Mitochondrial ATPase in the gills of the shore crab *Carcinus maenas*

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ABSTRACT: Posterior gills (No. 7 and 8) of shore crabs *Carcinus maenas* were homogenized and fractionated by means of differential and density gradient centrifugation. Employment of marker enzymes Na-K-ATPase and carbonic anhydrase for plasma membranes and cytochrome oxidase for mitochondria showed that these structural elements were separated. Ultramicroscopic investigations of combined fractions confirmed the presence of the respective mitochondrial and vesicular plasma membrane structures. An ATPase which did not depend on the presence of sodium (20 mM) ions in the incubation medium but on the presence of potassium (20 mM) ions only was found in the mitochondrial fractions. The mitochondrial ATPase was tightly bound to cellular particulates and activated approximately threefold by bicarbonate (20 mM) ions. The activity of this ATPase was nearly completely inhibited by oligomycin (1 µg ml^{-1}) and greatly inhibited by low levels (5 mM) of thiocyanate and calcium ions, the K_i for Ca^{2+} being ca 4 mM. The results obtained confirm literature data on high mitochondrial densities in crab gills and allow the assumption of significant rates of energy metabolism in these organs. Considering its properties the mitochondrial ATPase is clearly distinct from crab gill Na-K-ATPase and can be measured specifically in samples containing Na-K-ATPase. Mitochondrial ATPase is therefore considered a suitable and reliable marker enzyme for mitochondria.

INTRODUCTION

Crustacean gills are multifunctional organs located in the interphase between the animal and its environment. While particulate food is processed through the alimentary canals, and volume regulatory bulk water is released through the renal organs (Oglesby, 1981), the gills are the main sites of exchanges of respiratory gases (Burnett & McMahon, 1985; Böttcher et al., 1991), osmo- and ionoregulatory active ion uptake (Kirschner, 1979; Towle, 1981; Lucu, 1990), regulation of the acid-base status of the hemolymph (Truchot, 1979; Perry & Laurent, 1990) and excretion of nitrogenous metabolic end products (Kormanik & Cameron, 1981). All these functions have in common their participation in the processes of biological transport, their location in membranous structures and their close biochemical and physiological interrelation.

Accordingly, the branchial cells show all the features known to be characteristic of transporting epithelia, i.e. dense networks of invaginated plasma membranes in the apical membranes and considerably more pronounced ones in basolateral membranes. In

crustacean gills, the basolateral membranes are closely associated with high numbers of prolonged mitochondria. Mitochondrial densities and lengths become reduced in the regions beneath the apical membrane layers; while apical membranes themselves are seemingly not associated with mitochondria (Copeland & Fitzjarrel, 1968; Goodman & Cavey, 1990).

In contrast to the thoroughly investigated branchial ultrastructure and transport functions, especially those playing a role in energy-consuming osmoregulatory ion translocation, little is known about the energy providing processes of mitochondrial metabolism.

This publication describes the presence of mitochondrial ATPase in the gills of the shore crab Carcinus maenas including some properties of the enzyme and suggests its usefulness as a precisely measurable and reliable mitochondrial marker.

MATERIALS AND METHODS

Crabs

Shore crabs Carcinus maenas were obtained from fishermen at Kiel, Baltic Sea. Transport to the laboratory, maintenance, water quality, feeding and light conditions were identical to the procedures previously described (Siebers et al., 1983). Crabs were acclimated to a salinity of 10 ‰ for at least 1 month before experimental use. Experimentals were males of over 5 cm carapace width within the intermolt phase.

Homogenate preparation and centrifugation procedures

After removal of the carapace, the posterior gills (No. 7 and 8) were excised, blotted to near-dryness and weighed. 0.1 g tissue was homogenized for 45 sec with 0.9 ml TRIS/EDTA buffer (0.25 M sucrose, 0.02 M TRIS, 0.002 M Na_2-EDTA, pH 7.6) with a teflon-glass Potter-Elvehjem homogenizer at 400 rev · min^{-1} and 0 °C. Homogenates were then passed through 50 μm surgical gauze.

