Cytochemical Localization of Ca\(^{2+}\), Mg\(^{2+}\)-ATPase on the Limiting Membrane of Peroxisomes Isolated from Renal Tubules of Rats

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The cytochemical localization of Ca\(^{2+}\)-, Mg\(^{2+}\)-ATPase in peroxisomes isolated from rat renal tubules was investigated at the ultrastructural level. The reaction products were localized mainly on the cytoplasmic surface of the isolated peroxisomes, but also to some extent on the matrix side of the limiting membrane. Neither Ca\(^{2+}\)- nor Mg\(^{2+}\)-ATPase activity was affected by treatment with levamisole, an inhibitor of alkaline phosphatase. Ca\(^{2+}\)-ATPase activity was inhibited by thapsigargin and myricetin, but not by PCMB. Mg\(^{2+}\)-ATPase activity was inhibited by PCMB. It was inhibited slightly by thapsigargin, but was not affected by myricetin.

Key words: Cytochemistry, Ca\(^{2+}\)-, Mg\(^{2+}\)-ATPase, ATPase inhibitor, Isolated peroxisomes, Rat kidney

I. Introduction

Of the many enzymes localized in peroxisomes, the presence of ATPase has been demonstrated biochemically in rat liver peroxisomes [4]. Cytochemical identification of ATPase has been reported only in peroxisomes of yeast [5, 6], rat hepatocytes [12-14] and canine hepatocytes [7]. The main site of Mg\(^{2+}\)-ATPase localization is the outer surface, i.e. cytoplasmic side, of the limiting membrane, but to eliminate the effect of cytosol on the outer surface, isolated peroxisomes were utilized in the present study, since the precise site of Mg-ATPase activity remains to be elucidated.

This report describes the site of the cytochemical reaction of Ca\(^{2+}\)-, Mg\(^{2+}\)-ATPases on the limiting membrane of isolated peroxisomes from epithelial cells of rat renal tubules and the effects of inhibitors of alkaline phosphatase (ALPase) (levamisole), Ca-ATPase (thapsigargin and myricetin), and Mg-ATPase (p-chloromercuribenzoic acid; PCMB).

II. Materials and Methods

Chemicals

All chemicals were purchased from Kanto Chemical Co. (Tokyo, Japan) and Wako Chemical Co. (Osaka, Japan), except for 3-(morpholino)propane-sulfonic acid (MOPS), Nycodenz, ATP.2Na, levamisole, thapsigargin, myricetin and PCMB (Sigma Chemical Co., St. Louis, USA).

Isolation of peroxisomes

Male and female Wistar rats weighing 200–250 g were anesthetized with ether, and their kidneys were immediately excised and washed in an ice-cold solution containing 5 mM 3-(morpholino)propane-sulfonic acid (MOPS), 0.25 M sucrose, 1 mM EDTA and 0.1% (v/v) ethanol at pH 7.4. The cortex tissue was obtained by dissecting transverse sections of the decapsulated kidneys, and homogenized in 20 ml of the same buffer in a homogenizer. Isolated peroxisomes were obtained according to Serafini [18]; the light mitochondrial fraction was carefully toploaded on a discontinuous Nycodenz gradient composed of 1.5 ml 50% (w/v) Nycodenz solution, density = 1.21 g/ml + 7 ml 30% Nycodenz, density = 1.15 g/ml in 5 mM Tris-HCl, 3 mM KCl, 0.3 mM EDTA, 0.22 M sucrose, pH 7.4, and centrifuged in a fixed-angle rotor (RP 65; Hitachi Instruments) for 45 min at 40,000 rpm. Alternatively, the method of Zaar et al., [25] was used; the light mitochondrial fraction was suspended in 47% (wt/wt) sucrose in 5 mM MOPS buffer (pH 7.4), and then centrifuged for 5 hr in a fixed-angle rotor (RP 65; Hitachi Instruments) at 45,000 rpm. The resulting pellet was the peroxisome fraction.
Ca\(^{2+}\), Mg\(^{2+}\)-ATPase cytochemistry

For detection of Ca\(^{2+}\), Mg\(^{2+}\)-ATPase on the limiting membrane of peroxisomes, after fixation in cold 2% glutaraldehyde in 0.1 M sodium cacodylate at pH 7.4 for 45 min, and rinsing with cacodylate buffer containing 0.25 M sucrose, the fraction was incubated in the medium (Table 1) at 37\(^\circ\)C for 30 min. As a control, levamisole, an inhibitor of ALPase, thapsigargin and myricetin, inhibitors of Ca-ATPase, and PCMB, an inhibitor of Mg-ATPase, were added to the medium, or substrate-free medium was utilized. After incubation, the fractions were rinsed again with cacodylate buffer containing 0.25 M sucrose, followed by routine embedding with epoxy resin and subsequent sectioning. Ultrathin sections were contrasted with uranyl acetate before observation in a transmission electron microscope (Hitachi H700) at 80 kV.

