Subcellular Localization of Inorganic Pyrophosphatases in Rabbit Skeletal Muscle and Some Properties of a Microsomal Acid Pyrophosphatase

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Subcellular localization of muscle inorganic pyrophosphatase was examined using rabbit skeletal muscle homogenates. The pyrophosphatases were found to be contained in the microsomal, mitochondrial, and cytosol fractions. The microsomal and mitochondrial pyrophosphatases were most likely bound to the respective subcellular fractions. The pyrophosphatases associated with microsome and mitochondria showed their optimal activities at about pH 5.5 and 7, respectively. They were not dissociated from the particles by washing with salt solution or by ten times freezing-thawing. The activity of microsomal acid pyrophosphatase was not affected by Mg^{2+} , Ca^{2+} or EDTA, but that of the mitochondrial neutral pyrophosphatase was enhanced by the addition of Mg^{2+} . The microsomal acid pyrophosphatase was stable between pH values of 5.5 and 8.5 during storage at 4°C. The activity was inhibited by *p*-chloromercuribenzoate. The activity was irreversibly inhibited by sodium dodecyl sulfate, but reversibly inhibited by neutral salts and membrane solubilizing detergents such as Triton X-100, octaethylene glycol mono-*n*-dodecylether, and sodium cholate.

Pyrophosphate (PP) is a product of many biosynthetic reactions. PP has been suggested to regulate many metabolic reactions and to pull in the biosynthetic direction by hydrolysis of PP by inorganic pyrophosphatase (EC 3.6.1.1, PPase).^{1,2)} Not a few PPases are widely distributed in different cells where they enhance many PP producing reactions. There have been many studies on these enzymes isolated from a variety of sources.^{3,4)} They were found in mammalian cells of some organs such as liver,^{5,6)} brain,^{7,8)} and leucocytes⁹⁾ and their subcellular localization has been established in these organs. The PPase activity in mitochondria, lysosomes, or microsomes of rat liver^{6,10,11)} and in rat and bovine heart mitochondria^{11,12)} was also reported.

Little is known about the PPases in skeletal muscle cells. Previous studies had shown that PP added to muscle homogenates was hydrolyzed by the enzyme(s), which were present intrinsically in skeletal muscles.^{13,14)} The hydrolytic activity showed two pH optima at pH

5.5 and 7. Some properties of a purified neutral PPase in the cytosol fraction of muscle were already reported.¹⁵⁾ This paper presents the results of investigation on subcellular localization of these PPases in muscle and some properties of an acid PPase of the microsomal fraction.

MATERIALS AND METHODS

Materials. The sources of materials used in this work were as follows: dithiothreitol (DTT), sodium deoxycholate (DOC), sodium cholate, sodium salt glucose-6-phosphate (G-6-P), sodium salt β -glycerophosphate, ferrocytochrome *c*, and bovine serum albumin from Sigma Chemical Co., scintillation-grade Triton X-100 from Eastman Kodak Co., histidine, imidazole, Tris-(hydroxymethyl)aminomethane (Tris), *p*-chloromercuribenzoate acid (pCMB), sodium dodecyl sulfate (SDS), and sodium pyrophosphate (Na-PP) from Nakarai Chemicals Co., octaethylene glycol mono-*n*-dodecylether (C₁₂E₈) from Nikko Chemicals Co., and Milpaf filter paper from Azumi Roshi Co. All other reagents were of the purest grade commercially available.

Enzyme assay. The PPase assay was done by adding substrate after preincubation at 25°C for 7 min and the reaction was stopped by adding 2 ml of 16% trichloroacetic acid (TCA) to 2 ml of the reaction mixture. The PPase activity was determined by measuring the Pi liberated in the reaction mixture according to the method of Martin and Doty¹⁶⁾ with a slight modification.¹⁷⁾ The composition of the assay mixture is described in the legend of each figure. The specific activity was expressed in units (U) defined as μmol of Pi liberated in 1 hr by 1 mg of protein.

The G-6-P phosphatase activity in muscle samples was measured by the method of De Duve *et al.*¹⁸⁾ at 37°C in a reaction mixture containing 0.25 M sucrose, 9 mM G-6-P, 1 mM EDTA, and 20 mM histidine-NaOH buffer, pH 6.5. The reaction was stopped by the addition of TCA and the inorganic Pi content was determined in the filtrate.