In order to analyse the proportion of mitochondrial ATPase bound to cellular particulates, homogenates were centrifuged at 100,000 g for 1 h at 4 °C in an ultracentrifuge (L8-70 M, Beckman) with a 70.1 Ti rotor. The supernatants (SI) were decanted, and the pellets (PI) were resuspended, rehomogenized and centrifuged again to obtain supernatants SII and pellets PII. This procedure was repeated a third time (see Fig. 1). A second approach was performed in the same way except that the resuspended and rehomogenized pellets were additionally ultra-sonicated twice for 5 sec at 60 W with a pause of 10 sec (sonicator type GTS22/125, Sonotrode Tu 157/4; KLN Ultraschall, Heppenheim, FRG).

Subcellular components were fractionated by means of density gradient centrifugation. Homogenates were centrifuged at 750 g for 20 min at 4 °C. The resulting supernatant was stored and the pellet resuspended, rehomogenized and centrifuged again. The second pellet was discarded. The combined 750 g supernatants were centrifuged at 7500 g, the pellet was resuspended and placed on top of a 33.5 ml continuous sucrose gradient (10−40 %, w/w). Sucrose was dissolved in homogenizing medium. The gradient was centrifuged at 100,000 g for 1.5 h in a Beckman rotor SW 27. By means of a capillary
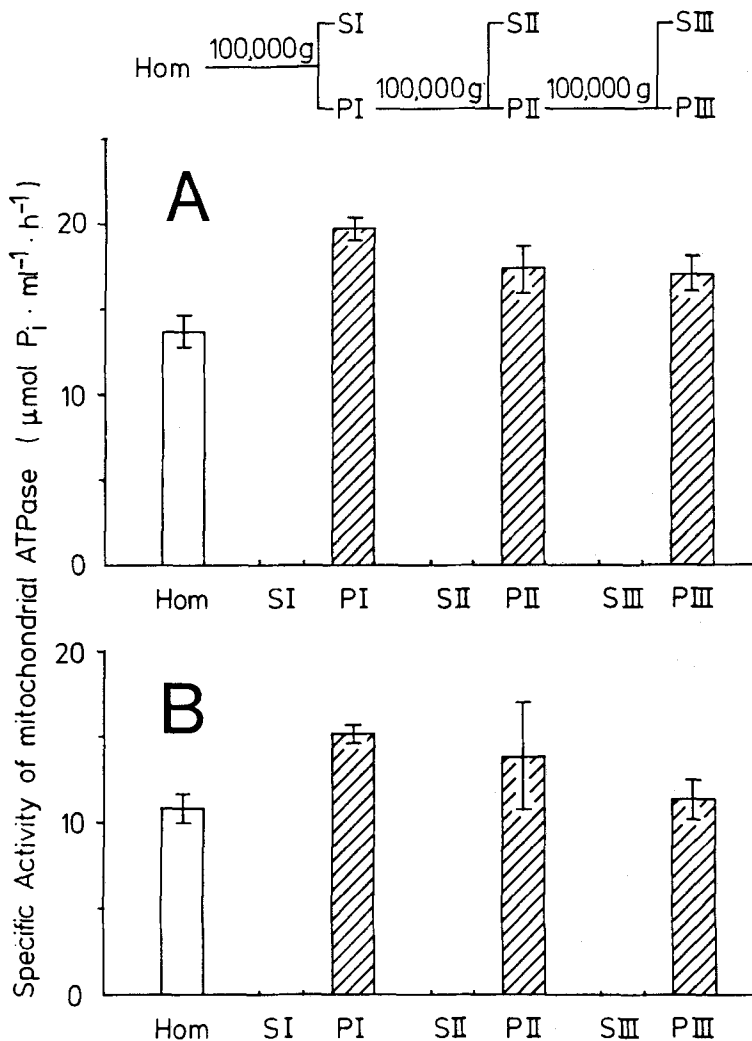
Fig. 1. *Carcinus maenas*. Mitochondrial ATPase in homogenates, supernatants (SI-SIII) and resuspended and rehomogenized pellets (PI-PIII) of one or repeated 100,000 g centrifugations. In the second and third centrifugation, resuspended and rehomogenized pellets of the previous centrifugation were used (A). Resuspended and rehomogenized pellets were additionally ultrasonicated and centrifuged again (B). Mitochondrial ATPase activities were calculated from the differences between the total ATPase activities and activities measured in the presence of 1 µg · ml^{-1} oligomycin. Non-specific (oligomycin-insensitive) activities were always less than 5 % of total activities. Bars represent means ± s.d. obtained from a sample size of 6 gills

tube immersed to the bottom of the centrifuge vial, 30 fractions of approx. 1.3 ml were withdrawn using a peristaltic pump and transferred to a fraction collector. Prior to further analysis the fractions were stored at −70 °C for no longer than 1 week.

