INORGANIC PYROPHOSPHATASE OF RAT LIVER MITOCHONDRIA

Correlation of Latency with Catalytic Properties and Intramitochondrial Location

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ABSTRACT

Stimulation of Mg^{2+}-dependent inorganic pyrophosphatase activity several fold by disruption of mitochondrial membranes does not appreciably alter the catalytic properties of the enzyme. Stimulation is due to increased accessibility of substrate to the enzyme, which is not solublized on activation. The enzyme is attached to the inside of the inner membrane, and under physiological conditions probably hydrolyzes only intramitochondrially-produced PP_i.

Disruption of the membranes of isolated rat liver mitochondria stimulates Mg^{2+}-dependent inorganic pyrophosphatase (PPase) activity (Enzyme Commission 3.6.1.1.) several fold (1). This latency is shared in varying degrees by other mitochondrial enzymes. We have compared some of the catalytic properties of the overt (intact mitochondria) and latent (disrupted mitochondria) PPase, and determined the intramitochondrial location of the activity. The results bear upon the question of the basis of the latency phenomenon, and reemphasize the importance of mitochondrial structural integrity in the maintenance of physiological enzyme activity.

METHODS

Liver mitochondria were prepared by the method of Schneider (2) from 200g Wistar rats which had been fasted overnight. Mitochondria were washed three times by resuspension in one-fourth of the original volume of medium (9 ml/g liver) followed by recentrifugation. For studies of stimulation and inhibition of PPase activity the suspending medium contained 0.88 M sucrose, 0.02 M Tris acetate (pH 7.2), and 0.1 mM ethylenediamine-tetraacetic acid (EDTA). For mitochondria which were to be subfractionated the suspending medium was 0.25 M sucrose.

Washed mitochondria were subfractionated by a separation technique which involves successively a mild osmotic shock, ATP-driven "contraction" of the inner membranes, and centrifugation of the insoluble membranous material through a discontinuous sucrose gradient (3). Mitochondria from 15 g of liver were centrifuged at 7000*g* for 10 min and the pellet was suspended in 18 ml of 10 mM Tris acetate buffer, pH 7.5, with a Teflon pestle fitted into the centrifuge tube. After 10 min at 0° the suspension was diluted with one-third vol of 1.8 M sucrose containing 2 mM adenosine triphosphate (ATP) and 2 mM $MgCl_2$. After 5 min further at 0°, the suspension was centrifuged at 44,000*g* for 15 min. The sedimented material, which contained approximately 90% of the Mg^{2+}-dependent PPase activity, was resuspended in 0.45 M sucrose using the Teflon pestle, and 10 ml of the suspension containing approximately 90 mg protein was layered on top of a sucrose bilayer composed of 5 ml of 0.76 M sucrose over 10 ml of 1.32 M sucrose. After centrifugation (in the same angle head rotor used in the mitochondrial preparation) at 44,000*g* for 3 hr, fractions were collected by applying vacuum to an horizontally bent-tip pipette successively lowered into the tube, which emptied into a test tube

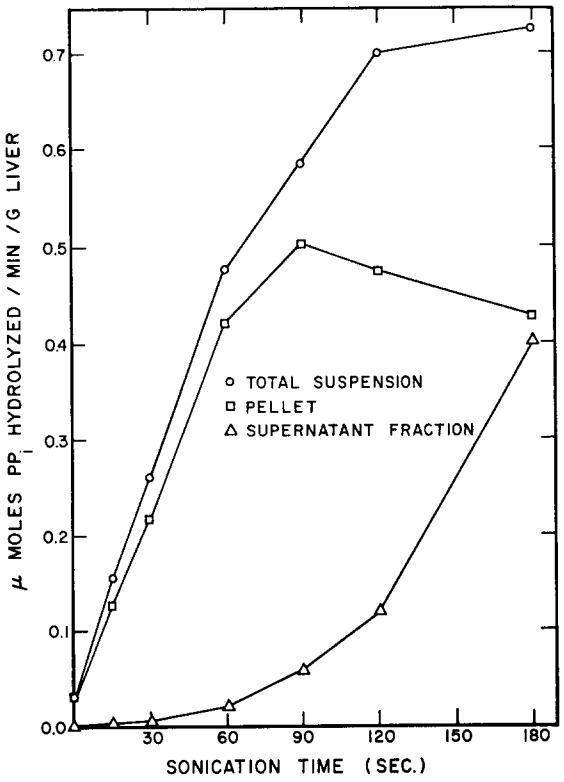

FIGURE 1 Time course of stimulation of PPase activity by sonication. Fresh mitochondria prepared in the 0.88 M sucrose media were, after the last wash, suspended in 0.25 M sucrose, approx. 10 ml per g of liver. The suspension was sonicated for 15 sec intervals at full power setting with a Branson Sonifier, Model S.125 (Branson Sonic Power, Danbury, Conn.). Aliquots were withdrawn at appropriate times during the cooling period between the repeated sonications. Insoluble membranous material was removed by centrifugation at 44,000 *g* for 30 min. PPase activity was measured in the supernatant and resuspended pellet fractions, and in the unfractionated sonicated mitochondrial suspension.

in a vacuum flask. The surface of the pellet was washed with 0.45 M sucrose and the pellet (Fraction 12) resuspended in 0.25 M sucrose for assay with the other fractions.