The β -glycerophosphatase activity in muscle samples was measured at 37°C in a reaction mixture containing 0.25 M sucrose, 20 mM β -glycerophosphate, and 0.1 M sodium acetate buffer, pH 5.0.^{19,20)}

The cytochrome *c* oxidase activity in muscle samples was measured by the method of Wharton and Tzagoloff²¹⁾ using a Hitachi recording spectrophotometer, model 323. The incubation mixture contained 0.25 M sucrose, 0.07% ferro cytochrome *c*, and 10 mM potassium phosphate buffer, pH 7.2. The change in optical density at 550 nm was followed at 25°C.

The cathepsin activity in muscle samples was measured by the method of Mycek²²⁾ at 37°C in a reaction mixture containing 0.25 M sucrose, 1% bovine serum albumin, and 0.15 M sodium acetate buffer, pH 5.0. The reaction was stopped by the addition of TCA, and the reaction mixture was filtered with Whatman No. 3 filter paper. The peptides produced in the filtrate were measured by the method of Lowry *et al.*²³⁾

Tissue fractionation. Homogenates of muscle from rabbits were fractionated by a differential centrifugation method similar to that of De Duve *et al.*¹⁸⁾ Two hundred grams of fresh minced muscle from rabbits was mixed with 800 ml of chilled 0.01 M histidine-NaOH buffer solution (pH 6.4) containing 0.25 M sucrose and 0.1 mM EDTA. The mixture was homogenized with a Hitachi model VA-835 homogenizer at 12,000 rpm for 1 min. The resulting slurry was centrifuged in the cold at $500 \times g$ for 10 min. The precipitate, which still contained a large amount of unbroken muscle cells, was rehomogenized in about the same quantity of the buffer and centrifuged. The sum of the supernatants of two centrifugation was centrifuged at $1000 \times g$ for 10 min. Observation under a microscope showed that there were a few fragmented myofibrils besides nuclei in the precipitate. From this supernatant, mitochondrial (M), lysosomal (L) heavy microsomal (Ms-1), intermediate microsomal (Ms-2), and light microsomal (Ms-3) fractions were isolated by differential centrifugation at $5,000 \times g$ for 10 min, $8,500 \times g$ for 10 min,

$12,000 \times g$ for 30 min, $20,000 \times g$ for 40 min, and $105,000 \times g$ for 60 min, respectively. These fractions were dispersed with the above buffer solution in a Teflon homogenizer of the Potter-Elvehjem type and centrifuged at $105,000 \times g$ for 30 min twice. Similarly, the nuclear fraction was dispersed with 3 volumes of the above medium and centrifuged at $1,000 \times g$ for 10 min. This washing procedure was repeated several times to obtain a rather light nuclear fraction.

A bulk microsomal fraction was prepared by a similar procedure as described above with slight modifications. To 100 g of fresh minced muscle, 300 ml of 0.01 M histidine-NaOH buffer (pH 6.4) containing 0.25 M sucrose was added, the sample was homogenized at 12,000 rpm for 2 to 3 min in a homogenizer cup equipped with an ice-jacket, and the slurry was centrifuged at $2,600 \times g$ for 20 min. The precipitate was rehomogenized as it seemed to contain a large amount of unbroken cells. The resulting supernatant was filtered through cheesecloth or Milpax filter paper, and centrifuged again at $10,000 \times g$ for 15 min to remove mitochondrial and lysosomal fractions. The microsomal fraction was collected by centrifugation at $100,000 \times g$ for 60 min or by acid precipitation from the supernatant of the centrifugation at $10,000 \times g$. In the latter case, the pH of the supernatant was adjusted to 5.4 with 3 N acetic acid. Immediately after the adjustment of pH, the turbid supernatant was centrifuged at $2,600 \times g$ for 20 min. The precipitated microsomal fraction was dispersed in a solution consisting of 0.25 M sucrose, 10 mM Tris-HCl buffer, pH 7.5, and 1 mM EDTA (Buffer A) using a Teflon homogenizer and the pH was adjusted to 7.5 with 0.1 N NaOH. After slow stirring for 1 hr at 4°C, the solution was centrifuged at $10,000 \times g$ for 60 min. This washing procedure by dispersing and centrifuging was repeated once more. The final washed microsomal precipitate was dispersed in Buffer A (washed microsomes) and stored in an ice-cold bath until used for further experimentation.

