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SUBCELLULAR LOCALIZATION OF INORGANIC PYROPHOSPHATASE IN RAT SALIVARY GLANDS

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Inorganic pyrophosphatase (EC 3.6.1.1) from cells of the sublingual and submandibular salivary glands of rat was found only in the cytosol and was absent in nuclei, mitochondria, lysosomes and microsomes.

The literature provides only few data on inorganic pyrophosphatase (EC 3.6.1.1). The present studies were aimed to elucidate the localization of this enzyme in subcellular structures of the submandibular and sublingual salivary glands of rat.

MATERIALS AND METHODS

Reagents. Sodium pyrophosphate (acid, pure), bovine serum albumin and sucrose were from Serva (Heidelberg, F.R.G.); MgCl₂ (1 m solution) was from B.D.H. Chemicals Ltd. (Poole, Dorset, Great Britain); sodium succinate, glucose-6-phosphate, β-glycerophosphate, cytochrome c, Tris and Triton X-100 were from Koch-Light Lab. Ltd (Colnbrook, Great Britain); 1,4-dithiothreitol (DTT) was purchased from Loba-Chemie (Vienna, Fischamend, Austria); EDTA, [ethylene glycol-bis(2-aminoethyl)-ether]-N, N'-tetraacetic acid (EGTA), L-glutamic acid (Na⁺) and atractyloside (K⁺) were from Sigma (St. Louis, Mo., U.S.A.); ADP and malic acid (Na⁺) were from Calbiochem (Los Angeles, California); mannitol was from U.C.B. (Brussels, Belgium). Other reagents, of analytical grade, were obtained from Ciech (Gliewie, Poland).

Animals. Wistar male rats weighing 180 - 200 g were sacrificed by rupture of the spinal cord. The sublingual and submandibular glands, encapsulated together in a connective-tissue sac, were immediately isolated and separated by the procedure described by Szymczyk & Jachimowicz (1971), whereupon they were washed with isotonic sucrose solution (+4°C).

Homogenates. The separated submandibular and sublingual glands were homogenized in a Potter-Elvehjem glass homogenizer with a Teflon pestle, then cooled...
with a water and ice mixture. Homogenates of the sublingual and submandibular salivary glands, 10% and 20% respectively, were prepared in a solution containing 0.32 M-sucrose, 10 mM-Tris/HCl buffer, pH 7.5, 5 mM-MgCl₂ and 1 mM-DDT.

**Separation of subcellular fractions.** For determination of the subcellular distribution of the total activity and protein, subcellular fractions were isolated from homogenates of salivary glands according to the modified method of Hogeboom (1960) and that of Pritchard *et al.* (1971). Moreover, particular fractions were isolated separately, directly from the homogenates, by methods assuring their higher purity (see below).

**Nuclei** were isolated from the homogenates according to Chauveau *et al.* (1956) and to Widnell & Tata (1964) as modified by Skrzypek-Osiecka *et al.* (1980). Crude nuclei were obtained from the homogenate by centrifugation at 600 g for 10 min and purified nuclei were obtained from crude nuclei by centrifugation through 2.2 M-sucrose at 100,000 g for 1 h. The nuclear pellet was suspended in a 0.32 M-sucrose solution containing 20 mM-Tris/HCl buffer, pH 8.0, 2 mM-MgCl₂ and 1 mM-DDT.

**Mitochondria** were prepared according to Horak & Pritchard (1971) and to Munkres *et al.* (1966). Homogenates of the sublingual and submandibular salivary glands, 5% and 10%, respectively, were prepared in homogenization buffer containing 0.23 M-mannitol, 0.07 M-sucrose, 0.5 mM-EDTA, 0.5 mM-EGTA, 10 mM-Tris/HCl buffer, pH 7.4. Homogenates were filtered through a double layer of nylon cloth, and then centrifuged at 2000 g for 5 min in a Sorvall centrifuge, to remove nuclei and cell membranes. The supernatant was centrifuged at 12,000 g for 10 min. The mitochondrial pellet was washed twice with the homogenization buffer, each washing being followed by centrifugation at 8000 g for 5 min.

After being suspended in the same buffer mitochondria were centrifuged in a 1.2 - 1.5 M-sucrose concentration gradient at 20,000 g for 30 min in a MLW Janetzki VAC 602 centrifuge with a swing-out rotor. The final mitochondrial pellet was suspended in 10 mM-Tris/HCl buffer, pH 7.5, containing 5 mM-MgCl₂ and 1 mM-DDT.