Phosphatase activities were measured as P_i released (4) at 30° from substrate in 0.25 M sucrose by about 2 mg of mitochondrial protein in a total volume of 1.1 ml. ATPase and routine PPase assays contained, in addition to 0.25 M sucrose, 2.5 mM substrate, 2.5 mM $MgCl_2$, and 0.2 M Tris acetate, pH 7.2. Glucose-6-phosphatase assays contained, in addition to sucrose, 25 mM glucose 6-phosphate, 0.1 M acetate (pH 5.5), but no $MgCl_2$. The amount of P_i release was proportional to incubation time and to amount of enzyme added. For determination of apparent Michaelis constants, Mg^{2+} and PP_i were varied together from 1 to 10 mM.

Cytochrome c was reduced with ascorbate (5). Cytochrome C oxidase was assayed at 550 mμ (6). Monoamine oxidase assays were initiated by addition of 0.1 ml of mitochondrial suspension containing about 0.1 mg protein to 0.9 ml of 5 mM benzylamine in 0.05 M phosphate buffer, pH 7.6. After 4 hr at 30° the reaction was stopped with 1 ml of 3 M perchloric acid. After centrifugation, 1.0 ml of the supernatant fraction was added to 1.0 ml of 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl. After 5 min the mixture was extracted with 4 ml of water-saturated benzene, and absorbance of the upper organic layer was read at 480 mμ. Nonenzymatic oxidation of benzylamine was not detectable. Protein was determined by the method of Lowry et al. (7), using crystalline bovine serum albumin as standard.

RESULTS AND DISCUSSION

The stimulation of Mg^{2+}-dependent PPase activity by sonication of intact mitochondria is illustrated in Fig. 1. Activation was evident at very short sonication times, and was largely complete before significant amounts of activity were rendered "soluble." Thus, latency is not due to the release of soluble enzyme which is more active than its membrane-bound form.

Some of the catalytic properties of the overt and latent enzymes are compared in Table I. In all conditions tested the enzymes gave virtually identical responses, suggesting that the overt and latent activities are not due to two different enzymes with different properties.

In contrast, other studies indicated that Mg^{2+}-dependent PPase and ATPase are not similarly sensitive to various inhibitors. For example, overt

Mg^{2+}-dependent PPase was stimulated 20% by 1 mM 2,4-dinitrophenol, and the corresponding ATPase was stimulated at least 800% under the same conditions. Inhibition by oligomycin (1 μg/ml) was 17% for PPase and 54% for ATPase.

TABLE I

Comparison of the Overt and and Latent Enzymes

Property	Overt	Latent
K_m	17 mM	12 mM
Inhibition: by		
1 mM NaF	92%	91%
0.1 mM PMB*	39%	38%
Omit Mg^{2+}	100%	99%

* p-Mercuribenzoate, preincubated 5 min before assay.

Thus the ATPase and PPase activities are probably due to different enzymes, and are not manifestations of a single enzyme with broad specificity.

In addition to sonication, a wide variety of other treatments, including osmotic shock (dilution with deionized water to 0.05 M sucrose 15 min before assay), freezing and thawing, and treatment with detergents (optimal concentrations were 0.04% Triton X-100, or 0.5% deoxycholate) increased Mg^{2+}-dependent PPase activity to approximately the same value, about a μmole PP_i hydrolyzed per min per gram of liver. The effects of the various treatments were not additive. Aging at 0° resulted in a slow spontaneous increase of PPase activity (Fig. 2), but the latent activity was concomitantly reduced. This spontaneous activation was minimal in the suspending medium which contained 0.88 M sucrose.

