules [12].

The tubule is under exquisite neuroendocrine control. Surprisingly, although much is known about the neuropeptides and biogenic amines that control fluid secretion in the Malpighian tubules [13], the salivary glands [14], or the alimentary canal [15], almost nothing is known about how different ions are regulated. The exception is that calcitonin-like peptides appear to have a natriuretic activity in Diptera [16] and locust [17].

Sodium and potassium

The two most abundant and dynamic ions in cells and biological fluids are sodium and potassium. Living cells maintain a critical internal balance, with high intracellular potassium and low intracellular sodium. The former sets the cell's resting potential, and the latter provides the ion gradient for a range of secondary active transports; this is widely described in text-books [18]. Dysregulation of the gradients of either of these two ions can result in paralysis or death, so these ions can be seen as essential nutrients with haemolymph levels that are tightly regulated, principally by the Malpighian tubules and hindgut. However, appropriate levels for haemolymph sodium and potassium vary with lifestyle and Order. Blood-feeding or predatory insects are seen as challenged by high dietary sodium levels, whereas plant feeders encounter relatively high dietary K^+. Whereas many species maintain sodium levels of 100-150 mM, and potassium levels of 10-20 mM, phytophagous lepidopteran larvae maintain a very high haemolymph K/Na ratio [19-21]. In *Manduca sexta*, photometric and electron-probe X-ray microanalysis both report haemolymph [Na] of around 15 mM, and [K] at 25 mM [21]. Such values would seem incompatible with our understanding of the operation of the nervous system; it has been hypothesised that in insects, the blood brain barrier or extra-axonal mucins produce a local environment that strongly buffered for Na^+ and K^+ [22-24].

Adaptation to extremes of heat or cold

The importance of ionic homeostasis in insects is clearly illustrated by chill coma. Most insect species have relatively tight thermal ranges; the upper thermal limit could be interpreted in terms of protein denaturation; but there is evidence that cold paralysis, or 'chill coma' may be caused by ionic dysregulation [25]. In cockroach, low temperatures induce a decrease in haemolymph [Na+] and increase in haemolymph [K+], concomitant with chill injury. Such a shift in [Na+]:[K+] ratio would be expected to induce paralysis. The extent of the ionic dysregulation impacts the time taken to re-establish homeostasis on return to normal temperature [26]. The decrease in [Na+]:[K+] ratio on chilling is associated with ion equilibration with the gut [26]. However, as all the key transporting epithelia (salivary gland, midgut, tubules and hindgut are topologically equivalent, chill coma could be caused by dysregulation of any, or all, these tissues. Indeed, there is evidence from *Drosophila* for the role of the Malpighian tubules in chill coma [27]. The capa neuropeptides promote diuresis in most insect species [13,28], and the CAPA receptor is expressed predominantly in the tubule [29]. Knockdown of CAPA receptor impact both desiccation and cold tolerance [27,30], suggesting that neuroendocrine control of the tubule may modulate survival under stress. Additionally (Fig. 2), cold-resistant *Drosophila* species showed enhanced ability to maintain ion homeostasis at low temperatures, compared to cold-sensitive species [31].

Marine, freshwater and terrestrial insects

Problems in ionic balance are particularly extreme in dilute or concentrated environments. Sea water is hyperosmotic to most insect haemolymph, and so the problem is to absorb water while keeping ions (notably sodium) out; no insect can spend their whole life-cycle in the open sea [32]. By contrast, freshwater insects have the opposite problem, and see ions as essential and scarce nutrients. Mosquito larvae have evolved a remarkable specialization to allow them to recover ions from very dilute freshwater; a set of anal papillae, which grow larger in more dilute water. These contain specialised ion transporting cells, analogous to chloride cells of fish [33,34]. Electrophysiological analysis confirms that papillae are the sites of active metal ion uptake [35,36], as well as excretion of the 'honorary metal' ion, ammonium [37].

Calcium

Calcium is a vital second messenger in all cells, and underpins operation of the nervous system and musculature. Although insects lack a calcareous skeleton, the eggshell contains relatively high levels of calcium, and so mated females are under particular stress to obtain sufficient calcium . At least one solution to this problem seems to be through storage excretion in the Malpighian tubules; spherical deposits ('Spherites') rich in calcium salts, are formed in both tubule and midgut cells [38], and are found in the tubule lumen [39]. The formation of the concretions depends on carbonic anhydrase [40], and on a secretory pathway Ca^{2+} ATPase, SPoCk [39,41]. Microarray comparison of anterior tubules (which form concretions, and can excrete calcium at prodigious rates: [42]) and posterior tubules (which do not), has produced a tentative model for the transport processes into specialized vacuoles that allows formation of the concretions [43]. These spherites appear to be similar to acidocalcisomes widely described in parasites [44]. Although calcium is a major spherite component, in termite, different subtypes of vesicle contain either calcium or zinc [45] (discussed below).