Determinations of marker enzymes, mitochondrial ATPase and protein

Na-K-ATPase present in homogenates and in the fractions of the density gradient was measured spectrophotometrically by monitoring the absorbance at 340 nm in a coupled assay using the modified procedure of Siebers et al. (1982). The final test volume of 1.3 ml, pH 7.25 consisted of 0.1 M imidazole/HCl, 75 mM NaCl, 64 mM NH_4Cl, 5 mM $MgCl_2$, 5 mM ATP-Na_2, 2 mM phosphoenolpyruvate (PEP), 0.33 mM NADH-Na_2, 12.2 U · ml^{-1} lactate dehydrogenase (LDH) and 0.36 U · ml^{-1} pyruvate kinase (PK). LDH and PK were suspended in 3.2 M ammonium sulfate. Assays were performed in the absence and presence of $5 \cdot 10^{-4}$ M ouabain which specifically and completely inhibits Na-K-ATPase. The reduction of total ATPase activity due to the presence of ouabain thus represents Na-K-ATPase activity.

Mitochondrial ATPase was measured in supernatants and pellets obtained by repeated 100,000 g centrifugations and in the fractions of the sucrose density gradient in a comparable coupled assay (340 nm, 25 °C). The final volume was 1.3 ml consisting of 50 mM HEPES, 30 mM TRIS, 100 mM KCl, 3 mM $MgCl_2$, 3 mM TRIS-ATP (Commercial TRIS-ATP or TRIS-ATP prepared from Ba^{2+}-ATP with TRIS/H_2SO_4), 2 mM PEP, 0.33 mM NADH-Na_2, 12.2 U · ml^{-1} LDH, 0.36 U · ml^{-1} PK. LDH and PK were suspended in 50 % glycerol. When oligomycin – an antibiotic specifically directed against mitochondrial ATPase (Lardy et al., 1975) – was applied in order to determine non-specific activities the assays including controls contained 0.5 % ethanol (supplied under agitation from a stock solution of oligomycin dissolved in pure ethanol), a concentration not affecting ATPase activity.

Activity of carbonic anhydrase (CA) was determined by the phenol red indicator method published by Maren (1960) and modified according to Bruns et al. (1986). Activity (A) of the enzyme was calculated by comparing the uncatalyzed reaction time (t_o) measured in seconds with the catalyzed reaction time (t_{enz}) according to the following equation:

$$A = t_o/t_{enz} - 1$$

Following the proposal by Maren (1954), 1 unit of CA activity was defined as the concentration of enzyme in the final assay volume required to halve the uncatalyzed reaction time.

Activity of cytochrome oxidase was determined by monitoring the oxidation of reduced cytochrome c at 550 nm (according to Wharton & Tzagoloff, 1967). Protein concentrations were analysed by means of a commercial assay (BCA-assay, Pierce).

Analytical grade inorganic salts, HEPES and imidazole were purchased from Merck (Darmstadt), NADH, LDH and PK, and Na-pyruvate from Boehringer (Mannheim) and oligomycin from Serva (Heidelberg). TRIS, sucrose, ATP (Ba^{2-}-, TRIS-, and Na^+-salt), PEP, and cytochrome c were purchased from Sigma (München).

