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THE OXALATE/SULFATE ANTIPORTER IN LOBSTER HEPATOPANCREAS:
INTERNAL AND EXTERNAL BINDING CONSTANTS

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Summary

Utilizing a purified basolateral plasma membrane vesicle (BLMV) preparation containing a sulfate/oxalate antiporter, it was demonstrated that sulfate exhibited similar binding characteristics to the transporter whether bound internally or externally. It was also demonstrated that oxalate had similar binding characteristics to the antiporter whether it was bound internally or externally. Oxalate had a greater affinity to the transporter than did sulfate. Several organic anions affected binding and, therefore, overall transport by the antiporter. Most notably, sulfate was the only anion that stimulated the transport rate of oxalate into BLMVs, which suggests a conservative binding specificity for the antiporter. 4-Acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS) and/or 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) inhibited the transport rate, confirming the existence of oxalate/sulfate exchange by the transporter. These results suggest that oxalate, not sulfate, regulates the transport rate because of its greater affinity to the transporter.

Key words: sulphate/oxalate exchange, hepatopancreas, binding constant, Atlantic lobster, Homarus americanus.

Introduction

Gastrointestinal and renal transport of the divalent anion sulfate across epithelial apical membranes has been investigated in various vertebrate groups, including mammals (Ahearn and Murer, 1984; Pritchard, 1987), teleost fish (Renfro and Pritchard, 1982, 1983) and the domestic chicken (Renfro et al., 1987). A number of mechanisms for brush-border carrier-mediated sulfate transport across epithelial membranes have been proposed, and they include sodium/sulfate cotransport (Ahearn and Murer, 1984; Lucke et al., 1979), anion exchange (Taylor et al., 1987) and pH-gradient-dependent transfer (Schron et al., 1985). These processes contribute to transepithelial regulation of sulfate, which maintains physiological levels of this anion and may affect acid–base balance and cell and plasma osmolarity.

Sulfate transport across serosal or basolateral plasma membranes has been investigated in a wide variety of cell types, including intestinal (Hagenbuch et al., 1985; Schron et al., 1985), renal (Pritchard, 1987; Renfro et al., 1987) and liver (Hugentobler et al., 1987) epithelia. The mechanisms involved in anion uptake differ considerably among the many systems that have been investigated. Transport can be coupled to proton (Hugentobler et al., 1987) and Na+ (Gerencser et al., 1996) cotransport or to anion exchange (Schron et al., 1985; Pritchard, 1987).

In crustaceans, the hepatopancreas is involved in both the digestion and absorption of nutrients (Gibson and Barker, 1979); some investigators have implicated this organ as a site of excretion as well (Dall, 1970). Recently, the use of isolated membrane vesicles has led to the definition of absorptive and exchange mechanisms for a number of solutes in the hepatopancreatic brush border (Ahearn et al., 1987; Ahearn and Clay, 1987a,b) and chloride/bicarbonate (Ahearn et al., 1987), sulfate/oxalate (Gerencser et al., 1995) and sulfate/bicarbonate (Gerencser et al., 1999) antiporters in the basolateral membrane. The present study uses lobster hepatopancreatic basolateral membrane vesicles (BLMVs) to assess the kinetic constants of an oxalate/sulfate transporter to characterize further its transport properties.

Materials and methods

Live Atlantic lobsters Homarus americanus (Milne-Edwards), each weighing 0.5 kg, were purchased from commercial dealers in Hawaii and maintained unfed at 10 °C for up to 1 week in filtered sea water. All animals in this study were either in intermolt or in early premolt, as assessed by the molt stage classification introduced by Aiken (1973).

The Na+ salt of $^{35}$SO$_4^{2-}$ was obtained from New England Nuclear. [$^{14}$C]oxalic acid was obtained from Sigma Chemicals.
Valinomycin, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS), 4,4'-disothiocyanostilbene-2,2'-disulfonic acid (DIDS), bumetanide, furosemide and other reagent-grade chemicals were purchased from Sigma Chemicals.