What function do these concretions serve? In *Drosophila*, the concretions are confined to the initial segment of the anterior tubule and appear to be stored, rather than excreted immediately. This suggests that they may form a pool that can be selectively reabsorbed by the lower tubule if required – for example, at times of higher calcium need, like egg production. Given that the vesicles contain calcium phosphates, it is interesting that there is a discrete alkaline phosphatase-rich region in the lower tubules of both *Drosophila* and other insects [46,47]; it is tempting to speculate that this enzymatic activity could assist in solubilizing concretions released from the initial segment at times of calcium demand.

Magnesium

Magnesium is an essential nutrient, which stabilises polyphosphates, for example ATP (which is strictly Mg.ATP), and polyphosphates involved in DNA and RNA synthesis. Magnesium is also used in over 300 enzymes. However, relatively little is known about magnesium homeostasis, but the renal tubules play a role. When dietary magnesium is increased, magnesium concentration in tubule stones increases [48], suggesting a protective role for stone deposition. The TRPM channel is protective against high Mg2+ levels, and knockdown in tubule causes symptoms similar to hypermagnesemia [49]. These results are consistent with classical studies in several species, suggesting that the tubules are key sites for magnesium excretion [50]Mitochondrial Mg levels are regulated by the carrier dMme1, an orthologue of the yeast mitochondrial magnesium carrier [51].

Zinc

Zinc is a critical element, forming active sites in a wide range of enzymes, notably metalloproteinases and peptidases, in carbonic anhydrase and superoxide dismutase, and of course the zinc fingers of many DNA-binding proteins [52]. Zinc transport and homeostasis has been well reviewed recently; [53,54]

Uptake in the *Drosophila* midgut depends on a basolateral zinc transporter, dZNT1; knockdown kills flies through zinc deficiency, whereas overexpression renders flies hypersensitive to dietary zinc [55-57]. Excretion in the tubules relies on Zip71B and ZnT35C, with reabsorption in the lower tubule mediated by dZnT1 [58]. Although calcium is the major metal in most kidney stones, it is common to find zinc enriched in the concretion, suggesting it might play a nucleating role [59,60]; consistent with this, knockdown of either Zip71B and ZnT35C reduces stone burden in *Drosophila* tubules [58].

Redox-active metals

Many metals are not simple charged balls, but can exist as ions of multiple valencies; for example, iron can shuttle between Fe^{2+} and Fe^{3+} states. This redox activity makes such elements vital prosthetic groups for many enzymes. However, such metals' ability to contribute to oxidative stress makes them poisonous in excess [61], requiring delicate control of their availability.

Iron

Iron is a vital nutrient, acting as cofactor in nearly a hundred enzymes, but one which is toxic in excess. Free iron levels are titrated by the ferritin storage complex, which can bind 4500 iron atoms per ferritin molecule [62]. Ferritin expression is conspicuous in the midgut, particularly in iron cells [62]; but flyatlas.org [29,63] confirms that ferritin is expressed at extremely high levels across all tissues studied, confirming a universal cellular need to buffer iron. Indeed, ferritin expression is essential for survival [64]. Inter-species comparisons showed that the ability to store iron varies between *Drosophila* species even when reared on the same diet; *D. erecta* accumulated 50% more, and *D. virilis* 30% less, iron than *D. melanogaster [65]*. A multi-element 'metallomic' approach was extended to the Tephritidae -major pests- and showed relatively consistent metal accumulation, despite 100 My of divergent evolution from the Drosophilidae [66].

Understanding of the uptake and regulation of iron, largely by the midgut, is likely to be helped by genetic studies in *Drosophila*, where it is possible to map a fairly complete pathway for iron absorption and buffering [67].

Given that high iron levels in diet can be expected to be challenging, haematophagous insects could expect to be particularly at risk. Indeed, it has been argued that haem toxicity has limited the evolution of blood-feeding, and insects show a broad spectrum of approaches to defend against dietary haem toxicity [68], from antioxidant enzymes to physical barriers.