Transmission electron microscopy

For electron microscopy the non-overlapping fractions obtained by density gradient centrifugation containing mitochondria (fractions 2–6, see Fig. 2) and plasma membranes (fractions 13–17) were fixed in 0.05 M Na-cacodylate buffered with 4 % glutaraldehyde, 400 mOsm · kg^{-1}, pH 7.3, by mixing identical volumes of suspensions of the gradient with
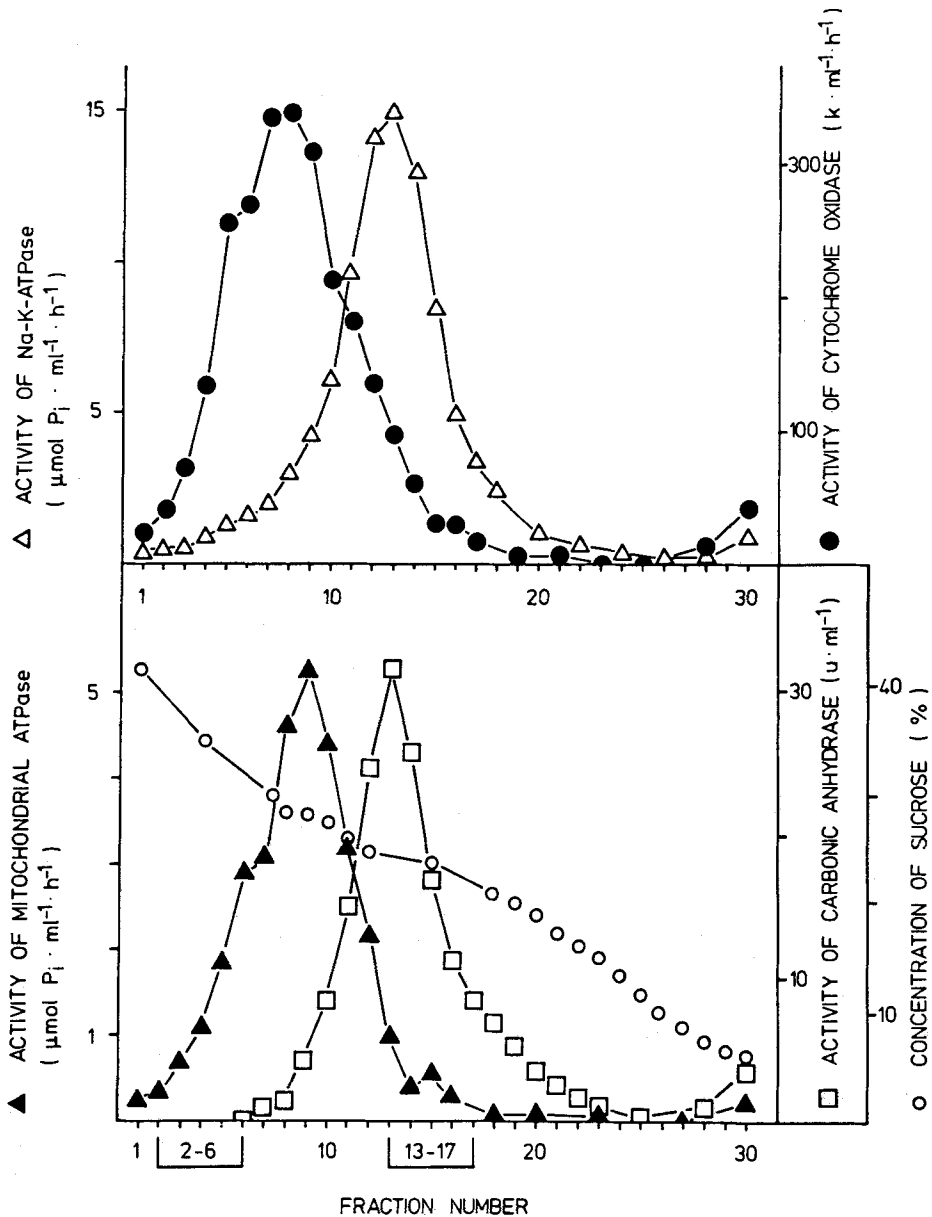
Fig. 2. *Carcinus maenas.* Fractionation of subcellular particulates of gill tissue by density gradient centrifugation. A homogenate prepared from posterior gills was centrifuged at 750 g. The supernatant was centrifuged at 7500 g, and the resuspended pellet was layered on top of a 10–40 % (w/w) continuous sucrose density gradient which was centrifuged for 1.5 h at 100,000 g (4 °C). Following centrifugation, 30 fractions were collected. Cytochrome oxidase and K+-dependent ATPase are characteristic of mitochondria; Na-K-ATPase and carbonic anhydrase are plasma membrane markers

the fixative to make up a final concentration of 2 % glutaraldehyde (1 h, room tempera-
ture). After centrifugation (55,000 g, 4 °C, 45 min) the pellets were resuspended in the
initial volumes of washing buffer (Na-cacodylate) and stored at 4 °C. Samples were
postfixed in 2 % osmium tetroxide (1 h, 4 °C, pH 7.3) and dehydrated in an ascending
series of alcohol up to 70 % ethanol. Specimens were then contrasted overnight with 1 %
phosphotungstic acid and 1 % uranylacetate in 70 % ethanol, completely dehydrated and
embedded in Epon 812. Ultrathin sections were cut using a Reichert ultracut E, stained
with lead citrate and viewed in a Siemens CT 150 TEM.