Other methods. Protein concentrations of the subcellular fractions were measured by the method of Lowry *et al.*²³⁾ with bovine serum albumin as a reference standard. Cholic acids were recrystallized from 70% ethanol.

RESULTS AND DISCUSSION

PPase activity in subcellular fractions of skeletal muscle

The subcellular fractions of skeletal muscle cells were prepared by differential centrifugation by the method of De Duve *et al.*¹⁸⁾ Figure 1 shows the plots of the PPase activity of the different subcellular fractions obtained from 200 g of the muscle as a function of pH. Nuclei and the small myofibril fraction had little PPase activity at pH 4.0 to 7.2. The PPase

FIG. 1. Plot of the Pyrophosphatases Activity of Subcellular Fractions of Rabbit Muscle Cells vs. pH.

○, Fraction containing mitochondria and lysosomes (14.4 mg protein/ml-20 ml) without MgCl_2 ; ●, fraction containing mitochondria and lysosomes with MgCl_2 ; □, fraction containing heavy microsomes (7.1 mg protein/ml-15 ml) with MgCl_2 ; △, fraction containing light microsomes (8.5 mg protein/ml-15 ml) with MgCl_2 ; ▲, fraction containing nuclei and fragmented myofibrils (20 mg protein/ml-240 ml) with MgCl_2 ; ○, soluble fraction (9.5 mg protein/ml-760 ml) without MgCl_2 ; ●, soluble fraction with MgCl_2 . Reaction mixture consisted of 0.25 M sucrose, 0.08 M buffer, 1 mM Na-PP, and 0 or 2.5 mM MgCl_2 . Buffers were: sodium acetate, pH 4~5.5; histidine-NaOH, pH 6.0~6.5; imidazole-HCl, pH 7.0~7.5; Tris-HCl, pH 7.2~8.5.

activity of mitochondria and the lysosome fraction had optima in neutral and acidic pH, in the presence and absence of MgCl_2 , respectively. The PPase activity of light and heavy microsomal fractions had an optimum at pH 5.5. The PPase of the soluble fraction had an optimum at pH 7.2, and MgCl_2 activated it as reported previously.¹⁵ These results show that neutral PPase is contained also in the mitochondrial or lysosomal fractions, whereas acid PPase is present in the lysosomal or microsomal fraction.

Activities of some marker enzymes were assayed to examine whether each fraction was finely separated by this method. Figure 2 shows the subcellular distribution of total acid PPase and marker enzyme activities. Cytochrome *c* oxidase, acid cathepsin (and β -glycerophosphatase), and G-6-P phosphatase were employed as marker enzymes for the mitochondrial, lysosomal, and microsomal

FIG. 2. Subcellular Distribution of Acid PPase and Some Marker Enzymes.

Acid PPase activity was assayed in the mixture containing 0.25 M sucrose, 0.5 mM EDTA, 1 mM Na-PP, and 0.1 M sodium acetate buffer, pH 5.5. Abbreviations and assay conditions for the marker enzymes are described in MATERIALS AND METHODS.

fractions, respectively. The specific activities were plotted on the basis of protein content according to the procedure of De Duve *et al.*¹⁸ Since nuclei could not be completely separated from fragmented short myofibrils and since the soluble neutral PPase activity was contained in the cytosol fraction (Fig. 1), these fractions were omitted from Fig. 2. Although much β -glycerophosphatase was also found in the microsomal fraction, cytochrome *c* oxidase and cathepsin were found exclusively in the mitochondrial and lysosomal fractions, respectively. Therefore, it was presumed that we succeeded in separating the subcellular fractions from the muscle homogenate. The intracellular distribution pattern of acid PPase was similar to that of G-6-P phosphatase, but

not to those of cathepsin and other marker enzymes. These results show a large amount of acid PPase is in the microsomal fraction.