Copper

Copper is also a redox-active metal, and so is also a valuable cofactor in dozens of copper enzymes. Copper can also be present in environmental excess: Bordeaux mixture ($CuSO_4$ + slaked lime) has been used as an agricultural fungicide for over a hundred years; studies have identified 'cuprophilic cells' in the *Drosophila* midgut [69,70]; and dysregulation of copper levels is damaging to the nervous system [71]. The relative ease with which candidate genes can be tested by reverse genetics in *Drosophila* has again allowed rapid progress in elucidating organismal copper homeostasis [72-76]. The copper-sensing metal-responsive transcription factor-1 (dMTF-1) is itself a copper enzyme, and mediates resistance to environmental copper, zinc and cadmium [74].

Molybdenum

Molybdenum is another example of a redox-active metal that is an essential nutrient because of its use as a prosthetic group in a range of oxidoreductases, from bacteria upwards. The molybdoenzymes are noteworthy because they have been identified by forward genetics in *Drosophila*, making an elegant epistatic series [77]. This element is formed into a prosthetic complex (MoCo) that is subsequently inserted into a range of oxidase enzymes, such as aldehyde oxidase [78] and xanthine oxidase [79]. The former catalyses a range of useful reactions (including the metabolism of acetaldehyde formed by metabolism of alcohol); and the latter is critical in purine metabolism. Most waste nitrogen in the body passes through hypoxanthine and xanthine to uric acid, and thence to allantoin or urea. Deficiencies in xanthine oxidase or its upstream synthetic enzymes lead to the formation of xanthine kidney stones and depletion of urate, quantified using metabolomics [80]; so genes implicated in molybdenum handling could be expected to show both a renal stone phenotype and a metabolomic signature.

Heavy metals

Many metals can be considered mainly as hazards. As in humans, for insects there is little good about lead, cadmium or uranium. It could be considered unusual in insect evolutionary history to encounter toxic levels of such elements; but this is now increasingly a problem for insects, particularly those with freshwater larval stages. There is a longstanding but fragmented literature on toxicology of metals in insects. Mining or industrial activities can produce remarkable levels of such elements in groundwater, and insects can be used as bioindicators for environmental pollution [81-85]. Caddis-fly larvae, with relatively slow growth over several years, are classic bioindicators, though it is now common to sample a 'basket' of insect species to provide a reliable index of water purity [82]. However, insects are relatively resistant to heavy metal toxicity [86] by mechanisms that are not well-understood; and it has been suggested that this makes them less suitable as biosentinel species [87].

Summary

It is thus clear that – as in all living organisms - metal ions are key to the successful operation of insects. We now know a lot about what they do, and which ones are more immediately essential to life. However, we still understand relatively little about how such levels are controlled to within particular limits, or how the actions of the key transporting epithelia are coordinated in order to produce a stable environment for life. However, the post-genomic technologies are now available to allow relatively rapid future progress in this exciting area.

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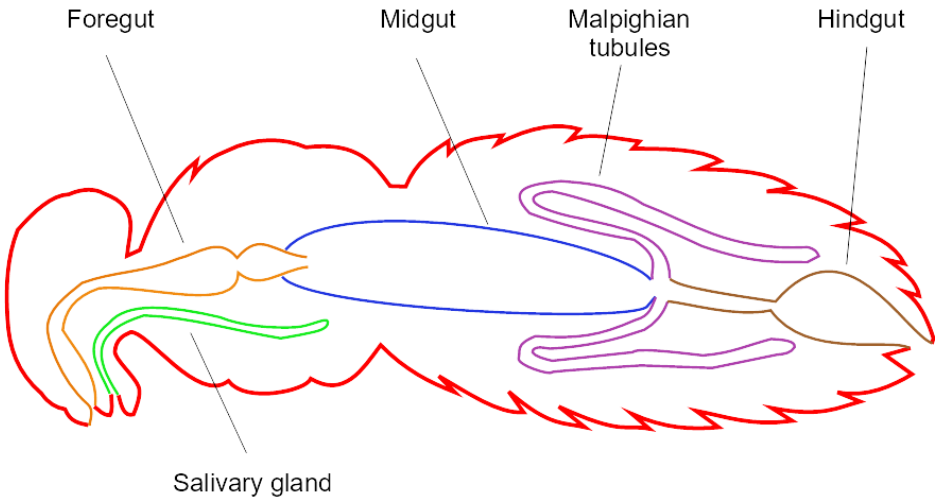
Figure legends

Fig. 1. Insect morphology and ions. (A) Insect schematic, showing the key transporting epithelia . (B) Minimal model for net epithelial transport of K^+ in insects. (C) Consensus model of transport genes strongly upregulated in salivary glands, midgut, hindgut and tubules. From [10].

