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Copper transport by lobster (*Homarus americanus*) hepatopancreatic mitochondria

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Summary

Mechanisms of copper transport into purified mitochondrial suspensions prepared from the hepatopancreas of the Atlantic lobster *Homarus americanus* were investigated. Mitochondria were purified by combining methods of differential and Percoll-gradient centrifugation, and copper transport was studied using the copper-sensitive fluorescent dye Phen Green. Copper transport by this mitochondrial preparation was kinetically the sum of saturable and non-saturable transfer components. Addition of 500 μmol l^{-1} Ca^{2+} or 500 nmol l^{-1} Ruthenium Red abolished the non-saturable copper transport component, significantly (*P*<0.01) reduced the apparent binding affinity of the saturable transport component, but was without effect (*P*>0.05) on the apparent maximal transport velocity of the saturable transfer process. The antiport inhibitor diltiazem (500 μmol l^{-1}) acted as a mixed inhibitor of the saturable transport mechanism, but had no effect on the non-saturable component of transfer. These results suggest that the non-saturable copper influx process was probably by way of the well-known Ruthenium-Red-sensitive Ca^{2+} uniporter and that the saturable transport component was probably due to a combination of both the Na^+-dependent, diltiazem-sensitive $1Ca^{2+}/2Na^+$ antiporter and the Na^+-independent, diltiazem-insensitive $1Ca^{2+}/2H^+$ antiporter. A model is discussed relating these mitochondrial copper uptake processes to the transfer of metal ions across the epithelial brush-border membrane.

Key words: copper, transport, hepatopancreas, mitochondria, brush border, lobster, *Homarus americanus*, Phen Green, fluorescent dye, Ruthenium Red, diltiazem.

Introduction

Mitochondria are multifunctional cellular organelles with both energetic and ion-sequestration functions. While considerable information exists about the ion-transporting functions of mammalian mitochondria (Carafoli, 1982; Gunter and Pfeiffer, 1990; Gunter and Gunter, 1994; Gunter et al., 1994), few comparative data are available for these organelles in invertebrate cells. This paucity of mitochondrial ion-transport information from invertebrates is particularly surprising because in some of these animals, such as the crustaceans, considerable ion fluxes occur through epithelial cells during the molt cycle and it is known from structural studies that epithelial mitochondria are a significant site of sequestration of divalent cations during this event.

In crustaceans, the role of mitochondria in Ca^{2+} regulation has been little studied since the early investigations of Chen and Lehninger (1973), Becker et al. (1974) and Chen et al. (1974). These studies investigated the effect of Ca^{2+} on the respiration of mitochondria isolated from crab hepatopancreas and found that the organelles were able to transport and store large amounts of Ca^{2+} and that the movements of the cation across mitochondrial membranes were influenced by inorganic phosphate, ATP, divalent cations and the uncoupling agent *p*-trifluoromethoxy cyanide phenylhydrazone (FCCP). In addition, ultrastructural studies identified intramitochondrial deposits of calcium phosphate associated with large movements of Ca^{2+} into the organelle.

Recently, the mechanisms of Ca^{2+} transport by lobster hepatopancreatic mitochondria were investigated (Klein and Ahearn, 1999). This study used $^{45}Ca^{2+}$ influx measurements to characterize both the uptake and efflux mechanisms for this divalent cation. The results suggested that, as with mammalian mitochondria (Gunter et al., 1994), the major uptake process was a Ruthenium-Red-sensitive uniporter, while efflux from the organelle was controlled by dual antiport processes: a Na^+-dependent, diltiazem-sensitive mechanism and a Na^+-independent, diltiazem-insensitive

process. Both influx and efflux processes were inhibited by cytoplasmic zinc.

Crustaceans often ingest considerable quantities of heavy metal ions from their diet and surroundings. At low concentrations, some of these metal ions, such as zinc, iron and copper, may serve important biological roles in enzyme activation or respiratory pigment synthesis, but at higher concentrations they become toxic and may impair nerve and muscle function. It is well known that invertebrates possess at least three epithelial detoxification mechanisms to regulate concentration of heavy metal ions in their bodies: (i) metallothioneins (Engel and Brouwer, 1989; Roesijadi, 1992); (ii) vacuolar concretion metal precipitates (George, 1983a,b; Viarengo et al., 1985, 1987; Vogt and Quinitio, 1994); and (iii) mitochondrial sequestration (Gunter and Pfeiffer, 1990; Gunter and Gunter, 1994; Saris and Niva, 1994; Viarengo, 1994). The specific membrane transport mechanisms responsible for isolating excess metals by way of vacuolar precipitates and mitochondrial sequestration are poorly understood.

In a more recent study, we investigated the carrier-mediated transport processes responsible for transferring copper into isolated lobster hepatopancreatic epithelial cells separated by centrifugal elutriation (Chavez-Crooker et al., 2001). This study did not address the fate of intracellular copper in any hepatopancreatic cell type following passage across the epithelial brush-border membrane. The present investigation extends our recent studies of hepatopancreatic epithelial copper uptake characterization and mitochondrial cation regulation by using the copper-specific fluorescent dye Phen Green to describe the mechanisms by which this heavy metal is accumulated within mitochondria as part of the detoxification processes present in this epithelium.

Materials and methods

Animals

Male Atlantic lobsters (*Homarus americanus* H. Milne Edwards, 500–700 g) were purchased from a local market and maintained in a seawater tank at 15 °C. All experiments were conducted with intermolt animals. Molt stage was estimated by determining the ratio between the mass of the stomach gastrolith (mg) and the carapace length (cm) (Aiken, 1973).

Mitochondrial purification

Purification of hepatopancreatic mitochondria followed the methods outlined by Klein and Ahearn (1999), as adopted from Chen and Lehninger (1973) with the addition of a Percoll gradient purification step for the final pellet (Symons and Jonas, 1987). A hepatopancreas was removed from an animal and minced into small pieces with a single-edged razor blade in ice-cold Buffer A (250 mmol l^{-1} sucrose plus 1 mmol l^{-1} EDTA) followed by homogenization (five strokes at 2800 revs min^{-1}) with a loose-fitting Teflon pestle in a volume of Buffer A that approximated the mass of the original tissue. The homogenate was diluted 10-fold in Buffer A and then distributed among 4–8 plastic centrifuge tubes followed by centrifugation (two 10-min low-speed spins at 750 ***g***) to remove dense nuclear and particulate material. Fat was removed from the top of the tubes, and the remaining supernatants were poured through two layers of cheesecloth. The filtered supernatants were centrifuged for 15 min at 9000 ***g*** to collect membranes and organelles. The resulting pellets were resuspended in 1 ml of ice-cold Buffer B (250 mmol l^{-1} sucrose, 25 mmol l^{-1} Hepes/Tris, pH 7.5), fully mixed using plastic serum pipettes, diluted to 30 ml in Buffer B, and centrifuged for 15 min at 7000 ***g***. The resulting pellets were resuspended in 1.0 ml of Buffer B using a plastic serum pipette, pooled, and distributed among centrifuge tubes containing 1.5 ml of iso-osmotic Percoll (9 parts Percoll to 1 part Buffer C; 2.5 mol l^{-1} sucrose plus 200 mmol l^{-1} Hepes, pH 7.5). Buffer B was added to the tubes to bring the final volume to 10 ml, and they were centrifuged for 60 min at 35 000 ***g***. Fractions (1 ml) of the resulting gradient were removed from the top of the tubes with a pipette and collected. Each band was cleaned of Percoll by dilution with ‘inside medium’ (composition varying between experiments) to 10 ml and centrifuged at 18 000 ***g*** for 15 min.