RESULTS

Mitochondrial localization of the K^+-dependent ATPase activity

Samples of crude homogenates were tested for ATPase activity in the presence of
100 mM KCl using the assay conditions presented in "Materials and Methods" for
mitochondrial ATPase (Fig. 1). The activities measured amounted to 14.3 ± 1.0 (A) and
11.1 ± 1.0 (B) μmol $P_i \cdot ml^{-1}$ homogenate $\cdot h^{-1}$. After 100,000 g centrifugations, once or up
to twice repeated, of the resuspended and rehomogenized (A) or additionally ultrasoni-
cated pellets (PI-PIII) (B), no activity could be detected in the supernatants (SI-SIII) (Fig.
1A and B). For repeated centrifugations the previous pellet was resuspended each time in
the original volume of the homogenate. Since concentrations of total proteins in the
resuspended pellets were reduced with regard to homogenates as a result of lacking
dissolved proteins contained in the supernatants activities are presented on a "per ml
basis". Therefore, the activities of supernatants and pellets shown in Figure 1 can be
directly compared. The results obtained imply that the ATPase, which is active in the
absence of sodium but in the presence of 100 mM KCl, is bound very tightly to cellular
particulates spun down at 100,000 g. Even strong mechanical handling of the samples
such as repeated resuspension, recentrifugation and ultrasonication of the pelleted
material did not bring detectable proportions into solution.

In order to find out the subcellular localization of the branchial K^+-dependent
ATPase, centrifugation (100,000 g, 1.5 h) of a 7500 g pellet in a continuous sucrose
gradient (10–40 %, w/w) was used. Thirty fractions obtained after centrifuging the
gradient were analysed for mitochondrial and plasma membrane marker enzymes. As
shown in Figure 2, the fractions containing the mitochondrial marker cytochrome oxidase
were clearly separated from the fractions containing the markers for plasma membranes,
Na-K-ATPase and carbonic anhydrase, though there was a zone of overlap. The ATPase
characterized by Na^+-independence but K^+-dependence was present in the mitochon-
drial fractions and has thus to be considered as mitochondrial ATPase.