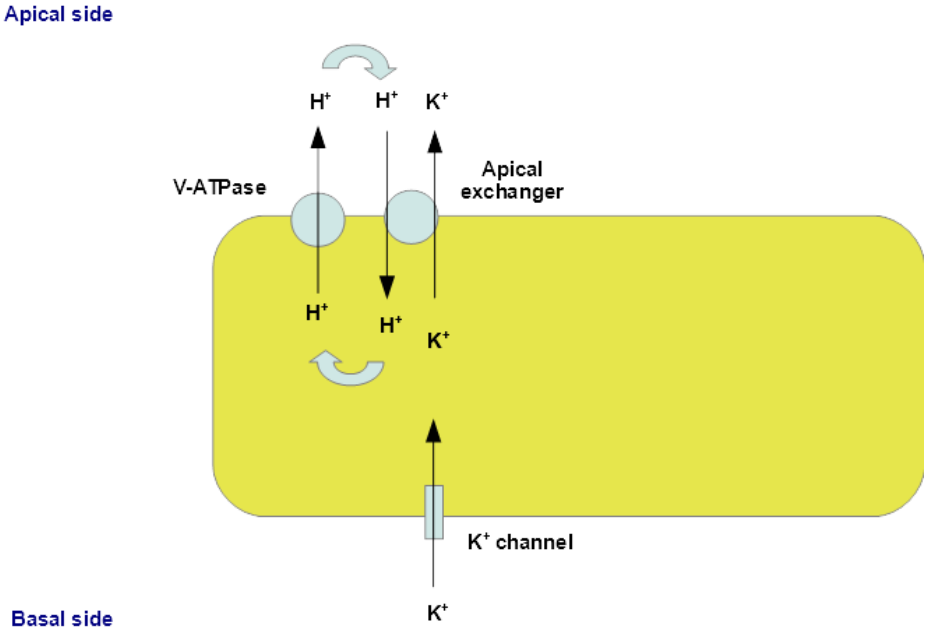
Fig. 2. Models for ion homeostasis in chill susceptible and chill tolerant insects. From [31].

Fig. 3. The molybdoenzyme cascade is conserved from bacteria to human. Black: enzyme name; red, human genetic disease; grey, gene names in bacteria; blue, gene names in *Drosophila*.