The mitochondrial fraction was collected from the Percoll gradient by pipetting off the top 8.5 ml and removing the dark brown band near the bottom of the tubes. The mitochondria were resuspended in appropriate ‘inside medium’ with 20 strokes of a 1 ml tuberculin syringe fitted with a 22-gauge needle and placed on ice until needed after dilution to a working concentration of 20–50 mg protein ml^{-1}. Protein was determined using the Bio-Rad protein assay with albumin standards in a Beckman DU-640 recording spectrophotometer.

Transport experiments

Transport assays were conducted by adding 1 part of mitochondria in ‘inside buffer’ to 8 parts of ‘outside buffer’. Outside buffers were composed of mannitol, sucrose, potassium gluconate, trimethylammonium (TMA^+) gluconate and Hepes/Tris adjusted to the appropriate pH (Klein and Ahearn, 1999). The copper-specific, fluorescent probe Phen Green and its acetoxymethyl ester (Molecular Probes, Inc.) were used in this study as described previously for whole-cell suspensions of the lobster hepatopancreas (Chavez-Crooker et al., 2001). Phen Green (at 1 μmol l^{-1}) fluorescence (excitation 490 nm; emission 520 nm) was measured at room temperature (24 °C) using hand-stirred samples in a Shimadzu spectrofluorometer (model FR-1501) connected to a DPU-411, Type II, thermal printer. Previous experiments with Phen Green and hepatopancreatic cell suspensions indicated that copper transport could be measured in dye-equilibrated cells following a quantitative decrease in fluorescence (e.g. signal quenching) as external metal ion concentration was increased (Chavez-Crooker et al., 2001). The resulting fluorescence quenching was measured as ΔF mg^{-1} protein 120 s^{-1} and was quantitatively related to the transport of copper across cell plasma membranes.

In the present study, copper transport experiments were started by equilibrating mitochondria with Phen Green (60 min at 24 °C in an orbital shaker at 100 revs min^{-1}). The preparation

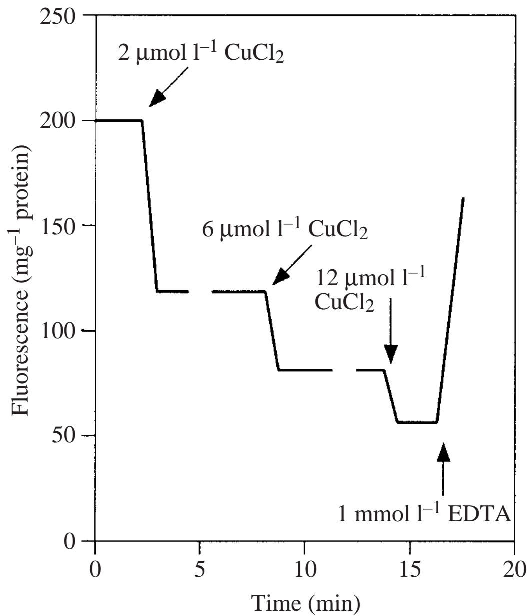

Fig. 1. Time course of Phen Green ($1\,\mu mol\,l^{-1}$) fluorescence quenching induced by addition of copper chloride at three different concentrations to buffer containing dye-equilibrated hepatopancreatic mitochondria. Arrows show the points where copper chloride was added to a stable fluorescence signal produced in the absence of the metal. This graph is representative of multiple similar recordings under experimental conditions reported in this study. Copper influx was estimated by the initial rate of fluorescence quenching observed over the first 120 s of incubation following addition of the metal. The excitation wavelength was 490 nm and the emission of the dye was recorded at 520 nm. Addition of $1\,mmol\,l^{-1}$ EDTA restored the fluorescent signal by complexing Cu^{2+} in solution.

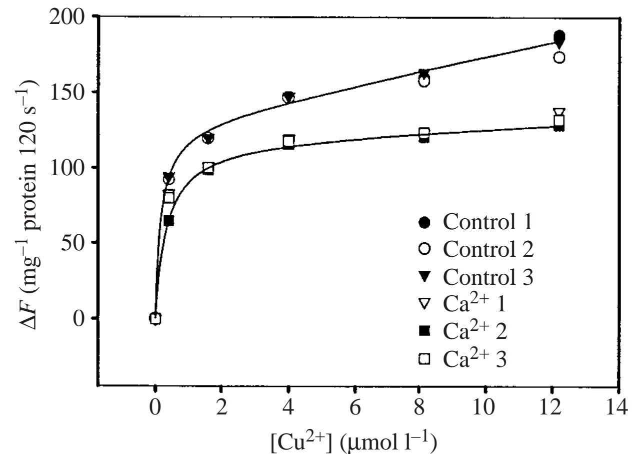

Fig. 2. Effect of $500\,\mu mol\,l^{-1}$ calcium chloride on the kinetics of Cu^{2+} influx (measured as $\Delta F\,mg^{-1}$ protein $120\,s^{-1}$, where ΔF is fluorescence quenching) in purified lobster hepatopancreatic mitochondrial suspensions. Concentrations of Cu^{2+} used in the experiment were 0.4, 1.6, 4.1, 8.2 and $12.2\,\mu mol\,l^{-1}$. Previous studies have shown that Ca^{2+} alone has no effect on the quenching phenomenon (Chavez-Crooker et al., 2001). Best-fit lines were drawn using SigmaPlot software, and the resulting kinetic constants are presented in the text. Individual triplicate values are displayed on the figure.

was then centrifuged for 10 min (at 9000 ***g***), and the resulting pellet was re-suspended in buffer and maintained on ice for further analysis. During an experiment, a sample of mitochondria was injected into a cuvette, housed in the spectrofluorometer, containing a physiological buffer without copper or another metal ion so that maximal fluorescence would be obtained. Once the dye was stable (at 100 % fluorescence), copper ions were added at different concentrations and the increase in fluorescence quenching (as $\Delta F\,mg^{-1}$ protein $120\,s^{-1}$) was monitored as an indication of metal transport across mitochondrial membranes.

Curve-fitting and the production of the resulting copper influx kinetic constants were performed using Jandel SigmaPlot 4.01 or 5.0 software. All values reported in this investigation were obtained from triplicate samples, and results are expressed as means ± 1 S.E.M.