The structural equivalents of the marker enzymes analysed in the fractions of the
sucrose density gradient was shown by transmission ultramicroscopy. Fractions 2–6 and
13–17 (see Fig. 2) were combined and prepared for TEM analysis as detailed in the
"Materials and Methods" section. The fractions used for TEM analysis were selected
within the zones of the gradient with minimal overlap, anticipating nearly pure mitochon-
dria in fractions 2–6 and plasma membranes in fractions 13–17 uncontaminated by
mitochondria. As shown in Figure 3, mitochondria (upper photo) contained only a few
plasma membrane elements and were clearly discernible from a relatively pure fraction
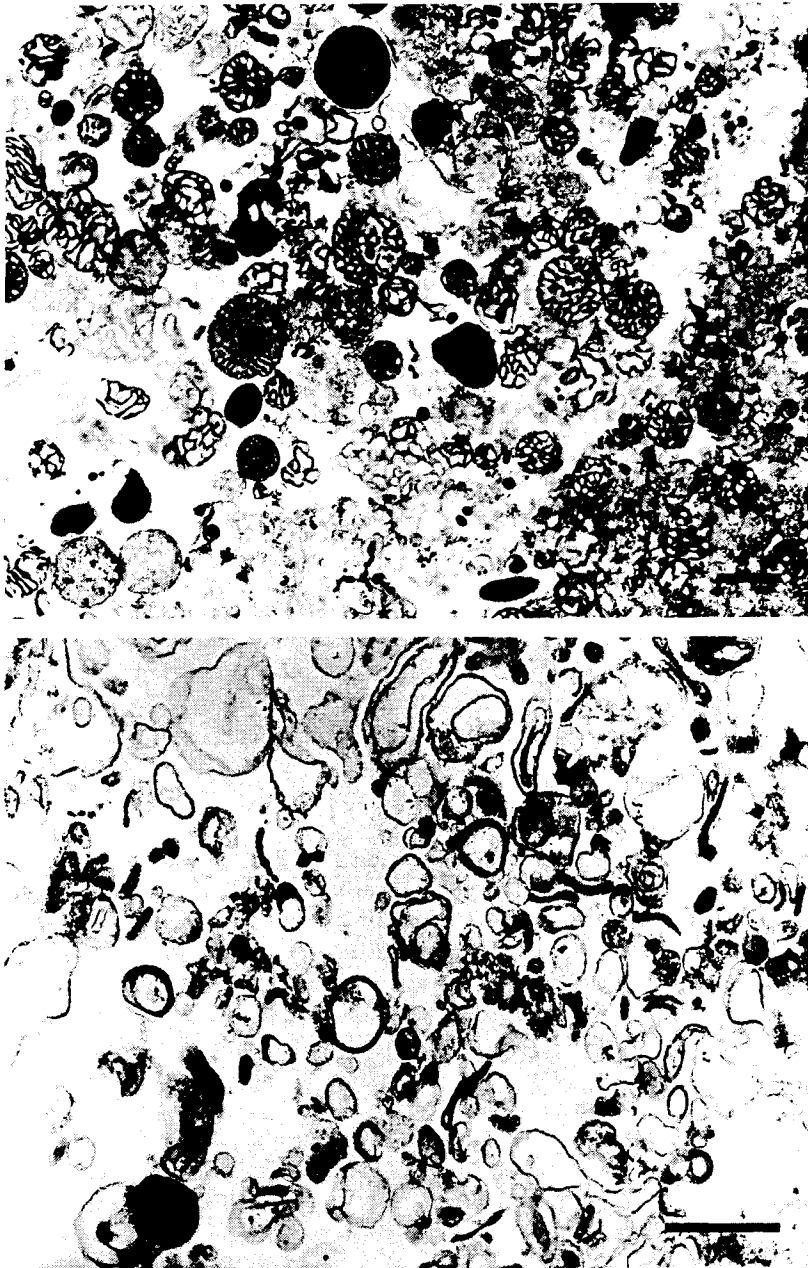
Fig. 3. *Carcinus maenas*. Electron micrographs of mitochondrial fractions 2–6 (upper photo) and plasma membrane fractions 13–17 (lower photo) obtained by density gradient centrifugation. The fractions were obtained in the same way as detailed in the legend to Fig. 2. Mitochondrial and plasma membrane fractions were pooled, fixed, stained, cut into ultrathin sections as outlined under "Materials and Methods", and viewed in a Siemens CT 150 transmission electron microscope. The horizontal bars amount to 0.5 μm

of vesiculating plasma membranes (lower photo). Some mitochondria showed structural damage which could not be prevented by varying the osmolalities of the fixation solutions. The damage is therefore not considered to be a result of unphysiological osmolalities but – as discussed by Graszynski (1970) – rather reflects the sensitivity of crustacean mitochondria to mechanical handling. A comparable shape of mitochondria in the gills of the chinese crab *Eriocheir sinensis* has been described in the publication by Péqueux et al. (1983).

Properties of mitochondrial ATPase

The pure mitochondrial fractions 2–6 (Fig. 2) obtained from the sucrose density gradient were used to analyse some properties of mitochondrial ATPase. As shown in Figure 4, activity amounted to only 1.5 μmol $P_i \cdot mg^{-1}$ mitochondrial protein $\cdot h^{-1}$ in the presence of 20 mM NaCl and was enhanced to reach values of ca 20 μmol $P_i \cdot mg^{-1}$ protein $\cdot h^{-1}$ when 20 mM KCl was employed in the incubation medium instead of NaCl. This finding shows that the activity of mitochondrial ATPase clearly depends on the cationic milieu of the incubation medium. When KCl was replaced by $KHCO_3$, activity was further greatly increased to levels of ca 70 μmol $P_i \cdot mg^{-1} \cdot h^{-1}$. Activities measured in the presence of NaCl and KCl imply that anions are not essential in determining enzymatic rates; however, HCO_3^- anions are obviously strong activators.