A


Foregut
Midgut
Malpighian tubules
Hindgut
Salivary gland

B


Apical side
H+
H+
K+
V-ATPase
Apical exchanger
H+
H+
K+
K+ channel
K+
Basal side

C

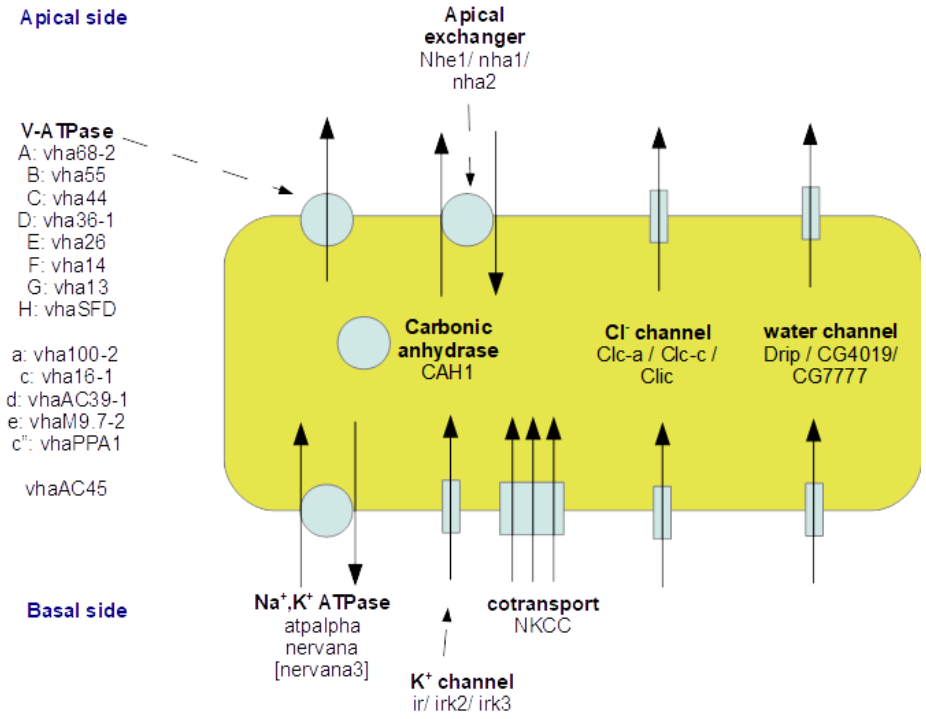

Apical side
Apical exchanger
Nhe1/ nha1/ nha2
V-ATPase
A: vha68-2
B: vha55
C: vha44
D: vha36-1
E: vha26
F: vha14
G: vha13
H: vhaSFD
a: vha100-2
c: vha16-1
d: vhaAC39-1
e: vhaM9.7-2
c'': vhaPPA1
vhaAC45
Carbonic anhydrase
CAH1
Cl- channel
Clc-a / Clc-c / Clic
water channel
Drip / CG4019/ CG7777
Basal side
Na+,K+ ATPase
atpalpha
nervana
[nervana3]
cotransport
NKCC
K+ channel
ir/ irk2/ irk3

Fig. 1.