Results

Time course of fluorescence quenching

Fig. 1 is a tracing of the time course of change in Phen Green fluorescence that occurred as a result of quantitative quenching of the signal following the addition of three copper concentrations (2, 6 and $12\,\mu mol\,l^{-1}$) to a spectrofluorometric cuvette containing dye-equilibrated hepatopancreatic mitochondria. The magnitude of quenching was closely correlated with the concentration of copper added to the external buffer, as found previously with isolated hepatopancreatic epithelial cells (Chavez-Crooker et al., 2001), and the rate of initial change in fluorescence over 120 s was used as a quantitative index of transmembrane copper transport. Addition of $1\,mmol\,l^{-1}$ EDTA, a copper chelator, restored the fluorescence signal, suggesting that complexes form between the chelator and copper in solution.

Effect of external Ca^{2+} on the kinetics of copper influx into hepatopancreatic mitochondria

Previous studies with isolated mitochondria from lobster hepatopancreas indicated that Ca^{2+} was transported into these organelles by a combination of a Ruthenium-Red-inhibited uniporter and a diltiazem-sensitive antiporter (Klein and Ahearn, 1999). To determine whether copper was transported by mitochondrial Ca^{2+} carrier proteins, a control experiment was first conducted measuring copper influx kinetics over periods of 120 s at a series of external copper concentrations (0.4, 1.6, 4.08, 8.15 and $12.2\,\mu mol\,l^{-1}$) (Fig. 2). As indicated by the results in this figure, Cu^{2+} appeared to be translocated to the mitochondrial interior by way of a combination of saturable and non-saturable transport components and the rate of transport followed the equation:

$$J_{Cu} = \{(J_{max}[Cu^{2+}]/K_{Cu}) + [Cu^{2+}]\} + K_D[Cu^{2+}]\,,$$

where J_{Cu} is total copper influx, J_{max} is apparent maximal

carrier-mediated transport, K_{Cu} is an apparent binding affinity constant, $[Cu^{2+}]$ is the external concentration of Cu^{2+} and K_D is the slope of the non-saturable influx component and may represent either diffusion or carrier-mediated transport by a low-affinity process that did not saturate within the range of concentrations selected. A best-fit curve was drawn through the data generated using Jandel SigmaPlot software. The kinetic constants for copper influx by the combination of these processes are as follows: K_{Cu}=0.17±0.04 μmol l^{-1}, J_{max}=129.8±5.9 ΔF mg^{-1} protein 120 s^{-1} and K_D=4.61±0.62 ΔF mg^{-1} protein 120 s^{-1} μmol^{-1} l Cu (N=3).

Addition of 500 μmol l^{-1} calcium chloride to the external medium at each copper concentration resulted in a significant decrease (P<0.01) in the rate of copper influx over the entire copper concentration range (Fig. 2). A best-fit curve is drawn through the experimental data in this figure, and the apparent kinetic constants for copper influx under these conditions are as follows: K_{Cu}=0.35±0.03 μmol l^{-1}, J_{max}=120.0±3.15 ΔF mg^{-1} protein 120 s^{-1} and K_D=0.95±0.31 ΔF mg^{-1} protein 120 s^{-1} μmol^{-1} l Cu (N=3). A comparison of the kinetic constants of the control and +500 μmol l^{-1} Ca^{2+} conditions suggests that addition of Ca^{2+} doubled (P<0.01) the apparent K_{Cu} (reduced the apparent copper binding affinity) and significantly (P<0.01) reduced non-saturable copper transport velocity by 80 %, without having a significant (P>0.05) effect on the apparent maximal carrier-mediated copper transport velocity (J_{max}). An increase in the apparent copper binding affinity as seen in the present set of experiments suggests that Ca^{2+} acted as a competitive inhibitor of carrier-mediated copper influx. The significant reduction of the non-saturable copper transport component in the presence of Ca^{2+} strongly supports the view that this transfer process was carrier-mediated, exhibiting a relatively low apparent copper binding affinity.

Effects of Ruthenium Red on copper influx kinetics into hepatopancreatic mitochondria

In a previous study of Ca^{2+} transport by lobster hepatopancreatic mitochondria, Ruthenium Red was shown strongly to inhibit the influx of this divalent cation through a well-described uniporter carrier mechanism (Klein and Ahearn, 1999). A Dixon plot of the interaction between Ruthenium Red and Ca^{2+} transport indicated that the dye was a strong competitive inhibitor of mitochondrial Ca^{2+} influx as a result of both dye and cation sharing a common binding site on the organellar membranes. Because Fig. 2 suggested that copper and Ca^{2+} may be transported into lobster hepatopancreatic mitochondria by two different carrier processes, it was important to establish whether one or both of these transport mechanisms were also sensitive to Ruthenium Red.

Before determining the effect of Ruthenium Red on copper influx into isolated mitochondria from lobster hepatopancreas, the possible quenching effect of Ruthenium Red on Phen Green fluorescence had to be quantified so that an appropriate concentration of the inhibitor could be determined that had a minimal effect on signal reduction from the fluorescent dye.

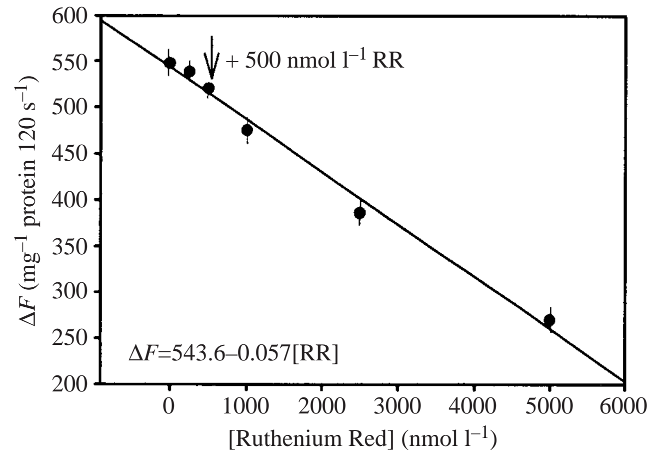

Fig. 3. Effect of Ruthenium Red on 1 μmol l^{-1} Phen Green fluorescence quenching. The activated form (which does not have to enter mitochondria and be activated by enzymes) of the dye was used in buffer containing the following concentrations of Ruthenium Red (RR): 0, 250, 500, 1000, 2500 and 5000 nmol l^{-1}. Fluorescence quenching (ΔF) was followed over 120 s after the addition of Ruthenium Red. Changes in initial fluorescence are presented in figure. Values are means ± S.E.M., N=3. From this figure, only 5 % of the initial signal was lost at a Ruthenium Red concentration of 500 nmol l^{-1}, but 50 % was abolished when the inhibitor concentration was raised to 5000 nmol l^{-1}. The regression equation is ΔF=543.6–0.057[RR], r^2=0.99, P=0.001.