**Lysosomes** were obtained exactly according to the method of Sawant *et al.* (1964). Homogenates of both kinds of salivary glands were prepared (1:8, w/v) in a solution containing 0.25 M-sucrose, 1 mM-EDTA, 10 mM-Tris/HCl buffer, pH 7.5, and then centrifuged. Fraction F₁ was the precipitate obtained by centrifugation of the supernatant at 16,300 g for 20 min, fraction F₁₁ resulted from centrifugation of fraction F₁ (suspended in a 0.3 M-sucrose solution) at 9500 g for 10 min, and fraction F₁₁₁ was obtained by centrifugation of fraction F₁₁ (suspended in a 0.7 M-sucrose solution) at 9500 g for 30 min. All three lysosomal fractions were suspended in 10 mM-Tris/HCl buffer, pH 7.5, containing 5 mM-MgCl₂ and 1 mM-DDT.

**Microsomes** were obtained according to Bergman *et al.* (1969). The sublingual and submandibular salivary glands were homogenized (1:8, w/v) in a solution containing 0.32 M-sucrose, 0.5 mM-CuCl₂ and 5 mM-Tris/HCl buffer, pH 7.4. Homogenates were filtered through a double layer of nylon cloth, and then centrifuged at 150 g for 10 min. The supernatant was centrifuged three more times: at 2000 g...
for 20 min, at 10 400 g for 15 min, and at 23 500 g for 60 min. The microsomal pellet obtained after the third centrifugation was suspended in 10 mM-Tris/HCl buffer, pH 7.5, containing 5 mM-MgCl₂ and 1 mM-DTT.

The cytosol fraction was obtained by centrifugation of the homogenates of both kinds of salivary glands at 20 000 g for 30 min, followed by centrifugation of the supernatant at 120 000 g for 90 min.

The degree of purity of the prepared fractions was controlled by determination of the activity of the following marker enzymes: succinate-cytochrome c oxidoreductase for mitochondria, acid phosphatase (EC 3.1.3.2) for lysosomes, and glucose-6-phosphatase (EC 3.1.3.9) for microsomes.

The degree of damage to mitochondria was examined by determining their "coupling state" from the respiratory control ratio according to Horak & Pritchard (1971). Moreover, use was made, in the presence of added ADP, of atractyloside, an inhibitor of nucleotide translocase. Measurements were taken using a Radelkis OH-102 polarograph (Budapest, Hungary) with a Clark oxygen electrode, at 25°C. The incubation mixture contained, in the final volume of 1.2 ml, 0.23 M-mannitol, 0.07 M-sucrose, 0.5 mM-EGTA, 20 mM-Tris/HCl buffer, pH 7.4, 5 mM-potassium phosphate buffer, pH 7.4, and 0.8 - 3.0 mg mitochondrial protein. The final concentrations of substrates in the incubation mixture were 4.5 mM-succinate, 1 mM-malate and 5 mM-glutamate. The final concentration of atractyloside was 1 μM, and that of ADP 0.25 mM.

The respiratory control ratio and the effect of atractyloside on the transition of mitochondria from State 3 to State 4 in the presence of ADP were calculated from the polarographic tracings according to Chance & Williams (1956).

The purity of cell nuclei preparations and their degree of damage were inspected under a light microscope after staining of nuclei with methylene blue on glass slides.

Determination of the enzymatic activities. The inorganic pyrophosphatase activity was assayed according to Irie et al. (1970) in the homogenates and subcellular fractions frozen previously at −20°C. After thawing, the fractions were homogenized in a Potter-Elvehjem homogenizer (glass-glass type) in the same solutions in which they had been suspended immediately after preparation. The activity of remaining enzymes was assayed in freshly prepared fractions: acid phosphatase by the method of Irie et al. (1970), succinate-cytochrome c oxidoreductase by the procedure of Green & Ziegler (1963), and glucose-6-phosphatase according to Harper (1963). The total enzymatic activity in the lysosomal fraction was determined after pretreatment of lysosomes with Triton X-100 in a final concentration of 0.2%. Enzymatic activities were expressed as μmol/mg protein per minute.

Determination of protein. Protein was assayed according to Lowry et al. (1951), with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

By the Hogeboom's method (1960) and by the procedure of Pritchard et al. (1971) in our modification as described under Materials and Methods it was demonstrated that no inorganic pyrophosphatase activity was present in the nuclei, mito-
Table 1

Distribution of the inorganic pyrophosphatase activity in subcellular fractions of salivary glands of rat

The total enzymatic activity in the homogenate was taken as 100%. Specific activity is expressed as μmol/mg protein per min. Total activity is expressed as μmol/min.

