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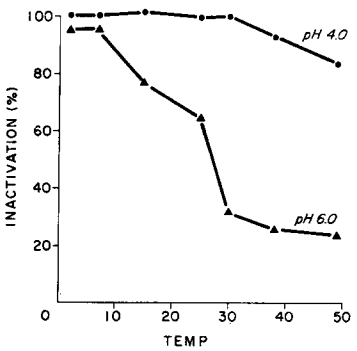
Activation and stability of lysosomal acid phosphohydrolase

NEIL AND HORNER[1] have drawn attention to some of the difficulties involved in the interpretation of tissue acid phosphohydrolase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) activity. A partial resolution of these difficulties occurred with the observation of DE DUVE *et al.*[2], on the association of hepatic "acid phosphatases" with the lysosome. While the lysosome concept has become established, detailed observations on the phenomena of enzyme sequestration and structure (particulate)-linked enzyme latency are meager. This communication examines the nature of hepatic α-naphthyl acid phosphohydrolase lability in activated and non-activated large granule fractions, and indicates the role played by metal ions in the inhibition of particulate and activated enzyme.

Adult Swiss albino mice were killed by decapitation and the liver homogenized for 20 sec in ice-cold 0.25 M sucrose. The large granule fraction was prepared from mouse liver 0.25 M sucrose–1 mM EDTA homogenates by differential centrifugation. The homogenate was spun at 7500 × *g*·min (centrifugal forces are expressed as time integrals (*g*·min) of the maximum field where $R_{max.} = 7.1$ cm)[3]. The supernatant was spun at 240 000 × *g*·min. The resulting pellet was resuspended in 0.3 M sucrose and centrifuged at 90 000 × *g*·min to obtain the large granule pellet which was resuspended in 0.3 M sucrose.

Total enzyme activity refers to enzyme measured in the presence of Triton X-100 (0.1–0.3%, v/v) whereas free activity represents untreated aliquots. α-Naphthyl acid phosphohydrolase activity was measured fluorimetrically[4]. β-Glycerophosphatase activity was measured after GIANETTO AND DE DUVE[5]. Liberated phosphate was determined by a modification of the method of LOWRY AND LOPEZ[6].

Preincubation of Triton X-100-activated, washed large granule fraction at



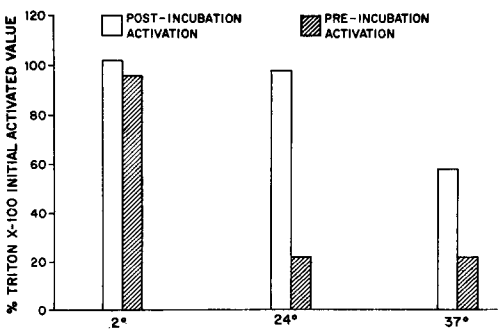


Fig. 1. Stability of hepatic phosphohydrolase as a function of pH and temperature of preincubation for 30 min in 0.2 M sodium acetate–KCl buffer. Degree of inactivation as percentage of activity at zero time, 24°, pH 6.0.

Fig. 2. Effect of preincubation at various temperatures in 0.2 M sodium acetate–KCl buffer, pH 6.0, for 20 min on thermolability of acid phosphohydrolase. Open columns demonstrate enzyme activity following Triton X-100 activation, post-incubation (*i.e.*, activation of incubated particulate enzyme after 20 min). Shaded columns represent the temperature-dependent loss of activity of the Triton X-100-activated enzyme during the 20-min preincubation.

TABLE I

PERCENTAGE INHIBITION OF α-NAPHTHYL ACID PHOSPHOHYDROLASE AND β-GLYCEROPHOSPHATASE ACTIVITY BY METAL IONS IN TRITON X-100 ACTIVATED LARGE GRANULE FRACTION

Aliquots of activated large granule fraction were preincubated at 2°, for 5 min in 0.2 M acetate–KCl buffer containing inhibitory ion. 0.1 ml removed for assay at 37°, 10 min in acetate–KCl buffer, pH 6.0. Values are means of 3 experiments.

Substrate	*Concn. of inhibitor in system*	*Inhibitory ion*			
		Hg^{2+}	Ag^{+}	Fe^{3+}	Cu^{2+}
α-Naphthyl acid sodium phosphate	Pre-incubation system (0.1 mM)	10	8	74	86
	Final assay system (0.05 mM)	89	43	81	90
β-Glycerophosphate	Pre-incubation system (0.1 mM)	0	5	+6	18
	Final assay system (0.05 mM)	33	18	+2	16

pH's 3.1–7.5, adjusted to isotonicity with 0.15 M KCl, demonstrated considerable inactivation at pH 6, but less than 10% inactivation at pH 4. The effect of the preincubation temperature on the rates of enzyme inactivation at pH 4.0 and pH 6.0 (Fig. 1) confirmed the pH dependence and illustrated the protective effect of temperatures less than 8° at pH 6.0. Moreover, the rapid rate of inactivation at 37° was amply illustrated following incubation at pH 6.0 in a Dubnoff metabolic shaker in an atmosphere of oxygen but not nitrogen.

The effect of Triton X-100 added after 20-min preincubation at 2°, 24° and 37° on the extent of activation of the large granule fraction and enzyme activated at zero time was compared (Fig. 2). The intraparticulate form of the enzyme was more thermostable.