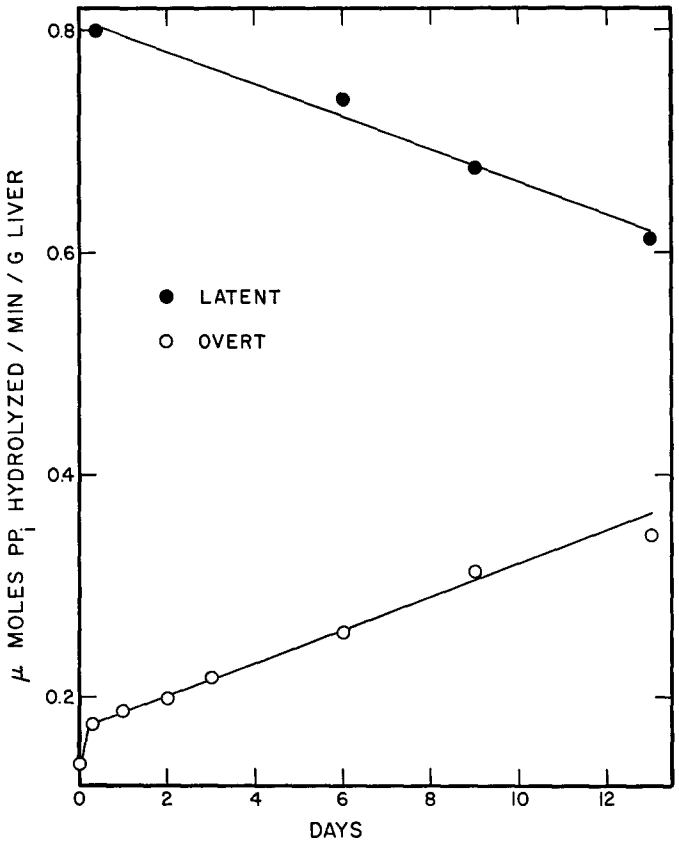

FIGURE 2 Effect of aging on Mg^{2+}-dependent PPase. Mitochondria prepared in the media containing 0.88 M sucrose were centrifuged and resuspended in 0.25 M sucrose to 5× the original volume. After various intervals at 3°, aliquots were assayed for Mg^{2+}-dependent PPase activity before and after sonication (1 min).

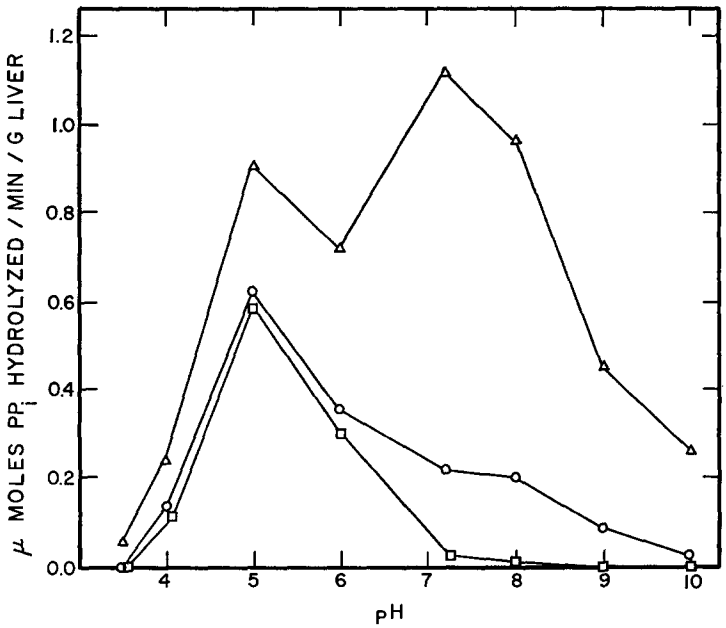

FIGURE 3 Effect of pH on PPase activities. Assays were standard except for alteration of the pH and omission of $MgCl_2$ where noted. □, overt activity, omitting $MgCl_2$; ○, overt activity in presence of $MgCl_2$; △, latent activity (1 min sonication) in presence of $MgCl_2$.

The variation of PPase activity of mitochondrial preparations as a function of pH under 3 different conditions is shown in Fig. 3. Unstimulated mitochondria assayed in the absence of added $MgCl_2$ exhibited a single optimum at pH 5. Addition of $MgCl_2$ had no effect on the acid PPase, but stimulated PPase activity in the neutral and alkaline range, and sonication specifically activated only the Mg^{2+}-dependent activity. The differential response to Mg^{2+} and sonication suggests the presence of two PP_i-hydrolyzing enzymes which are structurally and functionally independent. Unless specified otherwise, all PPase assays were done at pH 7.2 in the presence of $MgCl_2$, so the Mg^{2+}-dependent activity is effectively the only one measured.