III. Results

The peroxisomes isolated from renal tubules of rat showed Ca\(^{2+}\), Mg\(^{2+}\)-ATPase activity after incubation with the above medium (Table 1) for 30 min at 37\(^\circ\)C. The product of the enzyme activity was localized on the cytoplasmic surface and also the matrix side of the limiting membrane of the peroxisomes (Figs. 1–4). No transmembrane localization of reaction products was identified in this experiment at higher magnification (Figs. 2 and 4). At sites of degenerated limiting membrane, no reaction product of ATPase was localized (Fig. 3).

In control experiments, incubation in substrate (2Na-ATP)-free medium resulted in a negative reaction (Figs. 5 and 6).

Levamisole, an inhibitor of ALPase, did not abolish the reaction product of both Ca\(^{2+}\)- and Mg\(^{2+}\)-ATPase (Figs. 7 and 8). When thapsigargin, an inhibitor of Ca-ATPase, was added to the incubation medium, the reaction product of Mg\(^{2+}\)-ATPase activity was slightly decreased (Fig. 10), and that of Ca\(^{2+}\)-ATPase was drastically decreased (Fig. 9). Myricetin, another inhibitor of Ca-ATPase, also inhibited the Ca\(^{2+}\)-ATPase (Fig. 11), but not Mg\(^{2+}\)-ATPase (Fig. 12). On the other hand, PCMB, an inhibitor of Mg-ATPase, inhibited Mg\(^{2+}\)-ATPase (Fig. 14), but not Ca\(^{2+}\)-ATPase (Fig. 13).

These results clearly demonstrated that the outer surface of the membrane of isolated peroxisomes was the main site of Ca\(^{2+}\), Mg\(^{2+}\)-ATPase localization, although a small amount of reaction product was identified on the inner surface of the limiting membrane.

Table 1. Incubation medium for cytochemical localization of Ca\(^{2+}\), Mg\(^{2+}\)-ATPase.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>0.2 M Tris-HCl buffer at pH 9.0</td>
<td>13 ml (130 mM)</td>
</tr>
<tr>
<td>MgSO(_4), 7H(_2)O</td>
<td>49 mg (Mg: 10 mM)</td>
</tr>
<tr>
<td>(or CaCl(_2))</td>
<td>23 mg</td>
</tr>
<tr>
<td>ATP, 2Na (substrate)</td>
<td>30 mg (2.5 mM)</td>
</tr>
<tr>
<td>0.5% lead citrate dissolved in</td>
<td>5 ml (Pb: 2.5 mM)</td>
</tr>
<tr>
<td>50 mM NaOH</td>
<td></td>
</tr>
<tr>
<td>Dimethylosulfoxide (DMSO)</td>
<td>1 ml (5%: v/v)</td>
</tr>
<tr>
<td>2 mM 2,4-dinitrophenol (DNP)</td>
<td>1 ml (0.1 mM)</td>
</tr>
<tr>
<td>Total (final pH 8.8 to 9.0)</td>
<td>20 ml</td>
</tr>
</tbody>
</table>

Control medium:
Substrate-free
Levamisole (inhibitor of ALPase) | 10 mM
Thapsigargin (inhibitor of CaATPase) | 5 mM
Myricetin (inhibitor of CaATPase) | 5 mM
PCMB (inhibitor of MgATPase) | 10 mM

Fig. 1. Cytochemical localization of Ca\(^{2+}\)-ATPase in isolated peroxisomes from rat kidney, showing the enzyme reaction on the limiting membrane. \(\times 10,000\). Bars = 0.5 \(\mu\)m.

Fig. 2. At high magnification, Ca\(^{2+}\)-ATPase is localized on the outer surface (arrow) and also on the inner side (arrowheads) of the membrane of the isolated peroxisomes. \(\times 30,000\). Bars = 0.1 \(\mu\)m.

Fig. 3. Mg\(^{2+}\)-ATPase activity on the limiting membrane of peroxisomes. Note that partially ruptured limiting membrane has no reaction product (arrow). \(\times 10,000\). Bars = 0.5 \(\mu\)m.