<table>
<thead>
<tr>
<th>Subcellular fractions</th>
<th>Sublingual gland</th>
<th>Submandibular gland</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total protein*</td>
<td>Specific activity</td>
</tr>
<tr>
<td>Homogenate</td>
<td>90.0</td>
<td>1.70</td>
</tr>
<tr>
<td>Pellet I (nuclei)</td>
<td>30.5</td>
<td>0</td>
</tr>
<tr>
<td>Pellet II (lysosomes, mitochondria)</td>
<td>2.7</td>
<td>0</td>
</tr>
<tr>
<td>Pellet III (microsomes)</td>
<td>5.4</td>
<td>0</td>
</tr>
<tr>
<td>Supernatant I (cytosol, microsomes, lysosomes, mitochondria)</td>
<td>60.2</td>
<td>2.55</td>
</tr>
<tr>
<td>Supernatant II (cytosol, microsomes)</td>
<td>57.5</td>
<td>2.67</td>
</tr>
<tr>
<td>Supernatant III (cytosol)</td>
<td>53.4</td>
<td>2.88</td>
</tr>
</tbody>
</table>

* Obtained from 3.0 g of tissue (about 100 glands).
** Obtained from 5.0 g of tissue (about 50 glands).
chondria, lysosomes and microsomes of the sublingual and submandibular salivary glands (Table 1). In the cytosol contaminated with subcellular fractions (supernatant I and supernatant II) the total activity of the enzyme remained unchanged, irrespective of the subcellular fractions contaminating the cytosol, and it equalled the total activity of the enzyme in the uncontaminated cytosol fraction (supernatant III). The inorganic pyrophosphatase activity of the homogenate was quantitatively recovered in the cytosol of cells of both salivary glands.

Scheme 1. Centrifugation of salivary gland homogenates in a solution containing 0.32 M-sucrose, 10 mM-Tris/HCl buffer, pH 7.5, 5 mM-MgCl₂, 1 mM-DTT, according to the method of Hogeboom (1960) and the procedure of Pritchard et al. (1971), in our modification.

The subcellular fractions obtained by application of different rates of centrifugation in an isotonic sucrose solution (0.32 M-sucrose) were incompletely separated, and thus remained mutually contaminated; for better purification the particular subcellular fractions of the sublingual and submandibular salivary glands (Table 2) were separately isolated and purified. The purification of mitochondria, lysosomes and microsomes was paralleled by a rise in specific activity of marker enzymes. Purified morphologically intact cell nuclei exhibited no inorganic pyrophosphatase activity. In the case of the microsomal fraction, this enzyme was also absent. On the other hand, trace amounts of the activity of inorganic pyrophosphatase in mitochondria and lysosomes disappeared with the progress of purification of these two subcellular fractions.
Table 2

Purification of subcellular fractions of the sublingual and submandibular salivary glands

The particular fractions were isolated separately and purified as described in Methods. The activity of marker enzymes is expressed as µmol/mg protein per min.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific activity</th>
<th>Sublingual gland</th>
<th>Submandibular gland</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pyrophosphatase</td>
<td>succinate-cytochrome c oxidoreductase</td>
<td>acid phosphatase</td>
</tr>
<tr>
<td>Homogenate</td>
<td>1.700</td>
<td>0.013</td>
<td>0.003</td>
</tr>
<tr>
<td>Nuclei*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>crude</td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>purified</td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Mitochondria**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>crude</td>
<td>0.009</td>
<td>0.032</td>
<td>0.043</td>
</tr>
<tr>
<td>purified</td>
<td>0</td>
<td>0.125</td>
<td>0.023</td>
</tr>
<tr>
<td>Lysosomes***</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td>0.031</td>
<td>0.003</td>
<td>0.004</td>
</tr>
<tr>
<td>F2</td>
<td>0</td>
<td>0.018</td>
<td>0</td>
</tr>
<tr>
<td>F3</td>
<td>0</td>
<td>0.130</td>
<td>0</td>
</tr>
<tr>
<td>Microsomes****</td>
<td>0</td>
<td>—</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Fractions prepared according to:

* Chauveau et al. (1956) and Widnell & Tata (1964).
** Horak & Pritchard (1971) and Munkres et al. (1966).
*** Sawant et al. (1964).
**** Bergman et al. (1969).
Since the increase in specific activity of succinate-cytochrome c oxidoreductase does not prove unequivocally that the mitochondria obtained were functionally competent, the "coupling state" of mitochondria was also examined. For the sublingual gland, the value of respiratory control with succinate as substrate was 2.7, and with glutamate-malate as substrate, 4.5; the respective respiratory control values for the submandibular gland were 3.0 and 6.0. These results, as well as the atractysolide-induced transition of the mitochondria, in the presence of ADP, from state 3 to state 4 confirm that in the preparation obtained the mitochondria were functionally competent.

Thus, it was unequivocally demonstrated that inorganic pyrophosphatase present in cells of rat sublingual and submandibular salivary glands is located exclusively in the cytosol, unlike the enzyme from rat liver, found both in the cytosol and mitochondria (Irie et al., 1970).

REFERENCES


WEWNĄTRZKOMÓRKOWA LOKALIZACJA NIEORGANICZNEJ PIROFOSFATAZY W GRUCZOŁACH ŚLINOWYCH SZCZURA

Streszczenie

Nieorganiczna pirofosfataza (EC 3.6.1.1) z komórek gruczołów ślinowych podjęzykowych i podżuchwowych szczura występuje jedynie w cytosolu. Nie stwierdzono jej obecności w jądrach, mitochondriach, lisosomach i mikrosomach.

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