Rafter²⁴⁾ reported that a mitochondrial fraction prepared from mouse liver by centrifugation between $600 \times g$ for 15 min and $8,500 \times g$ for 15 min showed PPase activity, the maximum being at pH 5.3, without requiring Mg^{2+} ion. Nordlie and Lardy⁶⁾ reported that nuclear, mitochondrial, and microsomal fractions from rat liver contained significant amounts of PPase activity, that the activity of the nuclear fraction was due to contamination with erythrocytes, and that acid PPase appeared in the microsome fraction. Stetten and Burnett²⁵⁾ reported that PPase was contained in rat-liver microsomes, and was activated by treatment with ammonium or amino acid buffer at pH 9.5~9.8 or by treatment with DOC or Triton X-100. Soodsma and Nordlie²⁶⁾ detected a small amount of PPase activity in particulate fractions of rat heart at pH 7.5 in the presence of Mg^{2+} , besides Mg^{2+} -stimulated activity in the soluble fraction. Our results using rabbit skeletal muscles (Figs. 1 and 2) are consistent with those of Nordlie and Lardy. That is, the neutral PPase activity was found in the mitochondrial and soluble fractions in the presence of $MgCl_2$, and the bulk of acid PPase activity was in the microsomal fraction without latency.

Brightwell and Tappel¹⁰⁾ purified from rat-liver and kidney lysosomal fractions an acid PPase that could hydrolyze ATP and other nucleoside triphosphates, PP, thiamine diphosphate, and FAD. They also noticed that the activity of lysosomal PPase had latency, *i.e.*, the enzymatic activity could be released from the lysosomal fractions to a soluble state by freeze-thawing or sonication. Microsomal PPase obtained from rabbit muscle in this study did not show any latency even though freezing and thawing was repeated ten times.

With regard to mitochondrial PPase, Schick and Butler¹¹⁾ found the activity at pH 5 and pH 7~8 in the presence of Mg^{2+} in the intact

mitochondria (overt) from rat-liver. Mg^{2+} was not required for the activity of acid PPase. However, the Mg^{2+} -dependent neutral or alkaline PPase activity was stimulated several fold by disruption of mitochondrial membranes by sonication. They assumed from this latency that the enzyme was inside the inner membrane. Irie *et al.*²⁷⁾ partly purified two different alkaline PPases from mitochondria of rat liver. These were different with respect to Mg^{2+} requirement, substrate specificity, and inactivation by urea. Rabbit-muscle mitochondria in this study also showed neutral or alkaline PPase activity which was enhanced by the addition of Mg^{2+} (Fig. 1). The mitochondria were washed by dispersing 10 mM histidine-NaOH buffer (pH 6.4) containing 0.15 M NaCl and centrifuging. The washing did not remove the PPase activity from the fraction. The PPase was not released from mitochondria by ten freeze-thawing operations. These results suggest that an intrinsic PPase is associated with the mitochondrial membrane of rabbit muscle cells.

Effects of pH

The washed microsomal fraction contained

FIG. 3. Effects of pH on the Activity of Muscle Microsomal PPase.

The reaction mixture was 1 mM Na-PP, 5 mg/ml microsomal protein, and buffers. For pH control, 0.2 M sodium acetate (pH 4.0 to 5.5), 0.165 M histidine-NaOH (pH 6.16), and 0.2 M imidazole-HCl (pH 7.0) buffers were used. Preincubation was for 5 min at 25°C.

FIG. 4. Effects of pH on the Stability of Microsomal Acid PPase.

The microsomal fraction was incubated in a solution containing 5 mM MgCl_2 and 40 mM of various buffers for 44 hr ($\circ$), 90 hr ($\bullet$), and 136 hr ($\times$) at 4°C and the activity was then assayed at pH 5.5 in a reaction mixture containing 5 mM MgCl_2 , 1 mM Na-PP, and 0.2 M sodium acetate buffer. The reaction mixture was preincubated for 5 min at 25°C . Buffers used were sodium acetate (pH 4.0 to 5.5), histidine-NaOH (pH 6.16 to 6.5), imidazole-HCl (pH 7.0), and Tris-HCl (pH 7.5 to 8.5).

only acid PPase activity. Figure 3 shows that the pH-activity curve has a bell shape and the maximum activity is at about pH 5.5 in sodium acetate buffer. At pH 7.0, a little activity was shown. These results are in good agreement with those of Nordlie and Lardy⁶⁾ and are similar to those of rat-liver mitochondrial PPase in the absence of MgCl_2 .¹¹⁾ The data in Fig. 3 suggest that carboxyl and imidazolium groups may be involved in the active sites,²⁸⁾ but further studies on the pH-dependency of the maximum velocity or Michaelis constant will be required.