Fig. 4. Isolated peroxisomes from rat kidney have Mg\(^{2+}\)-ATPase on the outer surface (arrows) and also on the inner side (arrowheads) of the limiting membrane. \(\times 30,000\). Bars = 0.1 \(\mu\)m.

Fig. 5. A control section incubated in substrate-free medium. The fine structure of the limiting membrane of the isolated peroxisomes is negative for the Ca\(^{2+}\)-ATPase reaction. \(\times 36,000\). Bars = 0.1 \(\mu\)m.

Fig. 6. Negative reaction of Mg\(^{2+}\)-ATPase on the isolated peroxisomes incubated in substrate-free medium. \(\times 36,000\). Bars = 0.1 \(\mu\)m.

Fig. 7. The reaction product of Ca\(^{2+}\)-ATPase in the isolated peroxisomes is not inhibited by levamisole, an alkaline phosphatase inhibitor. \(\times 15,000\). Bars = 0.25 \(\mu\)m.

Fig. 8. Mg\(^{2+}\)-ATPase reaction product is also unaffected by levamisole. \(\times 15,000\). Bars = 0.25 \(\mu\)m.

Fig. 9. Inhibition of Ca\(^{2+}\)-ATPase on the isolated peroxisomes treated with thapsigargin, a Ca\(^{2+}\)-ATPase inhibitor. \(\times 15,000\). Bars = 0.25 \(\mu\)m.

Fig. 10. Mg\(^{2+}\)-ATPase on the isolated peroxisomes is not inhibited by thapsigargin. \(\times 15,000\). Bars = 0.25 \(\mu\)m.

Fig. 11. Ca\(^{2+}\)-ATPase in the isolated peroxisomes is inhibited by myricetin, a Ca\(^{2+}\)-ATPase inhibitor. \(\times 15,000\). Bars = 0.25 \(\mu\)m.

Fig. 12. The Mg\(^{2+}\)-ATPase on the isolated peroxisomes is not inhibited by myricetin. \(\times 15,000\). Bars = 0.25 \(\mu\)m.

Fig. 13. The Ca\(^{2+}\)-ATPase on the isolated peroxisomes is not inhibited by PCMB, a Mg\(^{2+}\)-ATPase inhibitor. \(\times 15,000\). Bars = 0.25 \(\mu\)m.

Fig. 14. Electron micrograph of the isolated peroxisomes showing inhibition of Mg\(^{2+}\)-ATPase on the limiting membrane by PCMB. \(\times 15,000\). Bars = 0.25 \(\mu\)m.
Figs. 1-4

Cytochemical Localization of Ca^{2+}-, Mg^{2+}-ATPase
Figs. 9-14
IV. Discussion

The necessity and presence of ATP on the limiting membrane of peroxisomes have been postulated by Whitney and Bellion [22]. Whether it exists on the outer surface, in a transmembrane location, or on the inner (matrix side) surface of the limiting membrane remains to be elucidated. It was revealed biochemically by del Valle et al., [4] that Mg\(^{2+}\)-ATPase activity corresponded to a protein of the peroxisomal membrane, exposed to the cytosol.

Either Mg\(^{2+}\)- or Ca\(^{2+}\)-ATPase activity has been cytochemically localized on peroxisomes of yeast [5, 6], rat hepatocytes [12–14] and canine hepatocytes [7]. In the present study of isolated peroxisomes from rat kidney it was clarified that both Ca\(^{2+}\)- and Mg\(^{2+}\)-ATPase were located on the outer surface, and also, to a lesser extent, on the inner side of the limiting membrane (Figs. 2 and 4). This result confirmed that the major reaction on the outer surface of limiting of isolated peroxisomes was not due to the influence of cytoplasmic membrane attached to the membrane. This result also confirms that the enzyme is not only localized on the cytosolic side or outside of the peroxisomal membrane, as previous experiments in this laboratory had indicated [7, 12], but also on the inner side.

There are two major conformational states of the enzymes, E1 and E2. The E1 conformation state has high affinity for Ca\(^{2+}\), but the E2 state has low affinity. Ca\(^{2+}\)-binding sites are exposed on the outer (cytoplasmic) side of the sarcoplasmic reticulum in the E1 state, but exposed to the inner side of the ER in the E2 state [23].