It was considered that the instability of α-naphthyl phosphohydrolase when fully released from particulate binding by Triton X-100 may be due to oxidation of terminal –SH groups. The effect of metal ions on the inhibition of α-naphthyl phosphohydrolase and β-glycerophosphatase (Table I), suggested that this was indeed the case. In particular, Fe^{3+} caused no significant inhibition of the enzyme with β-glycerophosphate as substrate, but markedly inhibited the activity with α-naphthyl acid phosphate; the inhibition caused by Cu^{2+} was significantly greater with α-naphthyl acid phosphate than with β-glycerophosphate as substrate, and was easily reversed in the former case by 5 mM L-cysteine. The rate of inactivation of α-naphthyl phosphohydrolase at 37° was increased in the presence of *p*-chloromercuribenzoate whereas ascorbate and L-cysteine protected the enzyme. Attempts to stabilize the Triton X-100-activated phosphohydrolase with 0.5 to 5 mM disodium EDTA or 5 mM 1,10-*o*-phenanthroline were unsuccessful, indicating that –SH group inactivation at pH 6 is not catalytically induced by trace metal ions. Moreover, the previously noted stability of the enzyme preincubated at pH 4, 37° was reversed in the presence of 1 mM EDTA (Fig. 3). Ascorbate (1 mM), reduced glutathione, and L-cysteine (5 mM each) reactivated the enzyme to varying degrees. Such reactivation was also noted following preincubation at pH 6.

Inhibition of α-naphthyl phosphohydrolase by Hg^{2+}, Fe^{3+} and Cu^{2+} is well documented, but applies to the activated enzyme. Evidence is lacking on the inhibitory action of these ions on intraparticulate enzyme. Aliquots of large granule

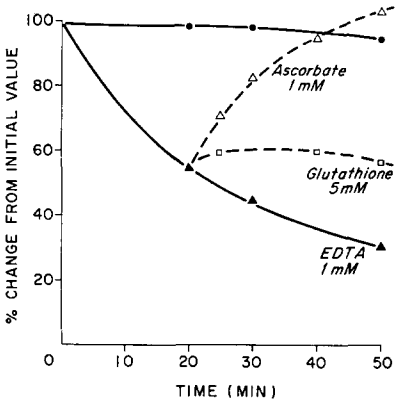


Fig. 3. Induced thermolability of Triton X-100-activated large granule α-naphthyl phosphohydrolase by disodium EDTA. Preincubation at 37°, pH 4.0. Control (●—●). Effect of ascorbate and reduced glutathione, added to EDTA fraction at 20 min also shown.

fraction were resuspended in 0.3 M sucrose, to which 0.1 mM inhibitory ions were added for 2 min at 2° or 24°. Portions were removed and assayed in a modified isotonic acetate–KCl system at pH 6.0 containing 1 mM EDTA. These assay tubes were in duplicate, one of which contained Triton X-100. The presence of EDTA in the final assay nullifies the inhibitory action of free ions transferred with the large granule fraction. The final reading (Table II) indicates enzyme inhibition that occurred while the enzyme existed in a particulate state. The small degree of inhibition by Cu^{2+} and Fe^{2+} is in marked contradistinction to that found in the activated fraction (Table I). Hg^{2+}, however, induced considerable phosphohydrolase activation (340%).

The sensitivity of hepatic acid α-naphthyl phosphohydrolase to *p*-chloromercuribenzoate, Cu^{2+}, Fe^{3+} and Hg^{2+} is consistent with a dependence upon sulphydryl groups[7]. The thermolability was dependent upon temperature and degree of oxygenation. Metal ions appear to play only a subsidiary role in the catalytic oxidation or irreversible inactivation of the terminal –SH group at pH 6.0. In contrast, no comparable thermal inactivation occurred during preincubation at pH 4. However, thermolability was induced at such pH in the presence of a chelator.

TABLE II

EFFECT OF INHIBITORY IONS, Cu^{2+}, Fe^{3+} AND Hg^{2+} ON PARTICULATE (NON-ACTIVATED) LARGE GRANULE FRACTION ACID PHOSPHOHYDROLASE

Aliquots of large granule fraction were preincubated with 0.1 mM ions at 24°. Assay in 0.2 M acetate–KCl, pH 6.0, in presence of 1 mM EDTA with and without Triton X-100 activation (0.2%, v/v). Values are means of 3 experiments.

Large granule fraction (90 000 × g·min)	*Inhibition (%)*		
	Cu^{2+}	*Fe^{3+}*	*Hg^{2+}*
Non-activated	8	2	+340
Triton X-100 activation in final assay	13	6	8

Reversal of the chelation-induced thermolability at pH 4 was accomplished by ascorbate, and –SH donating agents. Acid phosphohydrolase stability at pH 4.0 was dependent therefore upon the maintenance of reduced –SH groups, and secondarily dependent upon metal ion binding.

Particle sequestered acid phosphohydrolase was thermostable, even when pre-incubated at 37°. The terminal sulphydryl groups of the enzyme when intraparticulate are protected from inactivation, considerable enzyme activity being released by Triton X-100. These observations suggest that the lysosomal structure (membrane) confers thermostability on the phosphohydrolase by protecting the easily oxidized –SH groups. The lack of Cu^{2+} or Fe^{3+} inhibition of the intraparticulate enzyme is similarly explainable, but also suggests that the phosphohydrolase may be protected by covalent linkage to the membrane through the –SH group(s). Under these experimental conditions, we were unable to confirm the activating and solubilizing effect of Cu^{2+} and Fe^{3+} as observed by KOENIG AND JIBRIL[8].

The structure-linked latency of the sulphydryl-dependent acid phosphohydrolase of the large granule fraction is envisaged as being activated by Triton X-100 and mercuric ions. The activated enzyme is rapidly oxidized to the mercaptide form depending upon the pH and associated binding of protective metal groups, easily removed in the presence of chelating agents.

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