The data displayed in Fig. 3 indicate that, as Ruthenium Red concentration was increased from 250 to 5000 nmol l^{-1} in a spectrofluorometer cuvette containing 1 μmol l^{-1} Phen Green in transport buffer, the fluorescent signal was reduced in a linear fashion until, at the highest Ruthenium Red concentration used, approximately 50 % of the fluorescent signal was abolished. At a Ruthenium Red concentration of 500 nmol l^{-1}, only 5 % of the fluorescent signal was quenched, and this concentration of inhibitor was therefore used for subsequent experiments with copper. This concentration of Ruthenium Red was considered physiologically effective since the K_i for Ruthenium Red inhibition of Ca^{2+} influx by lobster hepatopancreatic mitochondria was found to be 1000 nmol l^{-1} (Klein and Ahearn, 1999).

Fig. 4 displays the results of an experiment examining the effects of 500 nmol l^{-1} Ruthenium Red on copper influx kinetics into isolated lobster hepatopancreatic mitochondria. As reported above, copper influx was biphasic under control conditions and appeared to be the sum of saturable and non-saturable processes over the range of copper concentrations selected. The kinetic constants for copper influx under control conditions were: K_{Cu}=0.63±0.15 μmol l^{-1}; J_{max}= 88.17±7.71 ΔF mg^{-1} protein 120 s^{-1} and K_D=3.05±0.67 ΔF mg^{-1} protein 120 s^{-1} μmol^{-1} l Cu (N=3).

In the presence of 500 nmol l^{-1} Ruthenium Red, the control biphasic influx curve was reduced to a single Michaelis–Menten hyperbolic relationship between the variables. A best-fit analysis of copper influx under these conditions resulted in the

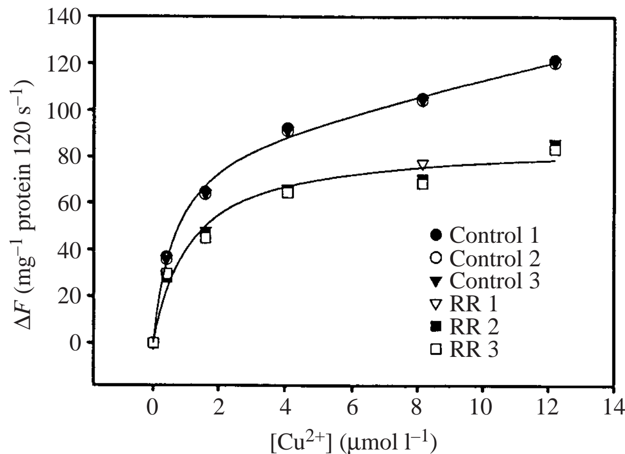

Fig. 4. Effect of 500 nmol l^{-1} Ruthenium Red (RR) on the kinetics of Cu^{2+} influx into purified mitochondrial suspensions from lobster hepatopancreas. Data are presented as in Fig. 2. Best-fit lines were drawn using SigmaPlot software, and the resulting kinetic constants are presented in the text. Individual triplicate values are displayed on the figure.

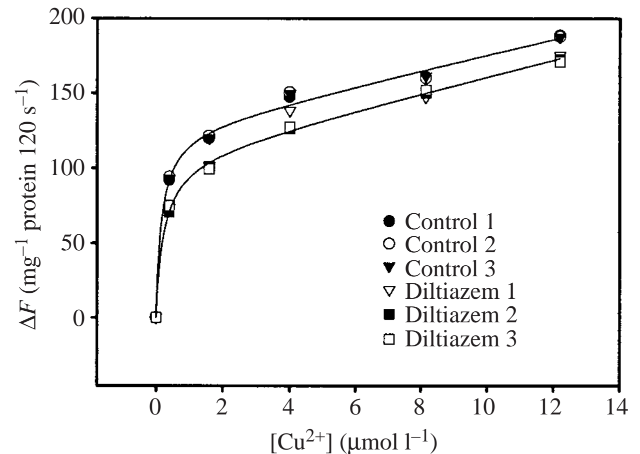

Fig. 5. Effect of 500 μmol l^{-1} diltiazem on the kinetics of Cu^{2+} influx into purified mitochondrial suspensions from lobster hepatopancreas. Data are presented as in Fig. 2. Best-fit lines were drawn using SigmaPlot software, and the resulting kinetic constants are presented in the text. Individual triplicate values are displayed on the figure.

following kinetic constants: K_{Cu}=1.05±0.34 μmol l^{-1} and J_{max}=82.94±6 ΔF mg^{-1} protein 120 s^{-1} (N=3). These results suggest that, in the presence of Ruthenium Red, the low-affinity, non-saturable copper influx component was abolished. In addition, the presence of Ruthenium Red resulted in an approximate doubling of the apparent affinity constant (P<0.01) of the saturable transport component, but had no significant effect (P>0.05) on apparent maximal copper influx by this transporter. These results are compatible with the view that Ruthenium Red acted on both apparent carrier-mediated transport processes responsible for copper uptake into lobster hepatopancreatic mitochondria.

Effects of diltiazem on copper influx kinetics into lobster hepatopancreatic mitochondria

Our previous experiments with hepatopancreatic mitochondria suggested that Ca^{2+} efflux from these organelles might occur through a diltiazem-sensitive, electroneutral $1Ca^{2+}/2Na^{+}$ antiporter (Klein and Ahearn, 1999). To assess whether copper was also transported by this cation exchanger, an experiment was conducted using Phen-Green-equilibrated mitochondria in the presence and absence of 500 μmol l^{-1} diltiazem and external concentrations of copper from 0.4 to 12.2 μmol l^{-1}, as previously employed in this study. As displayed in Fig. 5, the kinetic constants for copper influx under control conditions (lacking diltiazem) were K_{Cu}= 0.16±0.04 μmol l^{-1}, J_{max}=126.6±7.07 ΔF mg^{-1} protein 120 s^{-1} and K_D=5.04±0.75 ΔF mg^{-1} protein 120 s^{-1} μmol^{-1} l Cu (N=3). In the presence of 500 μmol l^{-1} diltiazem, the kinetic constants for copper influx were K_{Cu}=0.23±0.02 μmol l^{-1}, J_{max}=108.50±3.08 ΔF mg^{-1} protein 120 s^{-1} and K_D=5.44± 0.31 ΔF mg^{-1} protein 120 s^{-1} μmol^{-1} l Cu (N=3). Addition of diltiazem resulted in a significant (P<0.05) reduction in apparent copper binding affinity and a significant (P<0.05) decrease in apparent maximal transport velocity compared with the control condition, but had no significant (P>0.05) effect on the apparent non-saturable transport process. These results suggest that diltiazem acted as a mixed inhibitor of copper influx into hepatopancreatic mitochondria by the saturable transport system.

Discussion

Ca^{2+} uptake mechanisms of the crustacean hepatopancreas

The crustacean hepatopancreas is a multifunctional organ responsible for digestion and absorption of dietary nutrients, storage of glycogen and fat, ion transport and osmoregulation, synthesis and release of the oxygen-binding pigment hemocyanin and sequestration and detoxification of heavy metals. Among its many functions, this remarkable organ controls the uptake of divalent cations such as Ca^{2+} and Cu^{2+} from the gastrointestinal tract and stores them within organelles of the hepatopancreatic epithelium, incorporates them into biosynthetic pathways such as that for hemocyanin synthesis or transfers them through the cell layer to the hemolymph, where they may be absorbed by other cell types throughout the organism.