Leighton et al., [10] reported that enhancement of acyl-CoA oxidation by ATP was abolished when the membrane of isolated peroxisomes was ruptured. This indicated that an ATP complex was located in the limiting membrane. The lack of ATPase reaction product on the partially ruptured limiting membrane in this experiment (Fig. 3) may support the existence of an ATP-related complex in the limiting membrane.

It was possible to detect ALPase activity instead of ATPase with lead citrate incubation medium [24]. Levamisole, an ALPase inhibitor [1, 20], did not abolish the reaction in this experiment using lead citrate-containing medium, and it was evident that the enzyme was not ALPase (Figs. 7 and 8).

Both cytochemically and biochemically, it has been reported that PCMB inhibits Mg\(^{2+}\)-ATPase on chicken osteoclasts [1, 2]. By using this inhibitor, Ca\(^{2+}\)- was distinguished from Mg\(^{2+}\)-ATPase in this experiment. The enzyme was Ca\(^{2+}\)-ATPase when PCMB did not abolish the enzyme (Fig. 13).

Thapsigargin, a naturally occurring sesquiterpene lactone, is a -tetradecanoylphorbol-13-acetate-type tumor promotor. It is known to be the most potent inhibitor of the intracellular Ca\(^{2+}\) pump protein from skeletal muscle and cardiac sarcoplasmic reticulum, and brain microsomes [9, 16]. The inhibition induced by thapsigargin is proposed to involve discharge of intracellular Ca\(^{2+}\) by the release of stored Ca\(^{2+}\), so that it causes rapid inhibition of the Ca\(^{2+}\)-ATPase of rat liver microsomes [19]. Thapsigargin has also been reported to force the Ca\(^{2+}\)-ATPase into a conformation where Ca\(^{2+}\) binding is inhibited [17]. The inhibition of Ca-ATPase by this agent has also been demonstrated through ATP binding to Ca\(^{2+}\)-ATPase [3]. In the present study, thapsigargin inhibited Ca\(^{2+}\)-ATPase, although not totally (Fig. 9). As reported by Lytton et al., [11], thapsigargin inhibits only members of the sarcoplasmic or endoplasmic reticulum Ca-ATPase (SERCA) family. If so, the Ca-ATPase on the limiting membrane of isolated peroxisomes from rat kidney may be a member of the SERCA family.

Another mechanism of thapsigargin inhibition proposed by Wictome et al., [23] on the sarcoplasmic reticulum of rabbit muscle is that the agent binds strongly to ATPase and changes the Ca\(^{2+}\) binding to a conformation that can bind only one Ca\(^{2+}\) ion rather than the usual two.

Myricetin also inhibited Ca\(^{2+}\)-ATPase, but did not inhibit Mg\(^{2+}\)-ATPase (Figs. 11 and 12). This agent was recently reported to reduce the cellular content of superoxide anion in both resting and Ca\(^{2+}\)-loaded brain neurons [15]. Thiyagarajah et al., [21] reported that the ATP-dependent Ca\(^{2+}\) transport system located on the liver plasma membrane and endoplasmic reticulum was inhibited by myricetin. The inhibition induced by this agent could be caused by its interference with the binding of Ca\(^{2+}\) to the Ca\(^{2+}\)-binding site, although whether it interferes with the ATP-binding site is not clear.

In peroxisomes isolated from rat liver [10] ATPase was localized in the limiting membrane. Later on it was identified as, 70-kDa [8], one of the proteins of the limiting membrane of peroxisomes, which combines with ATP and supplies energy to transport action of fatty acid. Immunocytochemically, the 70-kDa peroxisomal membrane protein (PMP70) was localized outside and also inside the peroxisomal membrane using epitope selected antibody [8]. The present result of localization of reaction products of ATPase on the peroxisomal limiting membrane is an indication of ATPase and not necessarily ATP itself. A very close spacial relationship between the 70-kDa protein and the ATPase on the peroxisomal membrane implies that both substances may be in the same complex of substances if not identical to each other.

In conclusion, this study localized Ca\(^{2+}\)- and Mg\(^{2+}\)-ATPase on both the outer and inner surfaces of the limiting membrane of peroxisomes from rat kidney. Using some different Ca-, and Mg-ATPase inhibitors we were able to distinguish Ca\(^{2+}\)-ATPase from Mg\(^{2+}\)-ATPase on the limiting membrane. Both thapsigargin and myricetin, reported to be Ca\(^{2+}\)-ATPase inhibitors, cytochemically inhibited Ca\(^{2+}\)-ATPase on the limiting membrane.
V. References