Hepatopancreatic BLMVs were prepared from fresh tissue removed from individual lobsters. Each membrane batch was produced from 10 g fresh mass of tissue from one organ by a modification (Gerencser et al., 1995) of the method described by Ahearn et al. (1987). In the present study, hepatopancreatic tissue was homogenized for 30 s with a Polytron (Brinkmann Instruments) in 300 ml of bomb buffer (25 mmol l⁻¹ NaCl, 10 mmol l⁻¹ Tris-Hepes, pH 8.0 at 15 °C) containing 0.2 mmol l⁻¹ phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged for 15 min at 500 g in a Sorvall RC-5C high-speed centrifuge with an SS-34 fixed-angle rotor. After suction removal of a surface lipid layer, the supernatant was centrifuged for 33 min at 100 000 g in a Beckman L-8-55 ultracentrifuge with SW-28 swinging bucket rotor. The resulting pellet consisted of a dark, highly condensed central region and a fluffy, light external area. The light portion was separated from the remainder of the pellet and collected by pipette, suspended in 10 ml of 50 % sucrose in gradient buffer (12.5 mmol l⁻¹ NaCl, 10 mmol l⁻¹ Tris-Hepes and 0.5 mmol l⁻¹ EDTA, pH 7.4 at 15 °C) and resuspended by passage 10 times through a small-bore Pasteur pipette. The solution was brought to a final volume of 36 ml, placed in the bottom of four centrifuge tubes (9 ml each) and overlaid with successive 9 ml volumes of 40, 30 and 20 % sucrose in gradient buffer. The discontinuous sucrose gradient was centrifuged at 100 000 g for 93 min in a Beckman L-8-55 ultracentrifuge. Vesicles were collected from the 30–40 % sucrose interface and suspended in a small volume of inside buffer whose composition depended on the protocol. Vesicles were allowed to equilibrate in inside buffer at 4 °C for 2 h, and the solution was then centrifuged at 100 000 g for 33 min. Final vesicle pellets were suspended in a small volume of inside buffer and homogenized with 15 passes through a 22 gauge needle.

Spectrophotometric assays of marker enzymes were used to establish the relative purity of the membrane preparation. Alkaline phosphatase activity was determined using Sigma kit no. 104, leucine aminopeptidase activity was measured by the technique of Haase et al. (1978) and cytochrome c oxidase activity was assessed by the method of Cooperstein and Riley (1962) with the modifications described by Ames (1966).

Transport studies were conducted at 15 °C using the rapid filtration technique developed by Hopfer et al. (1973). For time-course experiments, 20 µl of vesicles was added to 180 µl of incubation medium containing either 0.1 mmol l⁻¹ ³⁵SO₄²⁻ or [¹⁴C]oxalate. At various incubation times, a known volume (20 µl) of reaction mixture was removed and plunged into 2 ml of ice-cold stop solution (composition varied with the experiment and generally consisted of incubation medium without sulfate) to stop the uptake process. The vesicle suspension was then rapidly filtered through a 0.65 µm Millipore filter and washed with another 7 ml of ice-cold stop solution. Filters were transferred to vials containing Beckman Ecolume scintillation cocktail and counted for radioactivity in a Beckman LS-8100 scintillation counter. Transport experiments (sulfate and/or oxalate) involving incubations of less than 10 s were conducted using a rapid-exposure uptake apparatus (Inovativ Labor, Adliswil, Switzerland). Uptake was initiated by mixing 5 µl of vesicles with 95 µl of radiolabeled incubation medium, and the filters were washed and counted for radioactivity as described above. Blank values were run for each condition by mixing stop solution, vesicles and radiolabeled incubation medium simultaneously; the resulting value was subtracted from corresponding experimental results before uptake was determined. Incubation and inside media varied between experiments and are described in the figure legends. Sulfate or oxalate uptake values are expressed as picomoles per milligram protein (Bio-Rad protein assay), using the specific activity of sulfate or oxalate, respectively, in the incubation medium.