Recently, we investigated the mechanisms of uptake and efflux of Ca^{2+} by lobster hepatopancreatic epithelium using purified brush-border and basolateral membrane vesicles (Ahearn and Zhuang, 1996; Zhuang and Ahearn, 1996, 1998). These studies described three brush-border membrane proteins involved in Ca^{2+} uptake from dietary elements: (i) an amiloride-sensitive, electrogenic $1Ca^{2+}/1H^{+}$ antiporter that was shared with Na^{+}; (ii) an amiloride-insensitive, electroneutral $1Ca^{2+}/2Na^{+}$ antiporter; and (iii) a verapamil-inhibited Ca^{2+} channel. On the basolateral membrane of this epithelium, three additional Ca^{2+} transport proteins were also described: (i) a high-affinity, ATP-dependent Ca^{2+} pump; (ii)

a low-affinity, electrogenic $1Ca^{2+}/3Na^{+}$ antiporter; and (iii) a verapamil-sensitive Ca^{2+} channel. Other authors have also reported the presence and characteristics of these Ca^{2+} transport systems in epithelia from freshwater crayfish (Wheatly, 1996, 1999; Wheatly et al., 1998, 1999), marine crabs (Flik et al., 1994) and the European lobster *Homarus gammarus* (Flik and Haond, 2000). It has been proposed that these six membrane proteins are responsible for most, if not all, Ca^{2+} movements into and through crustacean epithelia during feeding, excretion and ion regulation under conditions that the animals may encounter during their daily activities and during the different molt stages that they undergo periodically.

Heavy metal uptake mechanisms of the crustacean hepatopancreas

Heavy metals, such as zinc and cadmium, are known to share one or more of these Ca^{2+} transport proteins in epithelia from crustaceans and in related transporters in echinoderm epithelia (Ahearn et al., 1994; Zhuang et al., 1995). In addition, a recent study, using the copper-sensitive dye Phen Green and centrifugal elutriation to separate individual hepatopancreatic cell types cleanly into purified suspensions, showed that copper uptake into each of the four hepatopancreatic epithelial cell types occurred *via* a brush-border cation antiport process that was shared by Zn^{2+} and exchanged with intracellular Ca^{2+} or other cations (Chavez-Crooker et al., 2001). In this latter study, a model was suggested for the transapical uptake of heavy metals from dietary elements using one or both of the previously described Ca^{2+} antiporters shown to occur in this membrane. In a more recent study using Phen Green to investigate copper influx into individual lobster hepatopancreatic epithelial cells, amiloride was employed as a potential inhibitor of copper uptake by the amiloride-sensitive electrogenic $1Ca^{2+}/1H^{+}$ antiporter (P. Chavez-Crooker, N. Garrido and G. A. Ahearn, unpublished observations). No effect of this inhibitor was observed during copper influx into each of the four hepatopancreatic epithelial types, suggesting that the entry mechanism for this metal was probably *via* the brush-border electroneutral, amiloride-insensitive $1Ca^{2+}/2Na^{+}$ antiporter. None of these studies provided evidence for the intracellular fate of metals or Ca^{2+} after passage across the respective epithelial brush-border membranes.

Ca^{2+} and metal transport mechanisms of vertebrate mitochondria

One fate of Ca^{2+} and metal ions transported by hepatopancreatic epithelial cells might be the sequestration of these divalent cations in organelles such as the mitochondria, lysosomes or endoplasmic reticulum. In vertebrates, Ca^{2+} and a variety of other cations are known to be transported across mitochondrial membranes by several different proteins. The most thoroughly investigated process is the electrogenic, Ruthenium-Red-inhibited uniporter that transfers Ca^{2+} from the cytosol to the mitochondrial interior (Carafoli, 1982; Bronner, 1992; Gunter et al., 1994). Several studies have indicated that this uptake process also supports the transport of Zn^{2+} (Saris and Niva, 1994), Mn^{2+} (Pushkin et al., 1976; Baker and Schlaepfer, 1978), Sr^{2+} (Carafoli, 1965), Ba^{2+} (Akerman et al., 1977), Fe^{2+} (Romslo and Flatmark, 1973) and La^{3+} (Reed and Bygrave, 1974). Ruthenium Red is a non-competitive inhibitor of the vertebrate Ca^{2+} uniporter protein and, while reducing the rate of cation transfer across the membrane, does not interfere with the Ca^{2+} binding process (Reed and Bygrave, 1974). Several hypotheses have been proposed for the presence of a Ca^{2+} uniporter uptake process that transports divalent cations from the cytoplasm to sequestration sites within the organelle (Gunter et al., 1994). The suggested functions of this carrier include (i) regulation of cytosolic cation concentrations, (ii) the generation of a cation sink in times of excess cytoplasmic concentrations of substances that might otherwise be toxic, (iii) service as a reusable source of activator Ca^{2+} or essential metals needed for enzymatic function and (iv) regulation of mitochondrial free Ca^{2+} concentration to control the activation of Ca^{2+}-dependent dehydrogenases.

At least two characterized antiporter proteins occur on vertebrate mitochondrial membranes that control the rate of Ca^{2+} efflux from the organelle (and potentially that of other sequestered cations as well). One antiporter is Na^{+}-dependent and diltiazem-inhibited (Vaghy et al., 1982), while the other is Na^{+}-independent and not affected by diltiazem (Gunter and Pfeiffer, 1990). The diltiazem-sensitive exchanger displays a stoichiometry of $1Ca^{2+}/2Na^{+}$ and is therefore electroneutral. The Na^{+}-independent Ca^{2+} efflux antiporter also is electroneutral and exhibits a stoichiometry of $1Ca^{2+}/2H^{+}$. The driving force for these two electroneutral exchangers appears to be the transmembrane Ca^{2+} activity difference between the mitochondrial matrix and the cytoplasm, which is believed to be a factor of approximately 2 (Gunter and Pfeiffer, 1990; Gunter et al., 1994). Both efflux mechanisms display some specificity for other divalent cations. The $1Ca^{2+}/2Na^{+}$ antiporters can substitute Sr^{2+}, but not Mn^{2+}, for Ca^{2+} (Crompton et al., 1976; Gavin et al., 1990), while the $1Ca^{2+}/2H^{+}$ exchangers are able to transport Sr^{2+} (Gunter et al., 1988), Ba^{2+} (Lukacs and Fonyo, 1985) and Mn^{2+} (Gavin et al., 1990). While both antiporters exist in mitochondria from a wide variety of vertebrate tissues, the Na^{+}-dependent exchanger predominates in heart, skeletal muscle, brain and other tissues, while the Na^{+}-independent transporter predominates in liver and kidney (Wingrove and Gunter, 1986a,b).