The microsomal acid PPase was stable at pH values between 5.5 to 8.5 at 4°C , and the activity was not changed between pH 6.5 to 7.5 during incubation for 136 hr (Fig. 4).

Effects of divalent cations, EDTA, and ionic strength on the acid PPase activity

Unlike neutral water-soluble PPase A,¹⁵⁾ the acid PPase activity was not affected by the addition of divalent cations such as Mg^{2+} and Ca^{2+} , or by EDTA. These properties are similar to those of the enzymes in rat-heart microsomes,²⁶⁾ mouse-liver mitochondria,²⁴⁾

FIG. 5. Effects of Sulphydryl Reagents on the Activity of Muscle Microsomal Acid PPase.

Activity was assayed in the presence of 1 mg/ml microsomal protein, 0.25 M sucrose, 5 mM EDTA, 0.1 M sodium acetate buffer, pH 5.5, 1 mM Na-PP, and pCMB at 25°C after incubation at 4°C for 90 min and preincubation at 25°C for 5 min ($\circ$). Incubation mixture containing 2 mg/ml microsomal protein, 0.25 M sucrose, 5 mM EDTA, 0.1 M sodium acetate buffer, pH 5.5, and 2-fold concentration of pCMB shown on the abscissa was diluted to the above reaction mixture with ($\bullet$) or without ($\triangle$) 10 mM DTT after 17 hr at 4°C .

and in rat-liver and rat-kidney lysosomes.¹⁰⁾ Relative activity was slightly decreased in the presence of excessive divalent metals (20 mM). The activity of microsomal acid PPase decreased with increasing the concentration of NaCl in the reaction mixture.

Effects of sulphydryl reagents

As shown in Fig. 5, the microsomal acid PPase activity was inhibited over 80% after incubation for 17 hr at 4°C in the presence of 0.1 mM or more of pCMB, but this inhibition was suppressed by the addition of excess DTT. Probably the sulphydryl group(s) make some contribution to the active site of the enzyme, we have concluded pending the examination of the correlation between SH content and the activity.

Effects of detergents on the activity

Many surfactants have been used to solubilize enzymes which are bound to membranes. Among them, ionic detergents such as SDS and cholic acid, or nonionic detergents such as Triton X-100 and C_{12}E_8 are frequently

FIG. 6. Effect of Triton X-100 on the Activity of Microsomal Acid PPase from Rabbit Skeletal Muscle.

Microsomal fraction (10 mg/ml protein) was incubated in the solution containing 0.25 M sucrose, 5 mM EDTA, 10 mM Tris-HCl buffer (pH 7.2), and Triton X-100 shown on the abscissa (○) or 20-fold concentration of Triton X-100 shown (●) with occasional mixing for 20 hr at 4°C. The activity was measured at 25°C in the reaction mixture containing 1 mg/ml (○) or 0.5 mg/ml (●) microsomal protein, 1 mM Na-PP, 0.25 M sucrose, 5 mM EDTA, 0.1 M sodium acetate buffer (pH 5.5), and Triton X-100 at the final levels shown on the abscissa.

used.²⁹⁻³¹⁾ Before using these detergents, it is necessary to examine whether they denature the enzyme. Therefore, the effect of some detergents on the activity of microsomal acid PPase was examined. The acid PPase was irreversibly inactivated by the addition of SDS. However, the changes in the acid PPase activity by the addition of detergents such as Triton X-100, C₁₂E₈, sodium cholate, or DOC were reversible; relative activity decreased with increasing concentrations of Triton X-100, but the PPase activities were almost identical with those found on incubation in the solutions containing the low concentration of detergent when the PPase was transferred into solutions of low concentration of detergent from high concentrations after incubation for 20 hr at 4°C (Fig. 6).

Membrane-bound PPase in *Rhodospirillum rubrum* is coupled to the electron transport chain in chromatophores and it catalyzes the hydrolysis and synthesis of PP.³²⁾ The membrane-bound PPase in eucaryotes is closely related to PP synthesis coupled to the mito-

chondrial respiratory chain.³³⁾ Thus it is of considerable interest to study the role of microsomal PPase in muscle cells. Using the above detergents, we are now progressing in separating the acid PPase from the microsomal membrane.

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