Activities were inhibited greatly in the presence of additional 5 mM SCN^- and nearly completely in the presence of 1 μg $\cdot ml^{-1}$ oligomycin. Low concentrations of calcium ions inhibited the activity of crab gill mitochondrial ATPase (Fig. 5), halving enzymatic rates in the presence of approximately 4 mM Ca^{2+}.

DISCUSSION

The results obtained show that literature data on high densities of mitochondria in crab gills agree on the presence of a specific marker enzyme, the mitochondrial ATPase. The assays for this ATPase contained 0.66 mM Na^+ introduced by NADH-Na_2. At these low levels of Na^+, Na-K-ATPase activity was below its detection limit (Siebers et al., 1983). Using 1 μg $\cdot ml^{-1}$ oligomycin, a specific inhibitor of mitochondrial ATPase, activities were reduced by more than 95 %. This result shows that under the conditions employed (100 mM KCl, 0.66 mM Na^+ and 1 μg $\cdot ml^{-1}$ oligomycin in a parallel assay) oligomycin-sensitive ATPase can be measured specifically in samples containing high levels of Na-K-ATPase.

The activity of mitochondrial ATPase in posterior gills of shore crabs acclimated to brackish water of 10‰ S amounted to ca 13 μmol $P_i \cdot ml^{-1}$ homogenate $\cdot h^{-1}$ (Fig. 1). Taking into consideration that 1 ml homogenate contained 0.1 g tissue and that total protein concentrations were ca 25 mg $\cdot g^{-1}$ fresh weight, the activity of mitochondrial ATPase on a protein basis is 5.2 μmol $P_i \cdot mg^{-1}$ protein $\cdot h^{-1}$. According to Siebers et al. (1982), activities of Na-K-ATPase in the posterior gills of crabs acclimatized to brackish water were approximately 11 μmol $P_i \cdot mg^{-1}$ protein $\cdot h^{-1}$. The activity of mitochondrial ATPase is thus 47 % of the activity of Na-K-ATPase, a result indicating that both enzymes are present in the branchial tissues of shore crabs in comparable activities.

As outlined by Siebers et al. (1990), mitochondrial ATPase has previously been
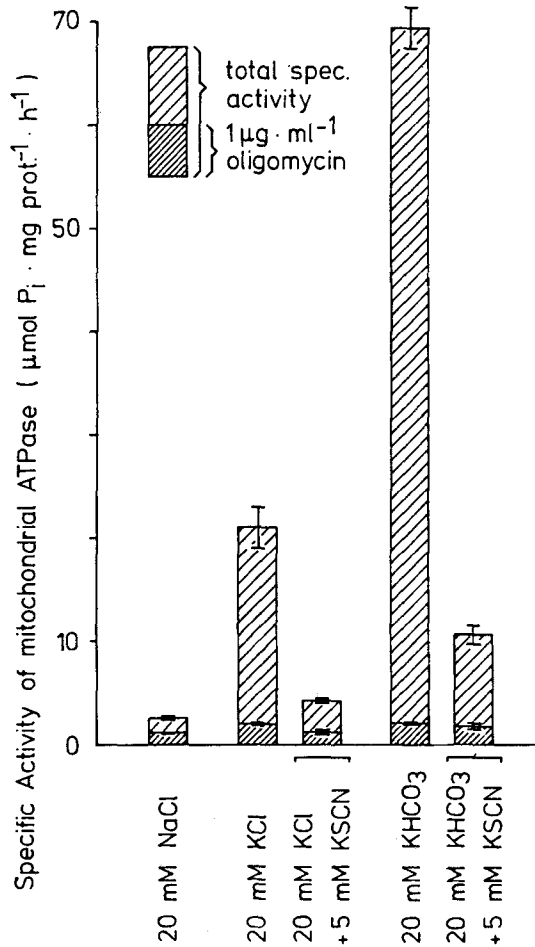
Fig. 4. *Carcinus maenas*. Properties of branchial mitochondrial ATPase. Samples of pooled mitochondrial fractions 2–6 (see Fig. 2 and upper photo, Fig. 3) obtained by density gradient centrifugation were analysed for ATPase activity. The fractions were obtained in the same way as detailed in the legend to Fig. 2. Activities were determined in the presence of 20 mM NaCl, KCl, or $KHCO_3$ in absence and in the presence of 5 mM KSCN or 1 µg · ml^{-1} oligomycin. Bars represent means ± s.d. obtained from a sample size of n = 5