Ca^{2+} and copper transport mechanisms in crustacean hepatopancreatic mitochondria

Recently, the Ca^{2+} transport mechanisms of lobster hepatopancreatic mitochondria have been characterized (Klein and Ahearn, 1999). This study showed that, as with vertebrate mitochondria, the crustacean organelles displayed a combination of a Ruthenium-Red-sensitive uniporter for Ca^{2+} uptake from the cytoplasm and two Ca^{2+} efflux mechanisms: a diltiazem-inhibited, electroneutral $1Ca^{2+}/2Na^{+}$ antiporter, and a diltiazem-insensitive, electroneutral $1Ca^{2+}/2H^{+}$ exchanger. Zinc appeared to interact with both uptake and efflux pathways.

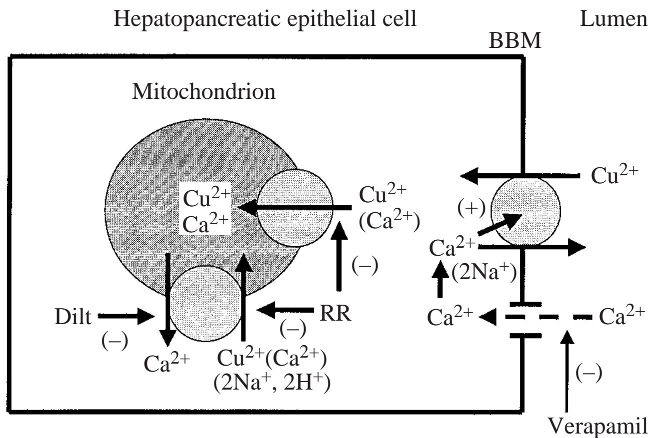

Fig. 6. Working model of Cu^{2+} uptake by lobster hepatopancreatic epithelial cells and sequestration within hepatopancreatic mitochondria by transport mechanisms described in the present investigation and reported previously (Klein and Ahearn, 1999; Chavez-Crooker et al., 2001). Cu^{2+} has previously been shown to be transported across epithelial brush-border membranes (BBMs) by an antiport process that exchanges external Cu^{2+} with intracellular cations such as Na^+ on an electroneutral, amiloride-insensitive $1Cu^{2+}/2Na^+$ antiporter. Ca^{2+} derived from the luminal medium may enter the cells through a verapamil-sensitive channel and either allosterically activate the exchanger or serve as a transport substrate. Intracellular Ca^{2+} and Cu^{2+} may enter hepatopancreatic mitochondria *via* three possible pathways: (i) an electrogenic, Ruthenium-Red-sensitive uniporter, (ii) a diltiazem-sensitive, electroneutral $1Ca^{2+}(1Cu^{2+})/2Na^+$ exchanger and a diltiazem-insensitive, electroneutral $1Ca^{2+}(1Cu^{2+})/2H^+$ exchanger. Ruthenium Red (RR) appears to interact with all three transport processes. Dilt, diltiazem.

In the present investigation, the mechanisms of copper uptake by lobster hepatopancreatic mitochondria were studied in an effort to determine whether any of the previously described Ca^{2+} transport systems of this organelle would accommodate the heavy metal. Fig. 6 is a working model of the combined results obtained in the present study, in our recent characterization of mitochondrial Ca^{2+} transport (Klein and Ahearn, 1999) and in our previous study examining the nature of copper influx into hepatopancreatic epithelial cells across brush-border membranes (Chavez-Crooker et al., 2001). As shown in this figure, heavy metals, such as copper, may enter hepatopancreatic epithelial cells by an antiporter carrier system in exchange for intracellular Na^+ or other cations such as Ca^{2+} (Chavez-Crooker et al., 2001). This transport system may be activated by Ca^{2+} derived from the exterior through verapamil-sensitive channels or Ca^{2+} may serve as an antiport substrate in exchange with the external cation. After copper, or another transportable heavy metal ion, has entered the cytoplasm, it may be translocated into mitochondria either through the Ruthenium-Red-sensitive uniporter or by way of one or both Ca^{2+} antiporters, displacing the normal substrates (e.g. $2Na^+$ or $2H^+$) for these exchangers.

Results from the kinetic analyses of copper influx into hepatopancreatic mitochondria (Figs 2, 4, 5) generally agree with previous studies characterizing the mechanisms of Ca^{2+} uptake by these organelles (Klein an Ahearn, 1999) and support the view that Ca^{2+} and copper share the same uptake processes. Klein and Ahearn (1999) described an apparent low-affinity, non-saturable, Ruthenium-Red-inhibited Ca^{2+} uptake process in hepatopancreatic mitochondria that did not saturate over a Ca^{2+} activity range of 14.06–450 $\mu mol\,l^{-1}$. Similarly, in the present investigation, mitochondrial copper influx kinetics suggested the presence of saturable and non-saturable transport components (Figs 2, 4, 5). Ca^{2+} and Ruthenium Red essentially abolished the non-saturable uptake process (Figs 2, 4), while diltiazem was ineffective as an inhibitor of this process (Fig. 5). It is likely, therefore, that this non-saturable uptake mechanism was the mitochondrial uniporter transport system and appeared to be capable of transporting Ca^{2+}, Cu^{2+} and Zn^{2+} (Klein and Ahearn, 1999).

Diltiazem produced a small, but significant, inhibition of copper influx through a mixed-type effect on the saturable influx process (Fig. 5). In addition, both Ca^{2+} and Ruthenium Red acted as competitive inhibitors of copper influx by the saturable transport mechanism (Figs 2, 4). It can, therefore, be postulated that this saturable uptake process is probably one or both of the previously characterized Ca^{2+} antiporters, can transport both Ca^{2+} and Cu^{2+} and is inhibited by both diltiazem and Ruthenium Red.

Ca^{2+} and Cu^{2+} uptake by hepatopancreatic mitochondria represent one possible means by which these cells are able to control the concentrations of these divalent cations in the cytoplasm and in the blood and, thereby, detoxify their potential debilitating effects on nerves and muscles. It is well known that Ca^{2+}, Mn^{2+} and Sr^{2+} may be stored at high concentrations in these organelles in combination with phosphate (Chen et al., 1974). This study and that of Klein and Ahearn (1999) suggest that both zinc and copper may also be accumulated within these organelles, thereby limiting their cytoplasmic and hemolymph concentrations.