Recently developed techniques allow essentially complete separation of functionally intact mitochondrial inner membranes from outer membrane fragments (3, 8). Subfractionation of mitochondria gave rise to the distribution patterns presented in Fig. 4. Fig. 4 A demonstrates, as expected, that the pellet (inner membrane fraction) contained at least 75% of the total cytochrome oxidase activity recovered. In contrast, 85% of the monoamine oxidase activity (outer membrane marker) was found in the interface fractions, indicating rather complete separation of the inner and outer membranes. About 80% of the applied protein was recovered in the pellet (inner membrane).

The distribution pattern (Fig. 4 C) for glucose-6-phosphatase coincided with that for Mg^{2+}-independent acid PPase, as expected from the evidence that these activities are due to the same microsomal enzyme (9, 10). Additional evidence that the acid PPase activity is due to contamination of the inner membrane fraction by microsomes was provided by the finding that the acid PPase activity of these mitochondrial preparations was stimulated twofold by a short exposure to pH 10, a characteristic of the microsomal enzyme (11), while the Mg^{2+}-dependent PPase activity was unaffected by this treatment, though even more strongly stimulated by a variety of other treatments, as mentioned above. Lysosomes contain a Mg^{2+}-independent PPase which is released in soluble form on osmotic shock (12). Since only the particulate residue of the shocked mitochondria was applied to the gradient, the acid PPase cannot be lysosomal in origin. The distinct separation of glucose-6-phosphatase from monoamine oxidase is not consistent with the notion (13) that monoamine oxidase is a microsomal, rather than mitochondrial, enzyme.

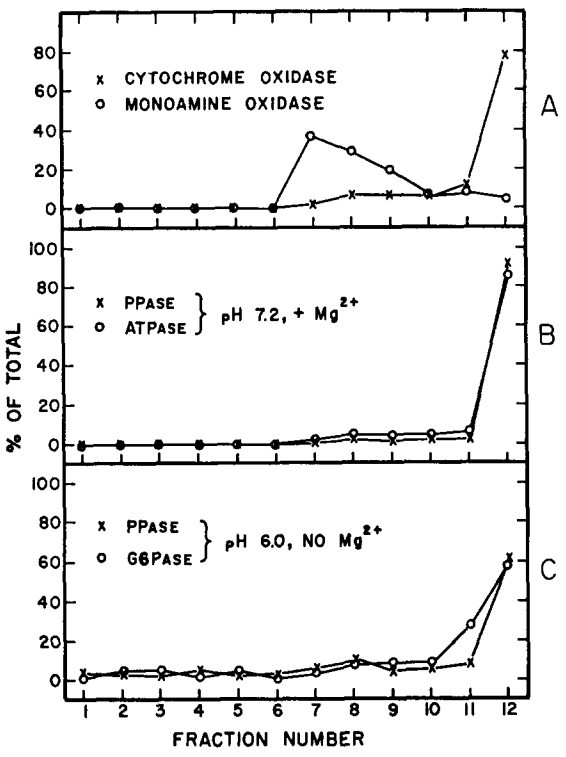

FIGURE 4 Separation of inner and outer membranes by centrifugation through a discontinuous density gradient (3). The separated outer membrane fragments, identified by their characteristic monoamine oxidase activity (3, 14), collect at the interface between layers of 0.76 M and 1.32 M sucrose (fractions 7 and 8); the denser inner membrane "ghosts", identified by cytochrome oxidase activity (14, 15), sediment through the 1.32 M sucrose and form a pellet (Fraction 12).

The above observations suggest that external PP_i is not hydrolyzed in vivo by morphologically intact mitochondria, i.e., that overt activity is due to damage to mitochondrial membranes during the isolation procedure. Variations in the level of overt activity in independent preparations presumably reflect differences in the integrity of mitochondrial structures; as noted above, disruption by several different techniques results in virtually the same specific activity. The mitochondrial outer membrane appears to be freely and rapidly permeable to externally added PP_i, as well as to sucrose and nucleotides (16, 17). The lack of ability of intact mitochondria to hydrolyze external PP_i suggests that the enzyme is located on the inside of the inner membrane, where endogenous PP_i is known to be produced by reactions such as activation of fatty acids for β-oxidation (18) or activation of amino acids for protein synthesis (19).

The Mg^{2+}-dependent PPase activity of the separated inner membrane does not appear to be latent; no stimulation was observed on sonication of these preparations. Osmotic shock, particularly in the presence of Tris buffer as was used here, results in nearly complete disruption of the inner membranes (20), so that no increase of activity would be expected on sonication of the preparation.

Journal Paper Number 3574 of the Purdue University Agriculture Experiment Station, Lafayette, Indiana.

This work was supported in part by the Indiana Elks through Purdue University's Cancer Research Committee.

Received for publication 7 January 1969, and in revised form 20 February 1969.

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