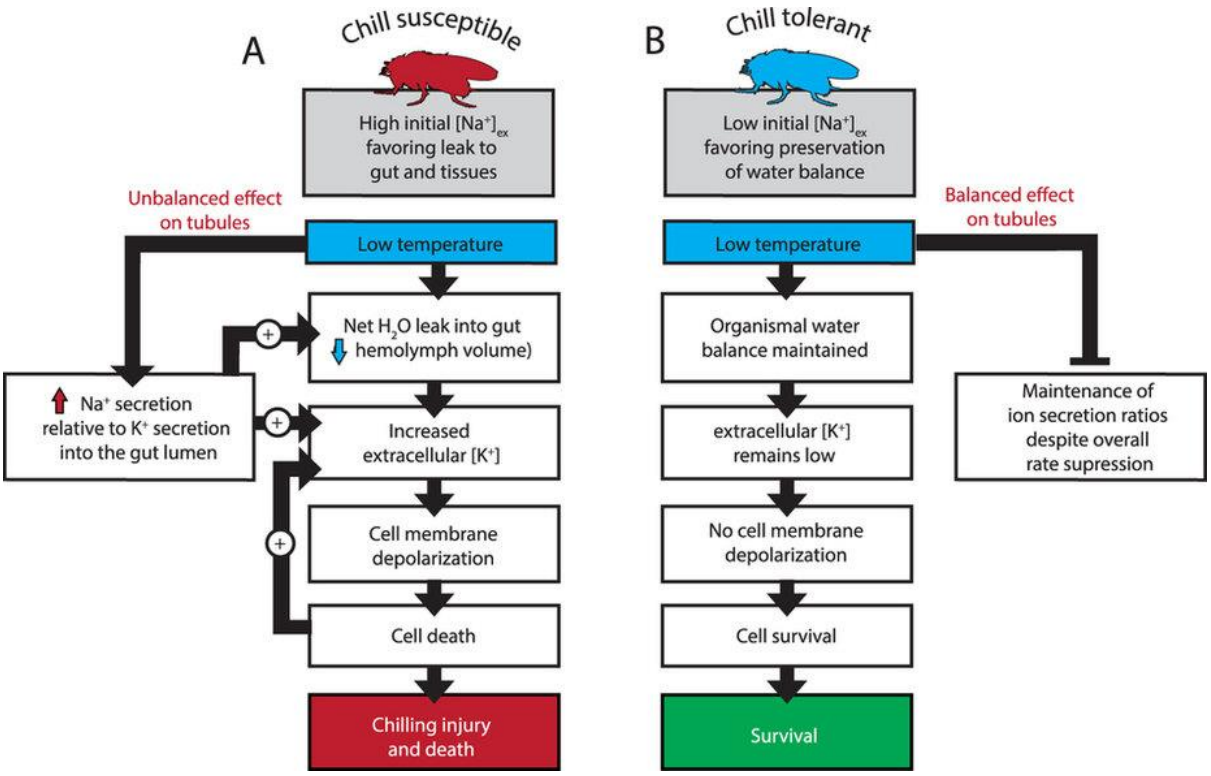

A
Chill susceptible
High initial [Na+]ex favoring leak to gut and tissues
Unbalanced effect on tubules
Low temperature
Net H2O leak into gut (hemolymph volume)
Na+ secretion relative to K+ secretion into the gut lumen
Increased extracellular [K+]
Cell membrane depolarization
Cell death
Chilling injury and death
B
Chill tolerant
Low initial [Na+]ex favoring preservation of water balance
Balanced effect on tubules
Low temperature
Organismal water balance maintained
extracellular [K+] remains low
No cell membrane depolarization
Cell survival
Survival
Maintenance of ion secretion ratios despite overall rate supression

Fig. 2.

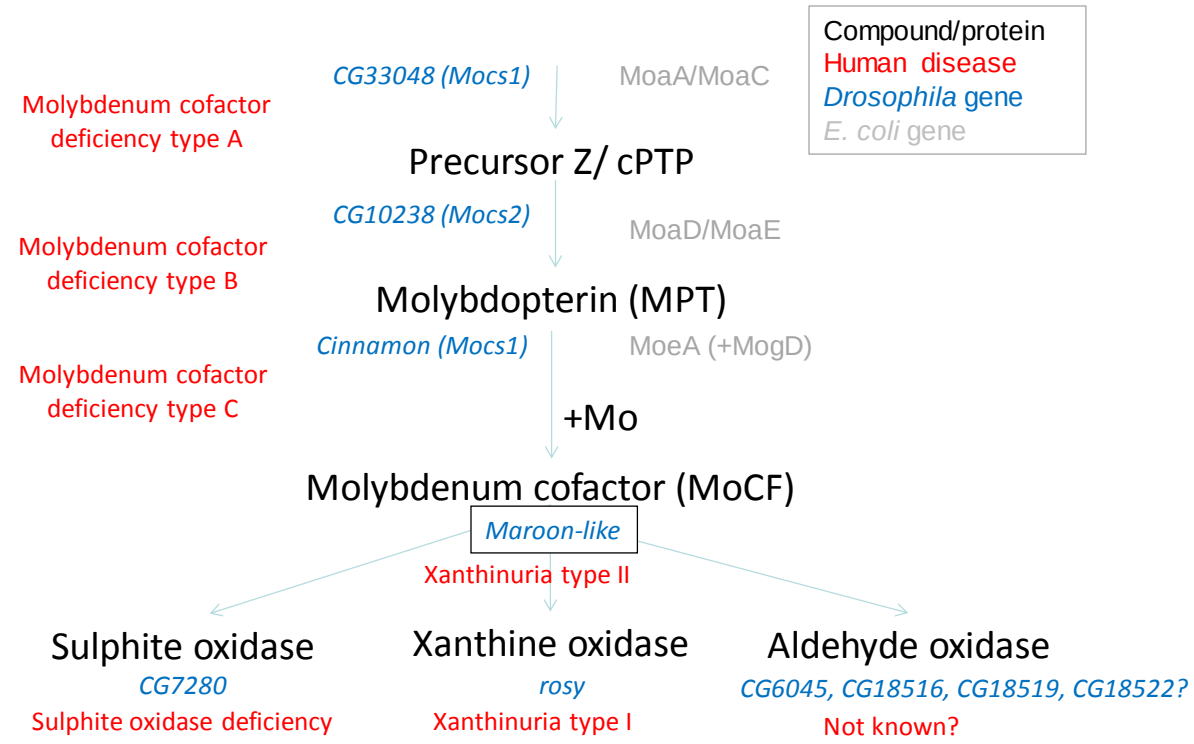

Compound/protein
Human disease
Drosophila gene
E. coli gene
CG33048 (Mocs1)
MoaA/MoaC
Molybdenum cofactor deficiency type A
Precursor Z/ cPTP
CG10238 (Mocs2)
MoaD/MoaE
Molybdenum cofactor deficiency type B
Molybdopterin (MPT)
Cinnamon (Mocs1)
MoeA (+MogD)
Molybdenum cofactor deficiency type C
+Mo
Molybdenum cofactor (MoCF)
Maroon-like
Xanthinuria type II
Sulphite oxidase
CG7280
Sulphite oxidase deficiency
Xanthine oxidase
rosy
Xanthinuria type I
Aldehyde oxidase
CG6045, CG18516, CG18519, CG18522?
Not known?

Fig. 3.