Unless indicated otherwise, valinomycin (50 µmol l⁻¹) and bilaterally equal K⁺ concentrations across the vesicular wall were present to short-circuit the membranes. Each experiment was repeated between three and five times using membranes prepared from different animals; there was no qualitative difference amongst these experiments. Within a given experiment, each point was determined from the mean of 3–5 replicate samples, and the experimental scatter between these replicates never exceeded 5 %. Differences between experimental and control means were analyzed using a Student’s t-test. Values are presented as means ± S.E.M.

Results

The degree of BLMV purity was assessed by comparing the activities of membrane-bound marker enzymes assayed in the hepatopancreatic homogenate with the activities of the same enzymes in the vesicle suspension (Table 1). The activities of the brush-border-localized enzymes alkaline phosphatase and leucine aminopeptidase were significantly decreased in the BLMV preparation. Similarly, cytochrome c oxidase, a mitochondrial marker enzyme, exhibited a decrease in activity in the BLMVs. However, the activity of Na⁺/K⁺-ATPase, a basolateral membrane marker enzyme, was found to be significantly enriched in the vesicle preparation. These observations suggest that the BLMVs used in this study had minimal contamination by brush-border or mitochondrial membranes.

The kinetic characteristics of sulfate/oxalate exchange were examined using two experimental protocols. Preliminary investigation determined that sulfate uptake into BLMVS was linear for the first 15 s at the concentration employed in this study (data not shown). Fig. 1 shows that sulfate influx was a hyperbolic function of internal oxalate concentration. Such a relationship can be described by a Michaelis–Menten carrier
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mechanism illustrating saturation kinetics. This process can be described by the equation:

\[ J = \frac{J_{\text{max}}[\text{Ox}]}{K_m + [\text{Ox}]}, \]

where \( J \) is total sulfate influx in pmol mg\(^{-1}\) protein 7 s\(^{-1}\), \( J_{\text{max}} \) is apparent maximal carrier-mediated influx, \( K_m \) is the apparent binding coefficient of oxalate to the vesicular interior and \([\text{Ox}]\) is the internal oxalate concentration.

A non-linear iterative best-fit computer program was utilized to analyze the data in Fig. 1 using equation 1. Apparent transport or binding variables derived in this manner were as follows: apparent \( K_m = 0.18 \pm 0.06 \) mmol l\(^{-1}\) and apparent \( J_{\text{max}} = 267.9 \pm 18.0 \) pmol mg\(^{-1}\) protein 7 s\(^{-1}\) (means \( \pm \) S.E.M., \( N = 5 \)).

Fig. 2 shows that oxalate influx was a hyperbolic function of internal sulfate concentration. Apparent sulfate transport or binding variables derived in this manner were as follows: apparent \( K_m = 7.1 \pm 0.1 \) mmol l\(^{-1}\) and apparent \( J_{\text{max}} = 296.3 \pm 18.1 \) pmol mg\(^{-1}\) protein 7 s\(^{-1}\) (\( N = 5 \)).

The third set of experiments assessed the role of external sulfate concentration on sulfate/oxalate exchange in BLMVs. Vesicles were loaded with 10 mmol l\(^{-1}\) sodium oxalate and incubated in media containing various concentrations of sulfate (0.25–30 mmol l\(^{-1}\)). The initial 7 s uptake was measured and found to be a hyperbolic function of external sulfate concentration. Fig. 3 shows this relationship, which again can be described by the Michaelis–Menten equation:

\[ J = \frac{J_{\text{max}}[S]}{K_m + [S]}, \]

Table 1. Activities of marker enzymes of lobster (Homarus americanus) hepatopancreatic basolateral membrane vesicles

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Homogenate activity ((\mu\text{mol mg}^{-1}\text{h}^{-1}))</th>
<th>BLMV activity ((\mu\text{mol mg}^{-1}\text{h}^{-1}))</th>
<th>Purification factor</th>
<th>( N )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>1.43±0.48</td>
<td>0.71±0.14*</td>
<td>0.50±0.16</td>
<td>7</td>
</tr>
<tr>
<td>Leucine aminopeptidase</td>
<td>698.6±115.6</td>
<td>237.1±40.0*</td>
<td>0.34±0.08</td>
<td>5</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>81.3±7.6</td>
<td>13.9±2.8*</td>
<td>0.17±0.03</td>
<td>5</td>
</tr>
<tr>
<td>Na(^+)/K(^+)-ATPase</td>
<td>3.12±0.59</td>
<td>58.9±4.3*</td>
<td>18.9±1.9</td>
<td>5</td>
</tr>
</tbody>
</table>

Values are means \( \pm \) S.E.M.; \( N \) is the number of preparations.