Other means may be employed by hepatopancreatic cells to limit intracellular concentrations of metals such as copper. Metallothioneins are low-molecular-mass proteins possessing high-affinity cation-binding sites that are synthesized in response to increased cellular concentrations of metals. Cytoplasmic metals may bind to these proteins during periods of excess dietary metal and contribute to their detoxification (Engel and Brouwer, 1989; Roesijadi, 1992; Viarengo et al., 1985, 1987). Electron micrographs of numerous epithelial cells of the gastrointestinal tract and kidneys of a variety of invertebrates show the presence of membrane-bound vacuoles within the cytoplasm that, upon examination by electron microprobe analysis, are found to contain concretions of calcium and several heavy metals including zinc and copper (Al-Mohanna and Nott, 1985, 1987; George, 1983a,b; George et al., 1982; Loret and Devos, 1992). In some instances, these metal-containing vacuolar concretions are stored in the respective epithelial cells and later released by exocytosis to the gastrointestinal lumen as the cell ages; they may eventually be found in the feces (Vogt and Quinitio, 1994). It is likely

that, through the combined contribution of heavy metal uptake and sequestration by the mitochondria, cytoplasmic metallothioneins and intracellular metal-containing concretions, cellular regulation of these potentially toxic elements can be managed.

Biosynthetic uses of transported copper by hepatopancreatic cells

An alternative fate of transported Cu^{2+} in hepatopancreatic epithelial cells, at low concentrations of the metal, is the inclusion of these ions in biosynthetic pathways for a variety of essential enzymes and the oxygen-binding pigment hemocyanin. In the last 10 years, a variety of elegant molecular biological methods (Spindler et al., 1992; Rainer and Brouwer, 1993; Durstewitz and Terwilliger, 1997) have shown the site of hemocyanin synthesis in crustaceans to be the hepatopancreatic epithelium. The biosynthetic characteristics of this important respiratory pigment, and inclusion of copper within its molecular make-up, is related both to the developmental stage of the organism, when animals undergo the transition from larval to adult blood types (Durstewitz and Terwilliger, 1997), and to the molting cycle, when large fluctuations in protein synthesis take place (Spindler et al., 1992). What is still unclear about the inclusion of copper within the biochemistry of hemocyanin synthesis is which type of hepatopancreatic epithelial cell is responsible for this activity. It is hoped that the cell purification methods described in our recent paper (Chavez-Crooker et al., 2001) may help define the locus of this biological process.

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References

Ahearn, G. A. and Zhuang, Z. (1996). Cellular mechanisms of calcium transport in crustaceans. *Physiol. Zool.* **69**, 383–402.

Ahearn, G. A., Zhuang, Z., Duerr, J. and Pennington, V. (1994). Role of the invertebrate electrogenic $2Na^+/1H^+$ antiporter in monovalent and divalent cation transport. *J. Exp. Biol.* **196**, 319–336.

Aiken, D. E. (1973). Proecdysis, setal development and molt prediction in the American lobster (*Homarus americanus*). *J. Fish. Res. Bd Can.* **30**, 1337–1344.

Akerman, K. E. O., Wikstrom, M. K. F. and Saris, N. E. (1977). Effect of inhibitors on the sigmoidicity of the calcium ion transport kinetics in rat liver mitochondria. *Biochim. Biophys. Acta* **464**, 287–294.

Al-Mohanna, S. Y. and Nott, J. A. (1985). The accumulation of metals in the hepatopancreas of the shrimp *Penaeus semisulcatus* de Haan (Crustacea: Decapoda) during the moult cycle. In *Marine Environmental Pollution* (ed. R. Halwagy, D. Clayton and M. Behbehani), pp. 195–209. Kuwait: Kuwait University Press.

Al-Mohanna, S. Y. and Nott, J. A. (1987). R-cells and the digestive cycle in *Penaeus semisulcatus* (Crustacea: Decapoda). *Mar. Biol.* **95**, 129–137.

Baker, P. F. and Schlaepfer, W. W. (1978). Uptake and binding of calcium by axoplasm isolated from giant axons of *Loligo* and *Myxieda*. *J. Physiol., Lond.* **276**, 103–125.

Becker, G. L., Chen, C. H., Greenawalt, J. W. and Lehninger, A. L. (1974). Calcium phosphate granules in the hepatopancreas of the blue crab (*Callinectes sapidus*). *J. Cell Biol.* **61**, 316–326.

Bronner, F. (1992). Current concepts of calcium absorption: an overview. *J. Nutr.* **122**, 641–643.

Carafoli, E. (1965). Active accumulation of Sr^{2+} by rat liver mitochondria. II. Competition between Ca^{2+} and Sr^{2+}. *Biochim. Biophys. Acta* **97**, 99–106.

Carafoli, E. (1982). Transport of calcium across the inner membrane of mitochondria. In *Membrane Transport of Calcium* (ed. E. Carafoli), pp. 109–139. London: Academic Press.

Chavez-Crooker, P., Garrido, N. and Ahearn, G. A. (2001). Copper transport by lobster hepatopancreatic epithelial cells separated by centrifugal elutriation: measurements with the fluorescent dye Phen Green. *J. Exp. Biol.* **204**, 1433–1444.

Chen, C. H., Greenawalt, J. W. and Lehninger. L. A. (1974). Biochemical and ultrastructural aspects of Ca^{2+} transport by mitochondria of the hepatopancreas of the blue crab (*Callinectes sapidus*). *J. Cell Biol.* **61**, 301–315.

Chen, C. H. and Lehninger, L. A. (1973). Respiration and phosphorylation by mitochondria from the hepatopancreas of the blue crab (*Callinectes sapidus*). *J. Biochem. Biophys.* **154**, 449–449.

Crompton, M., Capano, M. and Carafoli, E. (1976). The sodium-induced efflux of calcium from heart mitochondria. A possible mechanism for the regulation of mitochondrial calcium. *Eur. J. Biochem.* **69**, 453–462.

Durstewitz, G. and Terwilliger, N. B. (1997). Developmental changes in hemocyanin expression the the Dungeness crab, *Cancer magister*. *J. Biol. Chem.* **272**, 4347–4350.

Engel, D. W. and Brouwer, M. (1989). Metallothionein and metallothionein-like proteins: Physiological importance. In *Advances in Comparative and Environmental Physiology*, vol. 5 (ed. R. Gilles), pp. 53–75. Berlin: Springer-Verlag.

Flik, G. and Haond, C. (2000). Na^+ and Ca^{2+} pumps in the gills, epipodites and branchiostegites of the European lobster *Homarus gammarus*: effects of dilute sea water. *J. Exp. Biol.* **203**, 213–220.

Flik, G., Verbost, P. M. and Atsma, W. (1994). Calcium transport in gill plasma membranes of the crab *Carcinus maenas*: evidence for carriers driven by ATP and a Na^+ gradient. *J. Exp. Biol.* **195**, 109–122.

Gavin, C. E., Gunter, K. K. and Gunter, T. E. (1990). Manganese and calcium efflux kinetics in brain mitochondria. Relevance to manganese toxicity. *Biochem. J.* **266**, 329–334.

George, S. G. (1983a). Heavy metal detoxification in the mussel *Mytilis edulis* – Composition of Cd-containing kidney granules (tertiary lysosomes). *Comp. Biochem. Physiol.* **76C**, 53–57.

George, S. G. (1983b). Heavy metal detoxification in *Mytilis* kidney – An *in vitro* study of Cd- and Zn-binding to isolated tertiary lysosomes. *Comp. Biochem. Physiol.* **76C**, 59–65.