considered as plasma membranous Cl^--ATPase located in the gills of fishes and crustaceans (see also Kirschner, 1979). In addition to the work published in 1990, the present paper describes the mitochondrial ATPase as being strictly bound to cellular particulates represented by the combined mitochondrial fractions uncontaminated with plasma membranes (Fig. 2). This work directly compares biochemical data of partially purified mitochondria with morphological structures. Mitochondrial localization was demonstrated by transmission electron microscopy of the respective pooled density gradient fractions. These purified mitochondrial fractions also served to analyse the enzyme's
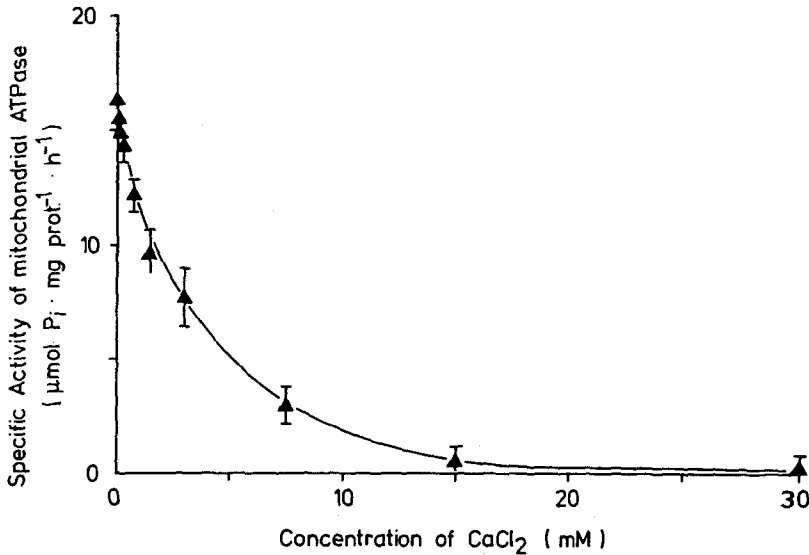
Fig. 5. *Carcinus maenas*. Inhibition of branchial mitochondrial ATPase by Ca^{2+} ions. Samples of pooled mitochondrial fractions 2–6 (see Fig. 2 and upper photo, Fig. 3) obtained by density gradient centrifugation were analysed for ATPase activity. The fractions were obtained in the same way as detailed in the legend to Fig. 2. Concentrations of Ca^{2+} ions varied between 0 and 30 mM. Activities were determined in the presence of 20 mM KCl and 1 µg · ml^{-1} oligomycin. Bars represent means ± s.d. obtained from a sample size of n = 5

properties such as alkali ion dependence, bicarbonate activation and inhibition by calcium ions, thiocyanate and oligomycin. Besides other activators, anions with 3 oxygen atoms have been shown to increase the specific activity of mitochondrial ATPase, HCO_3^-, HSO_3^- and $HSeO_3^-$ (Ebel & Lardy, 1975). Published properties of mitochondrial ATPase thus correspond to the properties of the crab enzyme found in the mitochondrial fractions of the density gradient.

Susceptibility of activity of the partially purified enzyme to the presence of oligomycin and Ca^{2+} ions implies that the enzyme in question is the mitochondrial ATP synthase (Lardy et al., 1975). Since in crab gills the majority of mitochondria is embedded in the dense network of basolateral plasma membrane invaginations, the sites of ATP synthesis in mitochondria and ATP utilization by Na-K-ATPase present in plasma membranes are located in close vicinity. The relation between ATP synthesis and consumption was shown by Siebers et al. (1990) using crude gill homogenates. They found out that in posterior gills of shore crabs *Carcinus maenas*, the activities of both enzymes were increased greatly in osmoregulating specimens maintained in brackish water of 10 ‰ S.

As both enzymes require different ionic milieus in their incubation media and as they are specifically inhibited by different antagonists (oligomycin and ouabain), they can be individually determined in the same sample. Therefore, the mitochondrial ATPase is considered as a reliable indicator of the presence of mitochondria and may thus be used as a mitochondrial marker enzyme.

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