Purification factors are means of individual BLMV activities/individual homogenate activities.

*Indicates a significant difference between homogenate and basolateral membrane vesicle (BLMV) activity (\( P < 0.05 \)).

Fig. 1. Effect of internal oxalate concentration on the influx of 0.1 mmol l\(^{-1}\) \(^{35}\)SO\(_4\)\(^{2-}\) into hepatopancreatic basolateral membrane vesicles (BLMVs). Vesicles were loaded with 100 mmol l\(^{-1}\) potassium gluconate, 0.10–10 mmol l\(^{-1}\) disodium oxalate and 90–100 mmol l\(^{-1}\) tetramethylammonium gluconate. Incubation medium contained 100 mmol l\(^{-1}\) tetramethylammonium gluconate, 100 mmol l\(^{-1}\) potassium gluconate and 0.1 mmol l\(^{-1}\) \(^{35}\)SO\(_4\)\(^{2-}\). All media contained 50 μmol l\(^{-1}\) valinomycin and 40 mmol l\(^{-1}\) Hepes-Tris at pH 7.0. \( J_{\text{max}} \), apparent maximal carrier-mediated sulfate influx; \( K_m \), apparent binding coefficient of oxalate to the vesicular interior. Values are means \( \pm \) S.E.M. (\( N = 5 \)).

Fig. 2. Effect of internal sulfate concentration on the uptake of 0.1 mmol l\(^{-1}\) \(^{14}\)Coxalate into hepatopancreatic basolateral membrane vesicles (BLMVs). Vesicles were loaded with 100 mmol l\(^{-1}\) tetramethylammonium gluconate, 80 mmol l\(^{-1}\) potassium gluconate and 10 mmol l\(^{-1}\) potassium sulfate. Incubation medium contained 100 mmol l\(^{-1}\) tetramethylammonium gluconate, 100 mmol l\(^{-1}\) potassium gluconate and 0.1 mmol l\(^{-1}\) \(^{14}\)Coxalate. All media contained 50 μmol l\(^{-1}\) valinomycin and 40 mmol l\(^{-1}\) Hepes-Tris at pH 7.0. Variables are defined in Fig. 1. Values are means \( \pm \) S.E.M. (\( N = 5 \)).
where \( K_m \) is the apparent sulfate concentration resulting in half-maximal uptake, \([S]\) is the external sulfate concentration, and the other symbols are as in equation 1. Transport or binding variables were calculated as above and were as follows: apparent \( K_m = 6.0 \pm 1.7 \text{ mmol l}^{-1} \) and apparent \( J_{\text{max}} = 302.3 \pm 37.0 \text{ pmol mg}^{-1} \text{ protein 7 s}^{-1} \) \((N=5)\).

Potential anion-exchange inhibitors of sulfate/oxalate antiport were tested in BLMVs loaded with 10 mmol l\(^{-1}\) potassium sulfate (Fig. 4). Inhibitors were added to the external medium at a concentration of 1 mmol l\(^{-1}\), while the control condition was sulfate/oxalate exchange in the absence of inhibitor. The potent stilbene compounds DIDS and SITS significantly reduced oxalate uptake, while neither furosemide nor bumetanide had any inhibitory effect.