George, S. G., Coombs, T. L. and Pirie, B. J. S. (1982). Characterization of metal-containing granules from the kidney of the common mussel, *Mytilus edulis*. *Biochim. Biphys. Acta* **716**, 61–71.

Gunter, K. K. and Gunter, T. E. (1994). Transport of calcium by mitochondria. *J. Bioenerg. Biomembr.* **26**, 471–485.

Gunter, T. E., Wingrove, D. E., Banerjee, S. and Gunter, K. K. (1988). Mechanisms of mitochondrial calcium transport. In *Cellular Ca^{2+} Regulation. Advances in Experimental Medicine and Biology*, vol. 232 (ed. D. Pfeiffer, J. B. McMillin and S. Little), pp. 1–14. New York: Plenum.

Gunter, T. E. and Pfeiffer, D. R. (1990). Mechanisms by which mitochondria transport calcium. *Am. J. Physiol.* **258**, C755–C786.

Gunter, T. E., Gunter, K. K., Sheu, S. S. and Gavin, C. E. (1994). Mitochondrial calcium transport: physiological and pathological relevance. *Am. J. Physiol.* **267**, C313–C339.

Klein, M. J. and Ahearn, G. A. (1999). Calcium transport mechanisms of crustacean hepatopancreatic mitochondria. *J. Exp. Zool.* **283**, 147–159.

Loret, S. M. and Devos, P. E. (1992). Structure and possible functions of the calcospherite-rich cells (R* cells) in the digestive gland of the shore crab, *Carcinus maenas*. *Cell Tissue Res.* **267**, 105–111.

Lukacs, G. L. and Fonyo, A. (1985). Ba^{2+} ions inhibit the release of Ca^{2+} ions from rat liver mitochondria. *Biochim. Biophys. Acta* **809**, 160–166.

Pushkin, J. S., Gunter, T. E., Gunter, K. K. and Russell, P. R. (1976). Evidence for more than one Ca^{2+} transport mechanism in mitochondria. *Biochemistry* **15**, 3834–3842.

Rainer, J. and Brouwer, M. (1993). Hemocyanin synthesis in the blue crab, *Callinectes sapidus*. *Comp. Biochem. Physiol.* **104B**, 69–73.

Reed, K. C. and Bygrave, F. L. (1974). The inhibition of mitochondria calcium transport by lanthides and Ruthenium Red. *Biochem. J.* **140**, 143–155.

Roesijadi, G. (1992). Metallothioneins in metal regulation and toxicity in aquatic animals. *Aquat. Toxicol.* **22**, 81–114.

Romslo, I. and Flatmark, T. (1973). Energy-dependent accumulation of iron by rat liver mitochondria. II. Relationship to the active transport of Ca^{2+}. *Biochim. Biophys. Acta* **325**, 38–46.

Saris, N. E. and Niva, K. (1994). Is Zn^{2+} transported by the mitochondrial calcium transporter? *FEBS Lett.* **356**, 195–198.

Spindler, K. D., Hennecke, R. and Gellissen, G. (1992). Protein production and the molting cycle in the crayfish *Astacus leptodactylus* (Nordmann, 1842). II. Hemocyanin and protein synthesis in the midgut gland. *Gen. Comp. Endocrinol.* **85**, 248–253.

Symons, L. J. and Jonas, A. J. (1987). Isolation of highly purified rat liver lysosomal membranes using two Percoll gradients. *Anal. Biochem.* **164**, 382–390.

Vaghy, P. L., Johnson, J. D., Matlib, M. A., Wang, T. and Schwartz, A. (1982). Selective inhibition of Na^+-induced Ca^{2+} release from heart mitochondria by diltiazem and certain other Ca^{2+} antagonist drugs. *J. Biol. Chem.* **257**, 6000–6002.

Viarengo, A. (1994). Heavy metal cytotoxicity in marine organisms: Effects on Ca^{2+} homeostasis and possible alteration of signal transduction pathways. In *Advances in Comparative and Environmental Physiology*, vol. 20 (ed. R. Gilles), pp. 85–111. Berlin: Springer-Verlag.

Viarengo, A., Moore, M. N., Mancinelli, G., Mazzucotelli, A., Pipe, R. K. and Farrar, S. V. (1987). Metallothioneins and lysosomes in metal toxicity and accumulation in marine mussels: the effect of cadmium in the presence and absence of phenanthrene. *Mar. Biol.* **94**, 251–257.

Viarengo, A., Moore, M. N., Pertica, M., Mancinelli, G., Zanicchi, G. and Pipe, R. K. (1985). Detoxification of copper in the cells of the digestive gland of mussel: The role of lysosomes and thioneins. *Sci. Total Environ.* **44**, 135–145.

Vogt, G. and Quinitio, E. T. (1994). Accumulation and excretion of metal granules in the prawn, *Penaeus monodon*, exposed to water-borne copper, lead, iron and calcium. *Aquat. Toxicol.* **28**, 223–241.

Wheatly, M. G. (1996). An overview of calcium balance in crustaceans. *Physiol. Zool.* **69**, 351–382.

Wheatly, M. G. (1999). Calcium homeostasis in Crustacea: the evolving role of branchial, renal, digestive and hypodermal epithelia. *J. Exp. Zool.* **283**, 620–640.

Wheatly, M. G., Pence, R. C. and Weil, J. R. (1999). ATP-dependent calcium uptake into basolateral vesicles from transporting epithelia of intermolt crayfish. *Am. J. Physiol.* **276**, R566–R574.

Wheatly, M. G., Weil, J. R. and Douglas, P. B. (1998). Isolation, visualization, characterization and osmotic reactivity of crayfish BLMV. *Am. J. Physiol.* **274**, R725–R734.

Wingrove, D. E. and Gunter, T. E. (1986a). Kinetics of mitochondrial calcium transport. I. Characteristics of the sodium-independent calcium efflux mechanism of liver mitochondria. *J. Biol. Chem.* **261**, 15159–15165.

Wingrove, D. E. and Gunter, T. E. (1986b). Kinetics of mitochondrial calcium transport. II. A kinetic description of the sodium-dependent calcium efflux mechanism of liver mitochondria and inhibition by Ruthenium Red and by tetraphenylphosphonium. *J. Biol. Chem.* **261**, 15166–15171.

Zhuang, Z. and Ahearn, G. A. (1996). Ca^{2+} transport processes of lobster hepatopancreatic brush-border membrane vesicles. *J. Exp. Biol.* **199**, 1195–1208.

Zhuang, Z. and Ahearn, G. A. (1998). Energized Ca^{2+} transport by hepatopancreatic basolateral plasma membranes of *Homarus americanus*. *J. Exp. Biol.* **201**, 211–220.

Zhuang, Z., Duerr, J. and Ahearn, G. A. (1995). Ca^{2+} and Zn^{2+} are transported by the electrogenic $2Na^+/1H^+$ antiporter in echinoderm gastrointestinal epithelium. *J. Exp. Biol.* **198**, 1207–1217.