Whether oxalate would exchange with a wide range of organic anions as transferable substrates was investigated by trans-stimulation with 5 mmol l\(^{-1}\) sulfate, oxaloacetate, succinate, \(\alpha\)-ketoglutarate, formate and citrate (Fig. 5). Only sulfate was able to stimulate 0.1 mmol l\(^{-1}\) \([^{14}\text{C}]\)oxalate uptake significantly more effectively than the response obtained in the presence of the non-exchangeable anion gluconate (control). This experiment indicates, that in BLMVs, the antiporter is relatively specific and will not accept similar dicarboxylic acids, or the tricarboxylic acid citrate, as a substrate.
Discussion

The present study provides corroborative evidence for the occurrence of a carrier-mediated, electroneutral sulfate/oxalate exchanger in BLVs isolated from lobster hepatopancreas (Gerencser et al., 1995). A number of marine invertebrates, including the lobster, have been shown to maintain plasma levels of sulfate below that in sea water (Robertson, 1949). This suggests that the organism is faced with a continuous need to eliminate sulfate against a concentration gradient. The mechanism elucidated here could be utilized as one means of removing sulfate from the blood to assist in maintaining anion homeostasis.

Sulfate/dicarboxylic acid exchange has been observed in the basolateral membrane of a variety of tissues such as the rat ileum (Hagenbuch et al., 1985), rat and fish kidney (Pritchard, 1987; Renfro and Pritchard, 1982) and elasmobranch liver (Hugentobler et al., 1987; Meier et al., 1987). As shown in Fig. 5, when BLVs are loaded with 5 mmol l\(^{-1}\) sulfate, there is stimulation of oxalate uptake over that of gluconate-loaded vesicles. Other sulfate/oxalate exchangers will accept similar dicarboxylic acids as trans-stimulators. Oxaloacetate and succinate were able to exchange with sulfate in the rabbit ileum (Schron et al., 1985) and in the liver of the skate Raja erinacea (Hugentobler et al., 1987). Lobster BLVs were found to be conservative in the acceptance of a suitable substrate and did not utilize oxaloacetate, succinate, formate, \(\alpha\)-ketoglutarate or citrate (Fig. 5). It appears that, in lobster hepatopancreas BLVs, the sulfate/oxalate exchanger does not extend its substrate specificity to other important physiological anions.

Sulfate/oxalate exchange was strongly inhibited by the stilbene derivatives DIDS and SITS (Fig. 4), which have been shown to inhibit anion/anion exchange in other systems (Gerencser et al., 1996; Ahearn et al., 1987). Furosemide and bumetanide will inhibit sulfate antiport in rat liver (Hugentobler et al., 1987) and rabbit ileum (Hagenbuch et al., 1985) BLVs, but were not effective in the lobster preparation (Fig. 4).

Sulfate influx into BLVs occurred by at least one carrier-mediated mechanism that exhibited Michaelis–Menten kinetics. When uptake was measured as a function of internal oxalate concentration, an apparent \(K_m\) of 0.18 mmol l\(^{-1}\) was observed, suggesting a relatively high affinity of the vesicular interior for the organic anion (Fig. 1). Sulfate association with the vesicle exterior was shown to have an apparent \(K_m\) of approximately 6 mmol l\(^{-1}\) (Figs 2, 3); this value is in agreement with the level of sulfate (18 mmol l\(^{-1}\)) reported in lobster plasma (Robertson, 1949). These observations suggest that the high sulfate concentration in lobster blood could act as the driving force for sulfate/oxalate exchange across the BLVs. In addition, the finding that oxalate has a binding constant (\(K_m\)) much lower than that of sulfate at both the internal and external sites of the carrier (Figs 1–3) indicates that oxalate, not sulfate, dictates the rate of the transporter because of its greater affinity for the antiporter (Neame and Richards, 1972). Oxalate binding to the antiporter therefore provides the rate-limiting step in the exchange of sulfate for oxalate.

The basolateral membrane sulfate/oxalate antiporter described in the present study cannot provide unassailable evidence for absorption or secretion of either sulfate or oxalate. However, a plausible explanation for the function of the antiporter is that intracellular sulfate binds Ca\(^{2+}\) under acute temporal conditions and stores Ca\(^{2+}\) as calcium oxalate (Gerencser et al., 1996; Yendt and Cohanim, 1985). It is known that many premolt crustaceans store Ca\(^{2+}\) as calcium oxalate in hepatopancreatic lysosomes and mitochondria for use during the molt cycle (Becker et al., 1974). The sulfate/oxalate antiporter could then be used for ridding the cell of excess oxalate as a cytoprotective mechanism after completion of the molting cycle.

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References


